ORIGINAL ARTICLE

Clinical Implications of High *MET* Gene Dosage in Non-Small Cell Lung Cancer Patients without Previous Tyrosine Kinase Inhibitor Treatment

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Introduction: Recently, two studies revealed that *MET* amplification was associated with secondary epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) resistance in non-small cell lung cancer (NSCLC) patients. But it remains uncertain whether *MET* amplification could be related to primary TKI resistance in NSCLC because of limited data.

Materials and Methods: *MET* gene dosage of the tumor tissues from 208 NSCLC patients was investigated by real time quantitative polymerase chain reaction and compared with molecular and clinical features, including *EGFR* mutations, *KRAS* mutations, *EGFR* gene copy numbers, and patient survivals. Three copies were used as the cutoff. Among them, 25 patients were also evaluable for EGFR TKI responsiveness.

Results: The proportion of high *MET* gene dosage was 10.58% (22/208) with higher incidence in squamous cell carcinoma (11.86%) and smokers (16.18%), although the differences with adenocarcinoma and nonsmokers were nonsignificant. Coexisting *EGFR* mutations were identified, and the incidence (8.54%) was similar to wild type (12.0%). High *MET* gene dosage was significantly associated with higher tumor stage (stage I + II versus stage III + IV; $p = 0.0254$) and prior chemotherapy for stage III + IV adenocarcinoma patients (35.71% versus 7.41%; $p = 0.0145$) but not correlated with primary TKI resistance. Among the 155 surgically resectable patients (stage I to IIIA), high *MET* gene dosage was significantly associated with shorter median survival (21.0 months versus 47.1 months; $p = 0.042$) by univariate analysis.

Conclusions: High *MET* gene dosage was not related to primary TKI resistance and the incidence was increased after chemotherapy, suggesting high *MET* gene dosage may also be related to chemotherapy resistance.

Key Words: *MET* amplification, *EGFR*, NSCLC, Tyrosine kinase inhibitor, Chemotherapy, Gene dosage.

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M ET gene (met proto-oncogene) was originally identified as a cellular counterpart of the chemically induced oncogene *tpr-met* isolated from a human osteosarcoma cell line.[1] The *MET* gene encodes c-Met protein, which is the receptor with highest affinity to hepatocyte growth factor (HGF).[2] HGF binding augments the intrinsic tyrosine kinase activity of c-Met, resulting in autophosphorylation of several tyrosine residues within the intracellular region.[3] Signaling via the c-Met–HGF/SF pathway has been shown to lead to an array of cellular responses including proliferation (mitosis), scattering (motility), and branching morphogenesis.[4] Moreover, the abrogation of c-Met signaling by RNA interference could lead to regression of the tumor and metastases.[5] c-Met overexpression had also been reported to be associated with poor clinical outcome in various cancers, including non-small cell lung cancers (NSCLC).[6–8] The above results are supportive for c-Met protein as an important human oncoprotein. *MET* gene also plays a central role in hereditary papillary renal carcinoma, which is causally related to gain-of-function germ line mutations in the *MET* gene tyrosine kinase domain.[9] Gain-of-function *MET* gene mutations at juxtamembranous domain, but not in the tyrosine kinase domain, have also been found in both small cell lung cancers (4/32, 12%),[10] and NSCLCs (0–13%).[11,12] But their clinical significance remained uncertain. In addition to mutations, several reports have shown genomic amplification of *MET* occur in 5 to 10% of gastric cancers and 4% of esophageal cancer.[13–15] As for NSCLC, before 2007, there was only one report about *MET* gene amplification (by single-nucleotide polymorphism array analysis) and the incidence was very low (1 in 70 NSCLC

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patients and none in 30 lung cancer cell lines).[16] In year 2007, two important studies revealed that *MET* gene amplification were associated with secondary tyrosine kinase inhibitors (TKI) resistance in NSCLC patients harboring epidermal growth factor receptor (*EGFR*) mutations and originally responsive to the EGFR TKI treatment.[17,18] *MET* gene analysis of tumor samples from multiple independent patient cohorts by Bean et al.[18] reported a significant difference in the proportion of *MET* gene amplification between the tumors from NSCLC patients with acquired resistance to TKI treatment (9/43, 21%) and tumors from TKI naïve NSCLC patients (2/62, 3%; $p = 0.007$). Thus, it would suggest c-Met protein could be a significant therapeutic target for NSCLC patients with secondary resistance to TKI but not for TKI-naive patients because of low incidence of *MET* gene amplification. Five recent studies[19–23] on TKI naive NSCLC patient cohorts also reported similar low incidence of *MET* gene amplification (1.4–11.1%) as Bean et al.[18] In contrast, one study from France revealed a much higher incidence of *MET* gene amplification (21%).[24]

Although *MET* gene amplification developed in patients with preexisting *EGFR* mutations after TKI treatment has been well recognized,[17,18,23] whether *MET* gene amplification could coexist with *EGFR* mutations in TKI naive patients was still controversial. Some studies revealed that all *MET* gene amplification did not coexist with *EGFR* mutations.[20,23,24] But Cappuzzo et al. and Kubo et al.[19,21,22] have reported presence of increased *MET* gene copy number together with *EGFR* mutations, suggesting that these two genetic aberrations are not mutually exclusive. It is also uncertain whether increased *MET* gene copy number could be related to primary TKI resistance in patient with NSCLC, which was mainly because of small patient number with increased *MET* gene copy number in each study. Kubo et al.[21] reported one patient with *MET* gene amplification and L858R mutation and was TKI resistant. Cappuzzo et al.[19] found four patients with high *MET* copy numbers ($\geqq 2.91$ copies) were still TKI responders. Among the four patients, one patient also had *EGFR* mutation.

Because there were no standard criteria or definition for *MET* gene amplification, it might be one reason why there were great variations of the incidence of *MET* gene amplification in the published studies and their clinical features. Among the eight published *MET* gene copy number studies in NSCLCs, six used quantitative real-time polymerase chain reaction (Q-PCR) to determine the *MET* gene copy number. Because the definition of gene amplification should be gene to the centromere ratio of at least two, it is probably more appropriate to use the term "high *MET* gene dosage" instead of "*MET* gene amplification" for studies using Q-PCR method, because Q-PCR could not determine the *MET* gene and centromere signal at the same time. In the gefitinib-resistant lung cancer cell lines with high *MET* gene amplification (HCC827 GR6 and H820), they usually have very high copy numbers (six-nine copies per nuclei).[17–19] However, such high copy number was rare in human NSCLC specimens and had not been used as the standard cutoff. Among the six *MET* gene studies using Q-PCR to determine the *MET* gene

copy number (gene dosage), two use the mean copy number + two standard deviation of all specimens as the cutoff, but the exact copy number data, including the cutoff were not shown.[23,24] Bean et al.[18] used three copies as the cutoff. Onozata et al.[20] used two copies as the cutoff because majority of tumors were all smaller than two copies. Kubo et al.[21] used five copies as the cutoff. The two reports from Cappuzzo et al.[19,22] used fluorescence in situ hybridization (FISH) to count the *MET* gene copy number of the tumor cells directly. They also use the mean copy numbers ($\geqq 2.91$ copies) as the cutoff in their first *MET* gene copy number study in NSCLC.[19] In their second study on 447 tissue array samples, they have analyzed various cutoff from two copies to six copies and used $\geqq$ five copies as the cutoff for survival analysis.[22]

To clarify the clinical implications of high *MET* gene dosage in NSCLC, we have performed *MET* gene dosage study by Q-PCR in 208 NSCLC patients without prior TKI treatment. We decided to use three copies as the cutoff for high *MET* gene dosage, because the cutoff in most of the published studies used, either by Q-PCR or FISH, were all below or equal to three copies.[18–20,23,24] In addition, because the tumor tissue always had small amount of normal stromal cells (two copies) component, the actual tumor copy number should be higher than the data obtained by Q-PCR using whole tissue DNA. Thus, tumors had *MET* gene dosage $\geqq 3$ copies by Q-PCR should have more than three copies per nucleus. Among the 208 patients in this study, TKI treatment responses were evaluable in 25 adenocarcinoma (ADC) patients. *KRAS* mutation, *EGFR* mutation, and *EGFR* gene copy number data were also available in most of these patients.

MATERIALS AND METHODS

Patients and Tissues

Patients Received Surgical Resection

Fresh frozen tumor specimens obtained soon after resection and stored at $-80°C$ from NSCLC patients receiving surgical resection and with signed informed consent at Chang-Gung Memorial Hospital from January 1996 to December 1998 and from May 2002 to May 2004 were obtained from the tissue bank of Chang-Gung Memorial Hospital for this study. The patients from May 2002 to May 2004 were the same patient group of our first *EGFR* mutation study.[25] The specimens from 1996 to 1998 were a retrospective application for the earliest fresh frozen NSCLC tumor tissue in the tissue bank, mainly for comparison of the survival differences of NSCLC patients of earlier years. The surgical specimens in the tissue bank from January 1999 to April 2002 were not applied for study because of limitation of research funds.

Patient with Advanced Stage Disease (Pathology Stage IIIB-IV)

Fresh frozen tumor specimens of 33 stage IIIb and stage IV lung ADC patients of a clinical trial testing gefitinib responsiveness in *EGFR*-mutated NSCLC patients were obtained by biopsy (one piece for pathology diagnosis and one piece for molecular study) with signed informed consent.

Most of these tissues were acquired from computed tomography (CT)-guided core needle biopsy or from wedge resection of lung at the time of diagnosis. Four samples were from craniotomy for brain metastasis, one was collected from pleural effusion, and one from neck soft tissue biopsy. None of these patients had received TKI treatment before the biopsy. Fourteen of these 33 patients had prior chemotherapy before biopsy.

Totally, 175 tumor specimens from surgical resection and 33 from biopsy were included in this study. All these 208 patients had no TKI treatment before the operation or biopsy. All clinicopathological information was obtained from the medical records. The therapeutic protocols of all patients were reviewed thoroughly for the analyses of patient survival. The stage listed was the pathology stage according to American Joint Committee on Cancer (AJCC) guidelines, 6th edition. Smoking status was defined as nonsmokers (never smoke or <100 lifetime cigarettes) and ever smokers. The death records were from the medical records or from Department of Health of Taiwan. For patient who received surgery, the overall survival (OS) was counted from the date of surgery until death. For patients received EGFR TKI treatment, the progression-free survival and OS were counted from the first day of TKI treatment. The study protocol had been reviewed and approved by the institutional review board of Chang-Gung Memorial Hospital and National Health Research Institutes.

Definition of Responsiveness to TKI Treatment

Among the 33 patients whose tumor specimens were obtained by biopsy, 25 of the 33 patients received TKI monotherapy treatment soon after the biopsy was done, and TKI treatment response was evaluated. For patients having prior antitumor treatment, they should have fully recovered from toxic effects of previous antitumor therapy, no chemotherapy within 1 month before the TKI treatment, and with adequate liver and renal function. For evaluation of the TKI treatment response of the patients, plain chest x-ray was taken every week for 4 weeks and at least monthly thereafter. CT scan was performed at base line, day 14, day 56, and every 3 months thereafter. Response was evaluated and classified as complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD), respectively, according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria. The response patterns were determined on day 56.

Quantitative Real-Time PCR (Q-PCR) for Evaluation of MET Gene Dosage

Frozen sections stained with hematoxylin and eosin was performed on all of the frozen tumor specimens first to check for the tumor percentage. If the tumor percentage was less than 60%, microdissection would be performed. Thus, we could be sure that all tumor tissue for DNA extraction had sufficient tumor component. Majority of tumor samples had a tumor percentage >80% in this study. DNA extraction from fresh frozen tumor tissue was then performed according to the protocol published previously.[25] Relative gene dosage of *MET* gene were determined by Q-PCR. *MTHFR* (5,10-meth-ylenetetrahydrofolate reductase, located at 1p36.3) was used as endogenous control gene as reported previously.[18] Expression were evaluated using the following primers:

MET-sense: 5′-CCATCCAGTGTCTCCAGAAGTG-3′;
MET-antisense: 5′-TTCCCAGTGATAACCAGTGTGTAG-3′;
MTHFR-sense: 5′-CCATCTTCCTGCTGCTGTAACTG-3′;
MTHFR-antisense: 5′-GCCTTCTCTGCCAACTGTCC-3′.

For this study, 20 ng of genomic DNA was amplified for 45 cycles (10 seconds at 95°C, 5 seconds at 65°C, and 5 seconds at 72°C) in a LightCycler 1.5 instrument (Roche Diagnostics GmbH, Mannheim, Germany), using the Light-Cycler FastStart DNA MasterPlus SYBR Green I kit (Roche Diagnostics) and 250 μM of primers. Each sample was performed in triplicate. The CT values were analyzed by LightCycler analysis software 4.05 (Roche Diagnostics). The DNA sample from peripheral blood of a healthy 24-year-old male volunteer (Taiwanese) was used as the reference sample (two copies). A549 lung cancer cell line had 1.5-fold increases (three copies) of *MET* gene dosage and was used as the positive control. This gene dosage (three copies per nucleus) of A549 cell line was confirmed by FISH using the Vysis LSI D7S522 (7q31)/CEP7 commercial probes (Abbott Molecular, Inc., Des Plaines, IL).

For all samples, triplicate cycle time (C_T) values were averaged and normalized to *MTHFR* using the reference DNA sample, and cutoff values for high *MET* gene dosage were determined by using positive controls. Fold changes were calculated using the equation $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = (C_T[MET]sample - C_T[MTHFR]sample) - (C_T[MET]reference\ DNA - C_T[MTHFR]reference\ DNA)$.[26] Gene dosage higher than 1.5-fold (three copies) was considered to have high *MET* gene dosage.[18]

MET Gene Copy Number Detection by FISH

The FISH detection was done on cultured A549 cell line on coating slides or 4-μm-thick paraffin-embedded formalin-fixed tissue sections on coating slides. The *MET* probe used was the Vysis LSI D7S522 (7q31)/CEP7 commercial probes (Abbott Molecular, Inc.). The deparaffinized formalin-fixed tissue sections were first pretreated with 100 mM Tris, 50 mM EDTA, pH 7.0 solution in 92°C for 15 minutes, and then washed with phosphate-buffered saline (PBS). For slides with cultured fresh A549 cell line, no pretreatment was used. The slides were then digested with 300 μL of Digest-all (Zymed, Inc., South San Francisco, CA) at 37°C for 10 to 20 minutes depending on the size of the tissue sections. The digestion was stopped by 10% neutral formalin at room temperature for 1 minute and washed with PBS again. Ten microliter of *MET* probe was applied to each dehydrated and air-dried slide, and denatured at 94°C for 3 minutes, then hybridized overnight at 37°C in VYSIS HYBrite (Abbott Molecular, Inc.). Posthybridization washes were performed with 0.5× standard saline citrate at 72°C for 5 minutes and then rinsed in PBS with 0.25% Tween 20 (Sigma-Aldrich Co., St. Louis, MO). The slides were then mounted with 10 μl of VECTASHIELD mounting medium (Vector Laboratories, Inc., Burlingame, CA) with DAPI. The FISH study results were evaluated with Leica DMR fluorescence micro-

scope (Leica Microsystems Nussloch GmbH, Nussloch, Germany) and counted by two scientists (S.F.H., Y.T.C.). At least 100 nonoverlapping and intact tumor nuclei were evaluated. The FISH images were taken by SPOT2 image system (DIAGNOSTIC instruments, Inc., IL).

EGFR and KRAS Mutation Analyses

For *EGFR* mutation, PCR amplification of the coding sequences from exons 18 to 21 followed by direct sequencing were performed. For *KRAS* mutation, PCR amplification and direct sequencing of exons 2 and 3 were performed. The methodology of the above two studies were the same as published previously.[25,27] The data of the *EGFR* mutations and *KRAS* mutations of the 175 surgical specimens had been included in our previously published studies.[27,29]

EGFR Gene Copy Number Detection by Chromogenic In Situ Hybridization

Determination of *EGFR* gene copy number was performed by chromogenic in situ hybridization (CISH) on paraffin sections of the tumor tissue. The methodology was the same as mentioned previously.[28] The *EGFR* probe used was purchased from Zymed, Inc., South San Francisco, CA. The *EGFR* gene copy number by CISH was performed in 150 patients and successful in 140 patients. All the 140 specimens were from patients who received surgical resection. The gene copy number was evaluated on at least 100 nonoverlapping and intact tumor nuclei per specimen on a cell by cell level. Because we have found significant association between high *EGFR* gene copy number (≥5 copies) and *EGFR* mutation previously,[27] we have used the same cut line for *EGFR* gene amplification to compare with the *MET* gene dosage data. The *EGFR* gene copy number data of these 140 patients have been included in our published study.[27]

Statistical Analysis

To examine the differences in the major clinicopathological features and molecular markers associated with high *MET* gene dosage, the frequencies and proportions are compared by conventional χ^2 association test or Fisher's exact test (when there is at least a cell frequency less than 5). A two-sided *p* value less than 0.05 was considered statistically significant. The differences in survivals were checked by logrank tests. Multivariate analysis of OS was performed using Cox's regression model.

RESULTS

Clinical Characteristics Associated with High MET Gene Dosage

The 208 NSCLC patients include 139 ADCs, 59 squamous cell carcinomas, and 10 other types of NSCLCs. High *MET* gene dosage were identified in 22 patients (10.58%). Only 4 of the 22 patients had more than 4 copies of *MET* gene. The major clinicopathological features and the molecular markers associated with high *MET* gene dosage were shown in Table 1. The proportion of high *MET* gene dosage in squamous cell carcinoma was 11.86% (7 of 59), which was similar to ADC (8.63%, 12 of 139). Only higher tumor stage

TABLE 1. Clinical Characteristics of *MET* Gene Copy Number Analysis in 208 NSCLC Patients

Clinical Characteristics	MET Gene Copy Number ≧3	MET Gene Copy Number <3	p
Total no. of patients (%)	22 (10.58)	186 (89.42)	
Gender (%)			
Male	14 (10.94)	114 (89.06)	0.8310
Female	8 (10.00)	72 (90.00)	
Median age (range), (yr)	65 (50–79)	63 (29–84)	
<65 yr/0 (%)	11 (10.58)	93 (89.42)	1.0000
≥65 yr/0 (%)	11 (10.58)	93 (89.42)	
Smoking history[a] (%)			
Never	10 (8.20)	112 (91.80)	0.0935
Former	11 (16.18)	57 (83.82)	
Histology type (%)			
Adenocarcinoma	12 (8.63)	127 (91.37)	0.4813
Squamous cell carcinoma	7 (11.86)	52 (88.14)	
Other	3 (30.00)	7 (70.00)	
Stage (%)			
I + II	7 (6.19)	106 (93.81)	0.0254
III + IV	15 (15.79)	80 (84.21)	
EGFR polysomy[b] (%)			
≥5	7 (10.94)	57 (89.06)	0.5381
<5	6 (7.89)	70 (92.11)	
EGFR mutation[c] (%)			
Mutant	7 (8.54)	75 (91.46)	0.4302
Wild type	15 (12.00)	110 (88.00)	
KRAS mutation[d] (%)			
Mutant	0 (0.00)	6 (100.00)	1.0000
Wild type	21 (10.5)	179 (89.5)	

[a] Eight patients without smoking history are not included.
[b] Sixty-eight patients without EGFR copy number data are not included.
[c] One patient without EGFR mutation data is not included.
[d] Two patient without KRAS mutation data are not included.
NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor.

(stage I + II versus stage III + IV) was significantly associated with high *MET* gene dosage (*p* = 0.0254). Because 14 ADC patients (stage IIIB or IV) in this series had received chemotherapy before the biopsy, we further analyzed the clinical characteristics and high *MET* gene dosage status in the 68 stage III and IV ADC patients (Table 2). The proportion of high *MET* gene dosage was significantly higher in patients with prior chemotherapy (5/14, 35.71%) than patients without (4/54, 7.41%; *p* = 0.0145). Among the six patients (five had prior chemotherapy) with specimens obtained from metastatic lesions (four from brain, one from neck, and one from pleural effusion), two had high *MET* gene dosage (2/6, 33%). These two patients had received prior chemotherapy.

Correlation of the MET Gene Dosage Results Between Q-PCR and FISH

MET FISH performed on A549 cell line demonstrated trisomy of both *MET* gene and chromosome 7 centromere signals (Figure 1*A*). *MET* FISH were also performed on 10 patients with MET gene dosage higher than 1.5-folds and 5 patients with *MET* gene dosage lower than 1.5-folds. Totally,

TABLE 2. *MET* Gene Copy Number Analysis in 68 Stage III–IV Adenocarcinoma Patients

Clinical Characteristics	*MET* Gene Copy Number ≧3	*MET* Gene Copy Number <3	p^a
Total no. of patients (%)	9 (13.24)	59 (86.76)	
Gender (%)			
Male	2 (6.06)	31 (93.94)	0.1515
Female	7 (20.00)	28 (80.00)	
Median age (range) (yr)	62 (50–79)	62 (39–82)	
<65 yr/0 (%)	6 (17.14)	29 (82.86)	0.4783
≧65 yr/0 (%)	3 (9.09)	30 (90.91)	
Smoking history (%)			
Never	7 (15.56)	38 (84.44)	1.0000
Former	2 (11.11)	16 (88.89)	
Not available	0 (0)	5 (100)	
Prior chemotherapy (%)			
Yes	5 (35.71)	9 (64.29)	0.0145
No	4 (7.41)	50 (92.59)	
EGFR mutation (%)			
Mutant	5 (11.63)	38 (88.37)	0.7155
Wild type	4 (16.00)	21 (84.00)	

^{a} *p* value from Fisher's exact test.
EGFR, epidermal growth factor receptor.

11 cases were successful in FISH (7 had gene dosages ≥1.5 folds and 3 had gene dosage lower than 1.5-folds) and 4 cases failed. All tumors with high gene dosage had more than three copies of *MET* gene per nucleus in more than 40% of tumor cells (Figure 1*B, C*). The tumors with *MET* gene dosage lower than 1.5-fold had two to three MET copies per nucleus (Figure 1*D, E*). The above results were consistent with our Q-PCR results. For the 33 patients with only biopsy specimens (obtained for clinical trial), only 5 of them had available paraffin sections. These biopsy specimens were quite small, easily lost during digestion and denature process. Thus, the FISH studies were not successful in these biopsy specimens, which included the two patients with highest gene dosages (increase for 8.805- and 2.960-folds).

EGFR and *KRAS* Mutations with High *MET* Gene Dosage

All except one of the 208 patients also had *EGFR* mutation data. The overall *EGFR* mutation rate was 39.6% (82/207) and the mutation rate in ADC was 56.5% (78/138). Seven of the 82 patients (7/82, 8.54%) with *EGFR* mutations also had high *MET* gene dosage. The association showed no significant difference with wild type *EGFR* (15/125, 12.0%; Table 1). *KRAS* mutation analysis was performed in 206 patients. Only six patients had *KRAS* mutations (2.9%), and none of them had high *MET* gene dosage (Table 1).

Increased *EGFR* Gene Copy Number and High *MET* Gene Dosage

For patients with or without high *EGFR* gene copy number (≧5 copies), the proportions of high *MET* gene dosage were similar and showed no significant differences (Table 1).

TKI Sensitivity and High *MET* Gene Dosage

Twenty-five patients received TKI monotherapy after the biopsy were evaluable for TKI responsiveness. All of them had ADC. There were 1 patient with CR, 16 patients with PR, 4 with stable disease, and 4 with PD. The status of *EGFR* mutation, high *MET* gene dosage and TKI response patterns are shown in Table 3. All three patients having both high *MET* gene dosage and *EGFR* mutations had PR after TKI treatment. The *MET* gene dosages of these three patients were 1.522-, 1.55-, and 1.6-folds, respectively (Figure 2). For the two patients with high *MET* gene dosage and no *EGFR* mutation, one had PD (the *MET* gene dosage was 1.765-folds), and one had PR (the *MET* gene dosage were 8.805-folds, equivalent to more than 17 copies). In total, there were five patients with high *MET* gene dosage, but only one had PD. Thus, high *MET* gene dosage did not show correlation with primary TKI resistance. *EGFR* mutations remained to be TKI response predictor, because 16 of the 21 patients with *EGFR* mutations had CR or PR, and only two patients had PD.

High *MET* Gene Dosage and Survival in Surgically Resectable (Stage I to IIIA) NSCLC Patients

We evaluated the high *MET* gene dosage and survival only for patients with surgically resectable disease (stage I to IIIA), because their therapeutic protocol were more uniform. Among the 175 patients receiving surgical treatment, 12 patients with stage IIIB and stage IV disease and 4 patients lost of follow-up were excluded. Totally 159 patients were eligible for survival analysis. Among them, two patients died of surgical complications and two patients died of other causes (written on the death records) were further excluded. Thus, totally 155 patients were included for the survival analysis. All except one patient were functional class 1 in performance status. The follow-up period ranged from 27 days to 158 months. None of these patients had received chemotherapy before operation. The survival data of these patients had been included in the survival analysis associated with *EGFR* mutation study previously.[29] Fifteen of the 155 patients (9.7%) had high *MET* gene dosage. The clinical and molecular characteristics of the 155 patients are shown in Table 4. The distribution patterns were similar to the 208 cases (Table 1), except that smoking status became significantly associated with high *MET* gene dosage ($p = 0.035$). This might be related to the higher percentage of squamous cell carcinoma (SCC) in these 155 patients, because SCC had higher incidence of high *MET* gene dosage (6/51, 11.8%) than ADC (5/93, 5.4%) and stronger association with smoking. But SCC alone had no significant difference with ADC in the proportion of high *MET* gene dosage ($p = 0.197$). The other types of NSCLCs, including adenosquamous carcinoma, sarcomatoid carcinoma, large cell carcinoma and lymphoepithelioma-like carcinoma, also had significantly higher proportion of high *MET* gene dosage (4/11, 36.4%) than SCC and ADC ($p = 0.008$), but the case number was too small for further analysis.
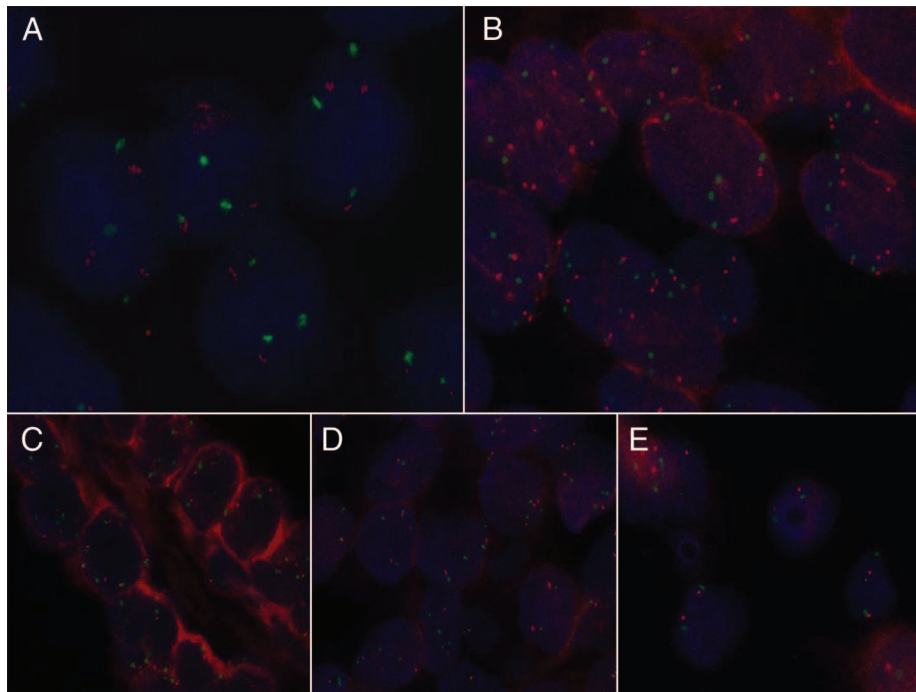
FIGURE 1. *MET* FISH study with the Vysis LSI D7S522 (7q31)/CEP7 commercial probes (Abbott Molecular, Inc., Des Plaines, IL). The *MET* gene had red signals and chromosome 7 centromere had green signals. *A*, A549 lung cancer cell line. The tumor nuclei had three signals of both *MET* gene and the centromere. *B*, This tumor was a squamous cell carcinoma with no *EGFR* mutation. The tumor nuclei had three to five signals of both *MET* gene and the centromere in more than 40% of tumor cells. The *MET* gene dosage of this tumor was 1.587-folds. *C*, This tumor was an adenocarcinoma (ADC) with *EGFR* mutation (del E746–A750). The tumor nuclei had four to seven signals of both *MET* gene and the centromere in more than 40% of tumor cells. The *MET* gene dosage of this tumor was 1.585-folds. *D*, This tumor was a squamous cell carcinoma with no *EGFR* mutation. The tumor nuclei had two to three signals of both *MET* gene and the centromere. The *MET* gene dosage of this tumor was 1.236-folds. *E*, This tumor was an adenocarcinoma with no *EGFR* mutation. The tumor nuclei had two signals of both *MET* gene and the centromere. The *MET* gene dosage of this tumor was 0.832-folds.

TABLE 3. High *MET* Gene Dosage and *EGFR* Mutation Status in TKI Treated 25 ADC Patients

Treatment Response	MET (+) EGFR (+)	MET (+) EGFR (−)	MET (−) EGFR (+)	MET (−) EGFR (−)
PR + CR (%)	3[a] (17.6)	1[b] (5.9)	13 (76.5)	0 (0.0)
SD (%)	0 (0.0)	0 (0.0)	3 (75.0)	1 (25.0)
PD (%)	0 (0.0)	1[c] (25.0)	2 (50.0)	1 (25.0)
Total number	3	2	18	2

[a] The *MET* gene dosage of these three patients were 1.522-, 1.550-, and 1.600-folds, respectively. The progression-free survival/overall survival were 6.87/15.87 mo, 11.93/22.07 mo, and 20.17/27.5 mo, respectively.

[b] The *MET* gene dosage of this patient was 8.805-folds. The progression-free survival was 2.47 mo, and overall survival was 6.0 mo.

[c] The *MET* gene dosage of this patient was 1.765-folds. The progression-free survival was 1.58 mo, and overall survival was 10.5 mo.

EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; ADC, adenocarcinoma; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease (PD), according to the sixth JUCC criteria.

FIGURE 2. *MET* gene dosages of the five adenocarcinoma (ADC) patients with higher than three copies together with normal and positive controls. Patients 1, 2, and 3 also had *EGFR* mutation. Each column represents the mean ± standard deviation for three independent experiments. The response to EGFR tyrosine kinase inhibitor (TKI) treatment and the gene dosage of each patient are shown on the top of each column. NC, normal control; PC, positive control (A549 cell line); PR, partial response; PD, progressive disease.

The survival analysis revealed a significant better survival for patient without high *MET* gene dosage by univariate analysis (Figure 3). The median survival for patients with and without high *MET* gene dosage were 21.0 and 47.1 months, respectively, ($p = 0.042$). However, by multivariate analysis, only patient age and disease stage were significantly associ-
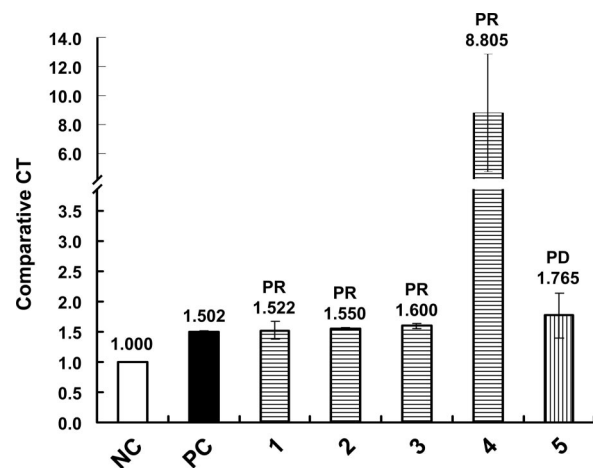
TABLE 4. *MET* Gene Copy Number Analysis in 155 Surgically Resectable NSCLC Patients

Clinical Characteristics	*MET* Gene Copy Number ≧3 (%)	*MET* Gene Copy Number <3 (%)	*p*
Total no. of patients	15 (9.7)	140 (90.3)	
Gender			
Male	13 (12.8)	89 (87.3)	0.089
Female	2 (3.8)	51 (96.2)	
Median age (range) (yr)			
<65 yr/0	6 (7.9)	70 (92.1)	0.589
≧65 yr/0	9 (11.4)	70 (88.6)	
Smoking history[a]			
Never	5 (5.5)	86 (94.5)	0.035
Former	9 (18.0)	41 (82.0)	
Histology type[b]			
Adenocarcinoma	5 (5.4)	88 (94.6)	0.008
Squamous cell carcinoma	6 (11.8)	45 (88.2)	
Other	4 (36.4)	7 (63.6)	
Stage			
I + II	7 (6.5)	101 (93.5)	0.072
IIIA	8 (17.0)	39 (83)	
EGFR polysomy[c]			
≧5	6 (12.0)	44 (88.0)	0.761
<5	6 (9.2)	59 (90.8)	
EGFR mutation[d]			
Mutant	3 (5.8)	49 (94.2)	0.389
Wild type	12 (11.8)	90 (88.2)	
KRAS mutation[e]			
Mutant	0 (0.0)	7 (100.0)	1.000
Wild type	15 (10.2)	132 (89.8)	

p value: all by Fisher's exact test.

[a] Fourteen patients had no smoking data were not included.

[b] If only compare the difference between adenocarcinoma and squamous cell carcinoma, *p* = 0.197.

[c] Forty patients without EGFR copy number data are not included.

[d] One patient without EGFR mutation data is not included.

[e] One patient without KRAS mutation data is not included.

NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor.

ated with survival (Table 5). This might be due to higher incidence of high *MET* gene dosage in stage IIIA patients, although the incidence of high *MET* gene dosage of stage I + II patients (7/108, 6.5%) versus stage IIIA patients (8/47, 17.0%) alone were still nonsignificant (*p* = 0.072). If we performed multivariate analysis by using *MET* gene copy number as a continuous variable, the results were quite similar. The patient age and disease stage remained the only two variables significantly associated with survival.

DISCUSSION

In this study, we investigated the *MET* gene dosage in 208 NSCLC patients and compared with various clinical features and molecular markers. The proportion of high *MET* gene dosage in this study was low (10.5%), which was similar to previous reports.[19–23] Coexisting *EGFR* mutations were observed, and the frequency (9.76%) was similar to wild type (11.2%), suggesting these two events occur independently. None of the patients with high *MET* gene dosage had *KRAS*
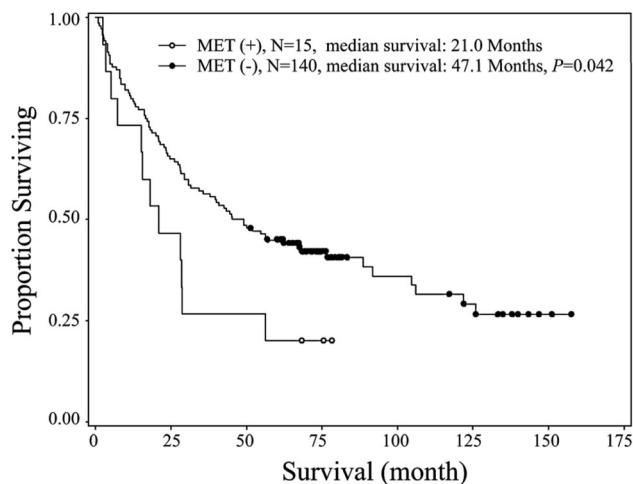
MET gene copies	Death/ Alive*	Median survival (95%CI)	HR (95% CI)	P**
≧3	12/ 3	21.0 months (15.2, 28.7)	1.86 (1.01, 3.43)	**0.042**
< 3	87/53	47.1 months (31.5, 76.7)	1.00	

*Number of patient, **by Log-rank test

FIGURE 3. Kaplan-Meier curves for overall survival (OS) of 155 NSCLC patients with and without high *MET* gene dosage, respectively. MET (+) for patient with *MET* gene copy number ≧3 and MET(−) for patients with *MET* gene copy number <3.

TABLE 5. Multivariate Survival Analysis of the 155 Surgically Resectable NSCLC Patients

Variables	HR (*p*)	95% CI
MET copy No. ≧3	1.84 (0.147)	0.81–4.19
vs. <3	1.00	
Gender = Male	0.79 (0.468)	0.41–1.50
vs. Female	1.00	
Age ≧65 yr	3.07 (<0.001)	1.80–5.25
vs. Age <65 yr	1.00	
Smokers	1.54 (0.190)	0.81–2.95
vs. Non smokers	1.00	
Pathology diagnosis		
Squamous cell carcinoma	0.63 (0.211)	0.30–1.30
Others	0.57 (0.406)	0.15–2.17
vs. Adenocarcinoma	1.00	
Stage = III	2.19 (0.009)	1.22–3.93
vs. I, II	1.00	
EGFR copy No. ≧5	1.06 (0.863)	0.55–2.02
vs. <5	1.00	
EGFR mutants	0.64 (0.250)	0.30–1.37
vs. wild type	1.00	
KRAS mutants	1.23 (0.689)	0.45–3.40
vs. wild type	1.00	

NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; HR, hazard ratio; CI, confidence interval.

mutations, which was also similar to previous reports.[19–23] There was no significant correlation between *EGFR* gene copy number changes and high *MET* gene dosage. The cutoff

of high *EGFR* gene copy number was defined as ≧5 copies in this study, which was different from the criteria (≧4 copies) used by most of the other reports on *EGFR* gene copy number study.

When compared with survival of the 155 surgically resectable patients, high *MET* gene dosage was significantly associated with shorter median survival (21.0 versus 47.1 months, $p = 0.042$) by univariate analysis (Figure 2), whereas the stage distribution between patients with and without high *MET* gene dosage was insignificant in this cohort (0.072). This result was similar to the recent study by Cappuzzo et al on a 447 patient cohort of western countries.[22]

We also analyzed the correlation between high *MET* gene dosage and TKI response in 25 patients without previous TKI treatment. Our data revealed that patient with high *MET* gene dosage could have either TKI response or TKI resistance (Table 3). In addition, when high *MET* gene dosage coexisted with *EGFR* mutations, all three patients in this series remained to be TKI responders. Thus, high *MET* gene dosage was not associated with primary resistance of TKI, especially when it was associated with *EGFR* mutations. It is intriguing why high *MET* gene dosage could be associated with secondary TKI resistance, but not for primary resistance. Because the mechanism of secondary TKI resistance caused by high *MET* gene dosage was due to phosphorylation of ERB3 and activation of PI-K3/AKT pathway.[17] One possible explanation for noncorrelation between high *MET* gene dosage and primary TKI resistance is that ErbB3 protein expression might be absent or very low in the TKI-naive NSCLCs. Thus, high *MET* gene dosage alone would not be able to provide survival advantage for these tumors. After long-term TKI treatment, both ErbB3 protein expression and high *MET* gene dosage might be gradually increased and resulted in secondary resistance.

When we analyzed the association between clinical characteristics and high *MET* gene dosage (Table 1), only tumor stage (stage I + II versus III + IV) was significantly associated with high *MET* gene dosage, which was quite consistent with the functional roles of *MET* gene, such as tumor cell migration and metastasis. Interestingly, when we further analyzed the high *MET* gene dosage in stage III and stage IV ADC patients, the proportion of high *MET* gene dosage was significantly higher in patients with prior chemotherapy (5/14, 35.71%) than patients without (4/54, 7.41%; $p = 0.0145$). This phenomenon has never been reported before. It could be because of tumor clone selection or adaptation to the toxic effect of chemotherapy. This could also explain why the clinical courses of NSCLC patients usually become more aggressive after development of chemotherapy resistance. Thus, to develop c-Met inhibitor is not only promising for treatment of secondary EGFR TKI resistance but might also useful for chemotherapy resistance.

ACKNOWLEDGMENTS

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