A Survey of c-MET Expression and Amplification in 287 Patients with Hepatocellular Carcinoma

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Abstract. *Background: c-N-Methyl-N'-nitro-N-nitrosoguanidine HOS transforming gene (c-MET) is a new potential drug target for treatment of patients with hepatocellular carcinoma (HCC), and a recent study of a c-MET inhibitor in such patients has shown promising results. In the present study, we investigated the incidence of c-MET overexpression and its prognostic impact. Materials and Methods: Tumor tissue microarrays were used to detect the expression of c-MET in samples from 287 patients with HCC who underwent surgical resection at Samsung Medical Center. We explored the relationships between c-MET overexpression and clinicopathological features of HCC, and investigated recurrence-free survival (RFS) and HCC-specific survival according to the level of c-MET expression. Additionally, we explored the correlation between c-MET protein overexpression, and MET mRNA expression and copy number variation. Results: Most patients in the present study were male (n=297, 82.6%), with Child-Pugh class A liver function (n=286, 99.7%) and hepatitis B viral infection (n=217, 75.6%). c-MET overexpression was observed in 80 patients (27.9%), and was not associated with Edmondson grade, tumor size, microvascular invasion, major portal vein invasion or stage. In addition, c-MET expression levels did not affect RFS or HCC-specific survival. c-MET expression was weakly correlated with c-MET copy number variation (r=0.255, p<0.001), but more than half of all patients with c-MET overexpression had a neutral c-MET copy number. c-MET protein expression was very weakly but significantly positively correlated with its mRNA expression (r=0.199, p=0.002). Conclusion: c-MET overexpression did not have any prognostic impact on recurrence or survival of patients with HCC undergoing surgical resection. However, 27.9% of patients who had c-MET overexpression could be considered candidates for treatment with c-MET inhibitor.*

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Primary tumors of the liver now represent the fifth most frequently diagnosed type of cancer worldwide, but the second most frequent cause of cancer death (1). Global incidence has risen to approximately 748,300 cases annually, with 695,900 cancer deaths reported in 2008. Of all primary liver cancers, hepatocellular carcinoma (HCC) represents the major histological subtype, accounting for 70 to 85% of the total liver cancer burden worldwide (2).

Surveillance programs of patients with cirrhosis enable for detection of HCC at early stages, when the tumors are amenable to curative treatment (60% in Japan, 25-40% in Europe and the United States) (3, 4). However, long-term prognosis after surgical resection of HCC remains poor due to the high rates of recurrence and lack of effective adjuvant therapies (5). Tumor recurrence complicates 70% of cases at five years, reflecting either intrahepatic metastases (true recurrences) or the development of *de novo* tumors (6-8).

In recent years, molecular-targeted therapy has offered new prospects and attracted a great deal of attention with regard to its use in the standardized treatment of HCC (9, 10). Systemic treatment with sorafenib, a multi-kinase inhibitor targeting RAF kinase and receptor tyrosine kinases (RTKs), including platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), and c-

KIT (a receptor specific for stem cell factor), is recommended for patients with more advanced HCC (11, 12). In addition, several other RTK-targeted drugs have been evaluated.

c-MET was discovered as an oncogene and encodes a tyrosine kinase-type growth factor receptor with an affinity for hepatocyte growth factor (HGF). The binding of active HGF to functionally established c-MET leads to receptor dimerization/multimerization, multiple tyrosine residue phosphorylation in the intracellular region, and downstream signaling for motility, proliferation, survival and morphogenesis (13, 14). De-regulation and activation of c-MET may result in unregulated cell growth and differentiation, thus contributing to malignant transformation (15). c-MET overexpression or enhanced activation relative to normal tissues has been noted in many types of human cancer such as gastric, colorectal, pancreatic, lung, head and neck, ovarian, renal, prostatic and breast cancer (16, 17). c-MET overexpression is observed in 20-48% of human HCC samples (18-20).

Recently, a phase II trial of tivantinib, a c-MET inhibitor, in patients with HCC was reported, and c-MET overexpression was proposed to be a predictive biomarker (21). We planned the current study to investigate: i) the incidence of c-MET overexpression in patients with HCC; ii) correlations between c-MET protein overexpression, mRNA expression and copy number variation (CNV); iii) the relationships between c-MET overexpression and clinicopathological features of HCC; and iv) tumor recurrence in and survival of patients with HCC according to c-MET overexpression.

Patients and Methods

Patients. A total of 287 consecutive primary HCC samples were collected from patients who underwent hepatectomy at the Samsung Medical Center (Seoul, Korea) from July 2000 to May 2006. None of the patients received preoperative chemotherapy. The Institutional Review Board of Samsung Medical Center approved this study (SMC 2013-05-018) and waived the need for written informed consent from the participants.

Clinical parameters, including age, sex, date of surgery, and tumor size, were obtained from pathology reports. The histopathological features of HCCs examined by pathologist were histological differentiation, microvascular invasion, major portal vein invasion, intra-hepatic metastasis and multicentric occurrence. HCCs were graded histologically according to the criteria of Edmondson and Steiner (22). Microvascular invasion was considered present when one or more endothelial cells or the *tunica media* of the vessel surrounded a neoplastic cell group. Intrahepatic metastasis and multicentric occurrence were matched to the criteria of the Liver Cancer Study Group of Japan (23).

Patient serum α-fetoprotein levels were evaluated, and three-phase dynamic computed tomography scans were performed at least once every three months after surgery. When tumor recurrence was suspected, precise diagnostic imaging was performed by magnetic resonance imaging. Recurrence-free survival (RFS) was defined from the date of resection until the detection of tumor recurrence. While HCC is the cause of death in most patients with the disease, some patients die of liver failure or from other causes in the absence of progressive HCC. We chose HCC-related mortality (disease-specific death) as the clinical endpoint for survival analysis, defined as follows: i) tumor occupying more than 80% of the liver, ii) portal venous tumor thrombus (PVTT) proximal to the second bifurcation, iii) obstructive jaundice due to tumor, iv) distant metastasis, or v) variceal hemorrhage with PVTT proximal to the first bifurcation (24).

Immunohistochemistry. Histological sections were examined by a pathologist, and representative tumor areas free from necrosis or hemorrhage were pre-marked in formalin-fixed paraffin-embedded blocks. Two 2.0-mm-diameter tissue cores were taken from the donor blocks and transferred to the recipient paraffin block at defined array positions.

Immunohistochemical staining was performed as previously described (25). Antigen retrieval was performed with 0.01 mol/l citrate buffer (pH 6.0) for 30 min in a pressure cooker. The sections were incubated for 30 min at room temperature with rabbit monoclonal antibody to c-MET (#8198, 1:100; Cell Signaling, Tech., Beverly, MA, USA). Negative controls (isotype-matched irrelevant antibody as primary antibody) were run simultaneously. To validate the concordance between tissue microarrays and whole tumor sections, we further detected c-MET expression for 40 corresponding whole tumor sections randomly chosen from the 287 cases.

All sections were scored by a pathologist who was blinded to the patient characteristics. The proportion of stained tumor cells was determined semi-quantitatively, and each sample was scored on a scale of 0-3 (0, <20%; 1, 20-60%; 2, 61-80%; 3, >80%). Duplicate tissue cores for each tumor showed high levels of homogeneity for the proportion of stained cells. In cases of differences between duplicate tissue cores, the higher score was used.

c-MET CNV and mRNA expression. Genomic DNA and total RNA were extracted from the sliced tissue specimens using the QIAamp DNA mini kit and RNeasy Plus mini kit (Qiagen, Hilden, Germany), respectively. RNA integrity was assessed using an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA). For the gene expression analysis, 287 tumor samples with an RNA integrity number greater than 5.0 were further analyzed. Normalized single nucleotide peptide (SNP) array intensity data were exported from Illumina Genome Studio and further processed using an in-house pipeline to obtain copy number segments and gene-summarized copy number estimates. Copy number gain and loss cutoffs were defined to be 2.3 and 1.7, respectively, based on an assessment of replicate samples from the same SNP arrays.

Total RNA was amplified and converted to biotinylated cRNA according to the manufacturer's protocol (Illumina Total Prep RNA amplification kit; Ambion). Two hundred nanograms of RNA was reverse transcribed. After second strand synthesis, the cRNA was transcribed *in vitro* from the resulting cDNA template in the presence of biotin-16-UTP. Labeled target cRNA was then hybridized to Human HT-12 v4 Bead Chips (Illumina, San Diego, CA, USA) using the Illumina Bead Chip HT-12 protocol. Bead Chips were scanned on the Illumina Bead Array Reader using Illumina Bead Array Reader image data acquisition software. We excluded the samples from the statistical analysis if less than 15% of probes had present calls using a cut-off of 0.05 for a present call. We also excluded the probes if they had present calls for fewer than 15% of samples. The intensities of the probes transformed by base 2 logarithm were normalized using the quantile normalization method (26).

Table I. *Patients' characteristics*.

	c-MET^{low} (N=207)	c-MET^{high} (N=80)	Total (N=287)	*p*-value
Gender				
Male	167 (80.7)	70 (87.5)	237 (82.6)	0.172
Female	40 (19.3)	10 (12.5)	50 (17.4)	
Age (years)	52 (17-74)	57 (29-76)	52 (17-76)	0.001
Etiology				
HBV	163 (78.7)	54 (67.5)	217 (75.6)	0.138
HCV	19 (9.2)	11 (13.8)	30 (10.5)	
Etc.	25 (12.1)	15 (18.8)	40 (13.9)	
C-P class				
A	206 (99.5)	80 (100.0)	286 (99.7)	0.999
B	1 (0.5)	0 (0)	1 (0.3)	
Edmondson grade				
1	17 (8.2)	12 (15.0)	29 (10.1)	0.065
2	169 (81.6)	65 (81.3)	234 (81.5)	
3	21 (10.1)	3 (3.8)	24 (8.4)	
Tumor size				
<5 cm	133 (64.3)	56 (70.0)	189 (65.9)	0.406
≥5 cm	74 (35.7)	24 (30.0)	98 (34.1)	
Microvascular invasion				
Absent	85 (41.1)	43 (53.8)	128 (44.6)	0.053
Present	122 (58.9)	37 (46.3)	159 (55.4)	
Major portal vein invasion				
Absent	196 (94.7)	78 (97.5)	274 (95.5)	0.527
Present	11 (5.3)	2 (2.5)	13 (4.5)	
Intrahepatic meta				
Absent	151 (72.9)	68 (85.0)	219 (76.3)	0.031
Present	56 (27.1)	12 (15.0)	68 (23.7)	
Multicentric occurrence				
Absent	195 (94.2)	73 (91.3)	268 (93.4)	0.367
Present	12 (5.8)	7 (8.8)	19 (6.6)	
AJCC T stage				
1	80 (38.6)	40 (50.0)	120 (41.8)	0.356
2	86 (41.5)	31 (38.8)	117 (40.8)	
3a	25 (12.1)	6 (7.5)	31 (10.8)	
3b	11 (5.3)	2 (2.5)	13 (4.5)	
4	5 (2.4)	1 (1.3)	6 (2.1)	
α-fetoprotein				
<400	126 (62.4)	64 (86.5)	190 (69.8)	<0.001
≥400	76 (37.6)	10 (13.5)	86 (31.2)	

Statistical analysis. Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA). The χ^2 test, Fisher's exact test, or ANOVA was used for comparison among groups. The RFS and HCC-specific overall survival (OS) were estimated by the Kaplan-Meier product limit method. The log-rank test was applied to compare survival between two groups. Univariate and multivariate analyses were based on the Cox proportional hazards regression model. Correlations between c-MET immunohistochemistry and its copy number were calculated using Spearman's correlation, whereas c-MET immunohistochemistry and its mRNA expression were calculated using Pearson's correlation. A *p*-value of less than 0.05 was regarded as statistically significant.

Results

Patients' characteristics. A total of 287 HCC specimens were included in this analysis. Patients were predominantly male (82.6%), and hepatitis B virus (HBV) infection (75.6%) was the major cause of tumor. HCCs were classified as Child-Pugh A (99.7%), Edmondson grade II (81.5%), and with high α-fetoprotein level (31.2%) (Table I). At the time of analysis, 189 patients (65.9%) had experienced HCC recurrence.

c-MET protein expression. Immunoreactivity for c-MET was observed in the cytoplasm of tumor cells with and without membranous expression. c-MET staining was score 0 for 116 (40.4%) patients, 1 for 91 (31.7%), 2 for 33 (11.5%) and 3 for 47 (16.4%) (Figure 1). Low c-MET protein expression (c-MET^{low}) was defined as immunoreactivity score of 0 or 1 and high c-MET expression (c-MET^{high}) as score of 2 or 3. We regarded c-MET expression as positive when the tumor exhibited high c-MET expression. Patients with c-MET^{high} tended to be older (median age, 52 *vs*. 57 years; p=0.001), with less intra-hepatic metastases (27.1% *vs*. 15.0%, p=0.031) and less increase in α-fetoprotein levels (37.6% *vs*. 13.5%; p<0.001) (Table I).

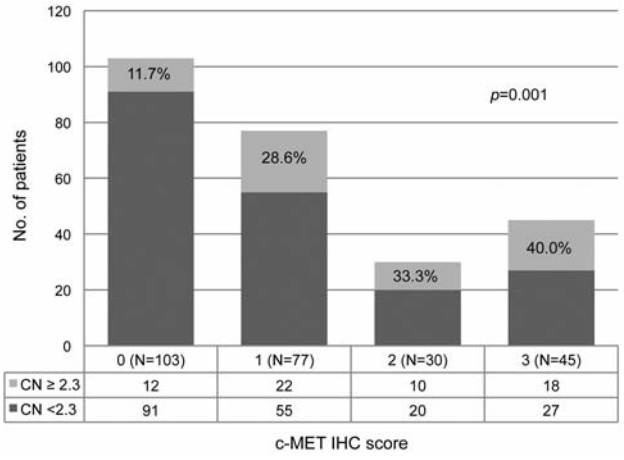
CNV and mRNA expression. Among 287 patients, 255 were evaluated for c-MET CNV. 62 patients (24.3%) had copy number gain (CNG) (Table II). Among patients with c-MET scoring 0, only 11.7% had CNG, whereas 40.0% of patients with c-MET scoring 3 had CNG. c-MET CNV was weakly-correlated to c-MET immunoreactivity (Spearman correlation coefficient, r=0.257, p<0.001).

A total of 237 patients were evaluated for *MET* mRNA expression. The mean mRNA expression (±standard deviation) was 8.33±0.72 in the c-MET^{low} group and 8.61±0.82 in the c-MET^{high} group (p=0.016). In Pearson's correlation, *MET* mRNA expression revealed a very weak but significantly positive correlation with c-MET immunoreactivity (r=0.199, p=0.002). Correlation between c-MET CNV and mRNA expression was moderate, with statistical significance (N=207; r=0.458, p<0.001)

Survival analysis. The median RFS across the whole population was 23.0 months [95% confidence interval (CI): 16.7-29.3 months]. There was no clear relationship between c-MET expression and median RFS (c-MET^{low} *vs*. c-MET^{high}, 19.3 *vs*. 34.7 months; p=0.490). Moreover, the median OS was not significantly different between the c-MET^{low} and c-MET^{high} groups (p=0.288) (Figure 2).

In multivariate analysis, only intrahepatic metastasis had an impact on HCC-specific OS (hazard ratio (HR)=3.608, 95% CI: 1.833-7.103; p<0.001). High Edmondson grade (HR=1.291, 95% CI: 1.009-1.651; p=0.042) and the presence of intrahepatic metastasis (HR=4.076, 95% CI:

Table II. *Correlation between MET immunohistochemistry and MET copy number.*



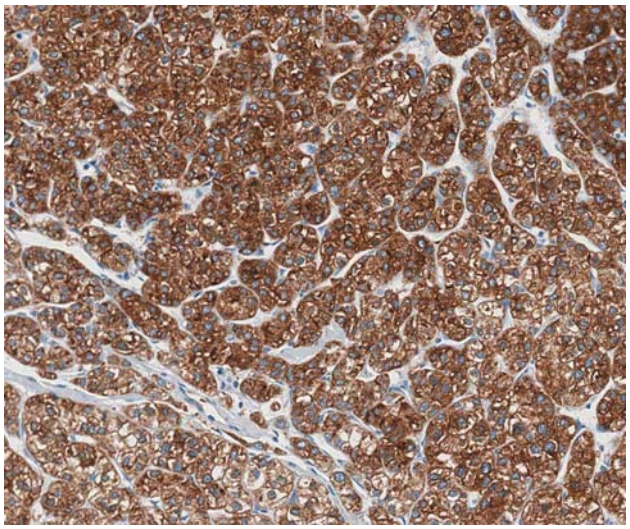


Figure 1. *Immunostaining of c-N-Methyl-N'-nitro-N-nitroso-guanidine HOS transforming gene (c-MET) showing cytoplasmic expression in hepatocellular carcinoma (×200).*

2.399-6.924; $p<0.001$) adversely affected RFS. c-MET expression did not significantly affect HCC-specific OS or RFS (Table III).

Further survival analysis based on c-MET copy number was performed, and c-MET copy number had no effect on RFS or OS, similar to those for c-MET protein expression (Figure 3).

Discussion

In this study, c-MET^{high} expression was found in 27.9% of patients with HCC, c-MET CNV was weakly-correlated with c-MET expression. Patients with c-MET overexpression tended to be older with less intra-hepatic metastases and less increase in α-fetoprotein levels, and c-MET overexpression had no influence on recurrence in and survival of patients who had undergone surgical resection for HCC.

The HGF/c-MET pathway promotes cell proliferation, inhibits apoptosis of tumor cells, stimulates cell motility and affects morphogenesis (17, 27). c-MET has been shown to be overexpressed in neoplastic tissues, and the extent of expression has correlated with disease extension and outcome in several tumor types (28, 29). c-MET overexpression was reported to be related to advanced disease stage and poor outcome in non-small cell lung carcinoma and breast and colon cancers (29-32). The investigation of c-MET expression in HCC showed that multiple nodular tumors or those with a high proliferative index had higher c-MET expression (33-35). There have been controversial results for other characteristics such as tumor size, level of differentiation, stage and invasion (20, 33-37). In terms of survival analysis, a few studies showed that high c-MET expression was related to shorter OS in patients after curative resection for HCC (35, 37, 38). Ke *et al*. (35) evaluated the role of overexpression of CD151 and c-MET in prognosis of HCC and reported that c-MET expression was associated with tumor size, number, differentiation, vascular invasion and TNM stage. Although c-MET expression had no impact on recurrence, it did affect overall survival (HR=0.758, $p=0.013$). A recent study in Japan showed that c-MET^{high} expression in HCC was significantly correlated with pathological vascular invasion and shorter RFS (39). The phase II trial of tivantinib in patients with advanced HCC reported that patients with c-MET^{high} expression had significantly shorter survival than those in the c-MET^{low} subgroup (median 3.8 *vs*. 9.0 months; $p=0.02$). Our finding that c-MET expression was correlated with some good prognostic factors, such as lower α-fetoprotein levels and less intrahepatic metastases, and c-MET expression did not impact on recurrence or survival, might be due to different patient characteristics, such as the proportion of HBV infection, and differences in ethnicity and clinical settings. In addition, our study patients underwent hepatectomy, but the tivantinib study included only patients with advanced disease.

c-MET CNG has been reported in stomach, colorectal and lung cancer, and *c-MET* copy number has been correlated with c-MET protein expression (40-43). In our study, *c-MET* CNG was more frequently observed in patients with c-MET overexpression [N=34 (18.9%) in c-MET^{low} and N=28 (37.3%) in c-MET^{high} groups]. However, among 75 c-MET^{high} patients, only 28 had a high copy number, with the other 47 having a neutral copy number, suggesting that mechanisms in addition to CNG, such as autocrine or paracrine HGF, ligand-independent interactions with other receptors, and regulation of epigenetic expression, may play

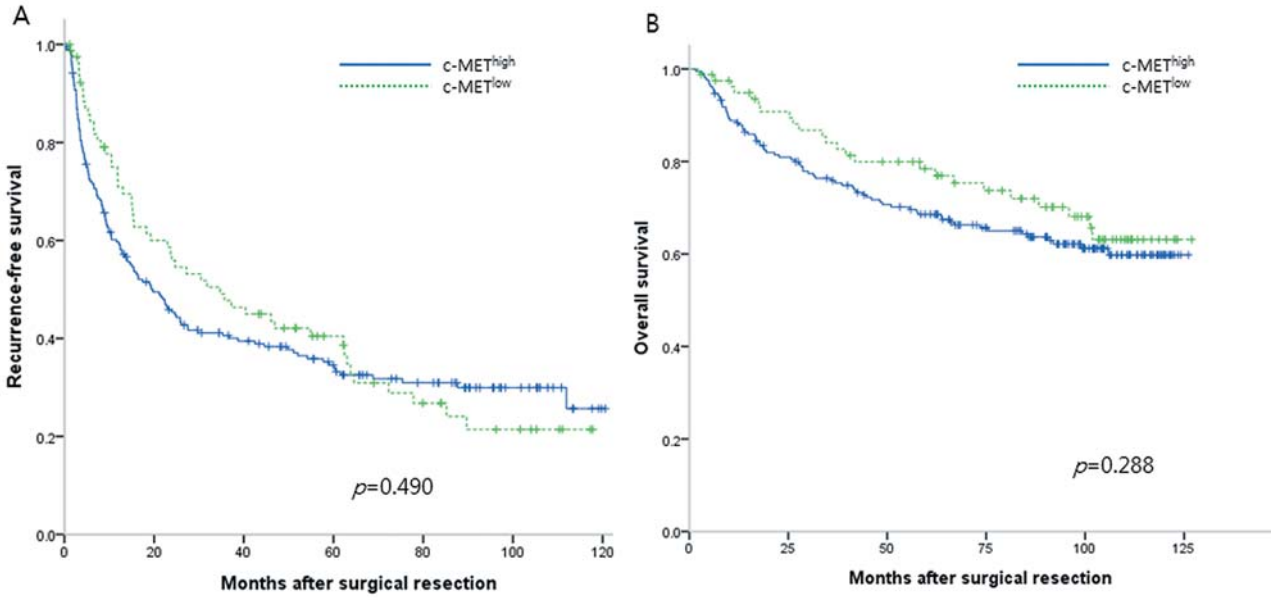


Figure 2. *Survival curves according to the level of c-MET expression: A: Recurrence-free survival and B: HCC-specific overall survival.*

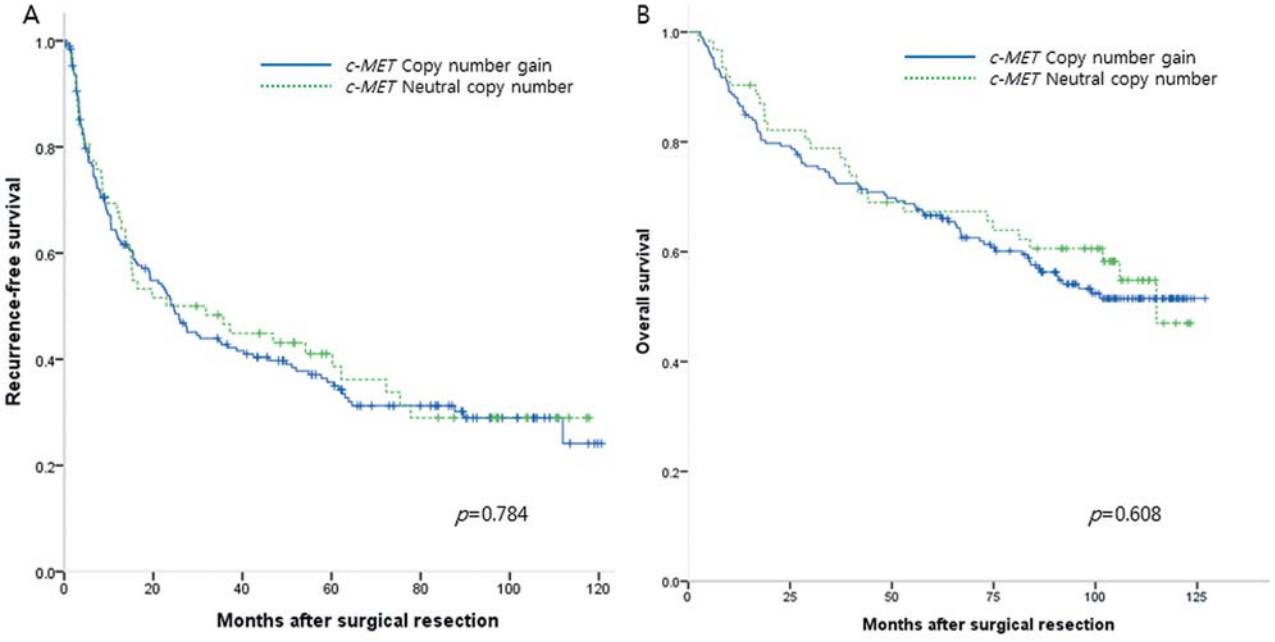


Figure 3. *Survival curves according to the level of c-MET copy number variation: A: Recurrence-free survival and B: HCC-specific overall survival.*

an important role in c-MET expression. A recent Japanese study of Kondo *et al*. demonstrated only one case of c-MET gene amplification among 44 HCC specimens by fluorescence *in situ* hybridization (39). This suggests that c-MET protein overexpression in HCC may occur by a different mechanism or process from that in gastric and lung cancer.

You *et al*. demonstrated that the c-MET inhibitor suppressed cell proliferation and induced apoptosis of c-MET-positive HCC cells, with no effect on c-MET-negative cells, and significantly inhibited the growth of c-MET-positive HCC tumors in a xenograft model (44). These *in vitro* and *in vivo* data combined with recent clinical result

Table III. *Univariate and multivariate Cox regression analyses for OS and RFS.*

Parameter	OS HR (95% CI)	*p*-Value	RFS HR (95% CI)	*p*-Value
Univariate				
Gender: Male/female	1.192 (0.707-2.011)	0.510	1.034 (0.716-1.494)	0.857
cMET: c-METhigh/c-METlow	0.883 (0.702-1.111)	0.290	0.946 (0.807-1.108)	0.490
Edmondson grade 3/1-2	1.642 (1.236-2.181)	0.001	1.488 (1.185-1.870)	0.001
HBV/*etc*.	1.264 (0.774-2.065)	0.350	1.495 (1.035-2.158)	0.032
Child-Pugh class: B/A	27.996(3.584-218.697)	0.001	8.883 (1.211-65.141)	0.032
α-fetoprotein: ≥400/<400	1.557 (1.029-2.358)	0.036	1.557 (1.029-2.358)	0.036
Size: ≥5 cm/<5 cm	2.935 (1.976-4.361)	<0.001	2.935 (1.976-4.361)	<0.001
Microvascular invasion: (+)/(–)	3.029 (1.934-4.743)	<0.001	3.029 (1.934-4.743)	<0.001
Major portal vein invasion: (+)/(–)	5.511 (2.846-10.675)	<0.001	5.511 (2.846-10.675)	<0.001
Intra-hepatic metastasis: (+)/(–)	5.554 (3.712-8.310)	<0.001	5.554 (3.712-8.310)	<0.001
AJCC stage 3-4/1-2	2.299 (1.867-2.831)	<0.001	2.299 (1.867-2.831)	<0.001
Multivariate				
cMET: c-MET^{high}/c-MET^{low}	1.099 (0.855-1.412)	0.461	1.095 (0.923-1.299)	0.299
Edmondson grade 3/1-2	1.279 (0.939-1.741)	0.119	1.291 (1.009-1.651)	0.042
Child-Pugh class: B/A	7.902 (0.901-69.321)	0.062	3.085 (0.506-32.975)	0.187
α-fetoprotein: ≥400/<400	0.853 (0.529-1.375)	0.514	1.1422 (0.813-1.605)	0.443
size: ≥5 cm/<5 cm	1.504 (0.850-2.663)	0.161	0.926 (0.601-1.427)	0.728
Microvascular invasion: (+)/(–)	1.240 (0.699-2.201)	0.463	1.151 (0.790-1.675)	0.464
Major portal vein invasion: (+)/(–)	1.196 (0.553-2.588)	0.649	0.940 (0.468-1.888)	0.863
Intra-hepatic metastasis: (+)/(–)	3.608 (1.833-7.103)	<0.001	4.076 (2.399-6.924)	<0.001
AJCC stage 3-4/1-2	1.099 (0.855-1.412)	0.461	1.077 (0.768-1.510)	0.667

of c-MET inhibitor (21) demonstrated c-MET to be a potential target of personalized treatment for HCC. However, which biomarker for the use of c-MET inhibitor is better, is unclear. There is insufficient evidence regarding the prognostic value of *c-MET* mRNA expression and gene amplification in HCC and this has never been investigated in a clinical trial of c-MET inhibitor. c-MET protein expression was evaluated in the tivantinib study, but the optimal method for evaluating c-MET protein expression remains controversial and needs further studies as have been camed out for Human epidermal growth factor receptor-2 (HER2) evaluation in breast cancer (45).

In conclusion, the present study demonstrated that c-MET overexpression was observed in 27.9% of patients with HCC. Although c-MET overexpression was not predictive of survival, this subset of patients may benefit from c-MET inhibitor treatment.

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