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Research Paper

Prognostic value of MET, cyclin D1 and *MET* gene copy number in non-small cell lung cancer

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Abstract

The aim of this study was to analyze the correlation of the expression of MET and cyclin D1 and *MET* gene copy number in non-small cell lung cancer (NSCLC) tissues and patient clinicopathologic characteristics and survival. Sixty-one NSCLC tissue specimens were included in the study. The expression of MET and cyclin D1 was evaluated by immunohistochemistry and *MET* gene copy number was assessed by quantitative real-time polymerase chain reaction (Q-PCR). Positive expression of MET and cyclin D1 protein and increased *MET* gene copy number occurred in 59.0%, 59.0% and 18.0% of 61 NSCLC tissues, respectively. MET-positivity correlated with poor differentiation ($P = 0.009$). Increased *MET* gene copy number was significantly associated with lymph node metastasis ($P = 0.004$) and advanced tumor stage ($P = 0.048$), while the expression of cyclin D1 was not associated with any clinicopathologic parameters. There was a significant correlation between the expression of MET and *MET* gene copy number ($P = 0.002$). Additionally, the expression of cyclin D1 had a significant association with the expression of MET as well as *MET* gene copy number ($P = 0.002$ and $P = 0.017$, respectively). MET-positivity and increased *MET* gene copy number were significantly associated with poor overall survival ($P = 0.003$ and $P < 0.001$, respectively) in univariate analysis. Multivariate Cox proportional hazard analysis confirmed that the expression of MET and *MET* gene copy number were prognostic indicators of NSCLC ($P = 0.003$ and $P = 0.001$, respectively). The overexpression of MET and the increased *MET* gene copy number might be adverse prognostic factors for NSCLC patients. The activation of the MET/cyclin D1 signaling pathway may contribute to carcinogenesis and the development of NSCLC, and may represent a target for therapy.

Keywords: MET, cyclin D1, *MET* gene copy number, prognosis, non-small cell lung cancer (NSCLC)

INTRODUCTION

Non-small cell lung cancer (NSCLC) accounts for 80-85% of primary lung cancer, which is one of the most important causes of cancer-related incidence and mortality worldwide[1]. Despite recent advances in lung cancer treatment by surgery, radiotherapy and chemotherapy, the prognosis of most patients with NSCLC is still poor. Thus, molecular targetted therapy based on a better understanding of the molecular mechanisms of NSCLC is urgently needed. Advances in the knowledge of the molecular mechanisms of

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NSCLC have highlighted several promising molecular targets, including the hepatocyte growth factor (HGF)/MET signaling pathway.

The *MET* gene is located on chromosome 7q21-31, and encodes a receptor tyrosine kinase for the HGF/scatter factor (SF). Binding of the HGF/SF to MET activates MET tyrosine kinase activity, which leads to the activation of a number of signaling pathways such as the phosphoinositide-3-kinase (PI3K), Ras-Rac/Rho, Ras mitogen-activated protein kinase (MAPK) and phospholipase C-γ signaling pathways in several types of human cancers, including NSCLC[2]. The constitutively activated HGF/MET signaling pathway results in tumor growth, angiogenesis and development of invasive phenotypes, making it an attractive target for potential anti-cancer treatment of NSCLC[3-6]. MET abnormalities in NSCLC include protein overexpression, gene mutation and gene amplification. Although MET is known to be overexpressed in tumor tissues relative to normal adjacent tissues, and its overexpression is associated with poor overall survival of NSCLC patients. The prognostic value of MET expression in NSCLC is still unclear[7-9]. *MET* gene copy number was found to occur in 1.1% to 21% of NSCLC patients by using different detection methods such as Q-PCR and fluorescent in situ hybridization (FISH). However, the clinicopathologic features and prognostic value of *MET* gene copy number remain controversial[10-14].

The aberrant expressions of cell cycle checkpoint proteins have been found to play a key role in NSCLCs due to their genetic or epigenetic alterations. Cyclin D1, a member of the G1 cyclin family, which reaches peak synthesis and activity in the G1 phase, is involved in the regulation of G1-to-S phase transition[15-16]. Cyclin D1 takes part in DNA repair by binding directly to RAD51, which drives the homologous recombination process[17]. Previous studies have demonstrated that sustained activation of MAPKs ERK1/2, downstream molecules of the HGF/MET signaling pathway, are required for enhancing the expression of cyclin D1 in the G1 phase in different types of cells[18-20]. Although the expression of cyclin D1 is evaluated in many human cancers including NSCLC and related to overall survival, the prognostic value of cyclinD1 in NSCLC is disputable[21-27]. Moreover, the combined effect of MET and cyclin D1 abnormalities on survival of NSCLC has never been reported and no conclusion has been reached.

In this study, we analyzed the expression of MET and cyclin D1 by immunohistochemistry, and the *MET* gene copy number by Q-PCR in NSCLC tissue specimens. The cut-off value for increased *MET* gene copy number was set at three copies, which is most frequently used in published studies either by Q-PCR or FISH[28-32]. Then, we investigated their association with patient clinicopathological parameters and survival.

SUBJECTS AND METHODS

Subjects

Sixty-one NSCLC patients who underwent tumor resection between 2004 and 2008 were recruited from the authors´ affiliated hospital. No patient received neoadjuvant chemotherapy or epidermal growth factor receptor (EGFR)-targeted therapy. Patients were followed closely until June 30, 2011, and the mean duration of follow-up was 29.6±14.7 months.

Tumor histological type and grade were assessed in accordance with the 1999 World Health Organization (WHO)´s histological classification standards for lung cancer. For squamous cell carcinoma, well-differentiated tumors had stratified pattern, various uniform or slight pleomorphic nuclei and significant keratinization, while poorly differentiated tumors showed solid growth and only had focal-stratified patterns and keratinization. For adenocarcinoma, well-differentiated tumors had a predominant lepidic pattern or predominant acinar pattern while poorly differentiated tumors had a solid growth pattern. Staging was based on the 2004 Tumor-Node-Metastasis (TNM) Guidelines of the National Comprehensive Cancer Network for NSCLC[33].

Clinicopathological data which included gender, age, smoking history, tumor histology and grade, pathological TNM designation, date and extent of surgery, and survival status were collected in this study and shown in ***Table 1***. The research protocol was approved by the institutional review board at the authors´ affiliated institution. Written informed consent was obtained from each patient.

Immunohistochemistry

Tissue specimens were fixed in neutral buffered formalin[10% v/v formalin in phosphate buffered saline, pH 7.4] and then embedded in paraffin. Tissue sections (4 μM) were prepared and mounted on positively-charged glass slides for immunohistochemical detection of MET and cyclin D1 protein. Rabbit anti-MET antibody and rabbit anti-cyclin D1 antibody (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used and a streptavidin-peroxidase kit was obtained from Golden Bridge International (Beijing, China). Protocols for immunohistochemistry were described previously[34]. An irrelevant rabbit antiserum served as a negative control. The sections were

Table 1 **Association of MET, cyclin D1 protein expression and *MET* GCN with clinicopathological data from NSCLC patients**

Variable	Total	MET protein expression			Cyclin D1 protein expression			*MET* GCN		
	(*n*)	- (%)	+ (%)	*P*	− (%)	+ (%)	*P*	< 3(%)	3 (%)	*P*
Total (*n*)	61	25(41.0)	36(59.0)		25(41.0)	36(59.0)		50(82.0)	11(18.0)	
Gender										
Male	47	21 (44.7)	26 (55.3)	0.444	20 (42.6)	27 (57.4)	0.883	38 (80.9)	9 (19.1)	0.984
Female	14	4 (28.6)	10 (71.4)		5 (35.7)	9 (64.3)		12 (85.7)	2 (14.3)	
Age (years)										
< 60	29	13 (44.8)	16 (55.2)	0.749	11 (37.9)	18 (62.1)	0.841	23 (79.3)	6 (20.7)	0.857
⩾ 60	32	12 (37.5)	20 (62.5)		14 (43.8)	18 (56.2)		27 (84.4)	5(15.6)	
Smoking history										
Yes	39	16 (41.0)	23 (59.0)	1.000	15 (38.5)	24 (61.5)	0.793	29 (74.4)	10 (25.6)	0.087
No	22	9 (39.1)	13 (60.9)		10 (45.5)	12 (54.5)		21 (95.5)	1 (4.5)	
Histology										
AC	28	11 (39.3)	17 (60.7)	1.000	10 (35.7)	18 (64.3)	0.610	24 (85.7)	4 (14.3)	0.714
SCC	33	14 (42.4)	19 (57.6)		15 (45.5)	18 (54.5)		26 (78.8)	7 (21.2)	
Differentiation										
Well differentiated	28	17 (60.7)	11 (39.3)	0.009	13 (46.4)	15 (53.6)	0.592	26 (92.9)	2 (7.1)	0.088
Poorly differentiated	33	8 (24.2)	25 (75.8)		12 (36.4)	21 (63.6)		24 (75.8)	9 (24.2)	
T-status										
T1-2	48	18 (37.5)	30 (62.5)	0.456	19 (39.6)	29 (60.4)	0.913	40 (83.3)	8 (16.7)	0.899
T3-4	13	7 (53.8)	6 (46.2)		6 (46.2)	7 (53.8)		10 (76.9)	3 (23.1)	
Lymph node metastasis										
No	32	16 (50.0)	16 (50.0)	0.214	15 (46.9)	17 (53.1)	0.470	31 (96.9)	1 (3.1)	0.004
Yes	29	9 (31.0)	20 (69.0)		10 (34.5)	19 (65.5)		19 (65.5)	10 (34.5)	
TNM stage										
Ⅰ + Ⅱ	45	19 (42.2)	26 (57.8)	0.973	18 (40.0)	27 (60.0)	1.000	40 (88.9)	5 (11.1)	0.048
Ⅲ + Ⅳ	16	6 (37.5)	10 (62.5)		7 (43.8)	9 (56.3)		10 (62.5)	6 (37.5)	

GCN: gene copy number; AC: adenocarcinoma; SCC: squamous cell carcinoma; TNM: tumor-node-metastasis; NSCLC: non-small cell lung cancer.

counterstained with Mayer´s hematoxylin. The stained tissue sections were reviewed and scored for both the percentage of positive cells and the intensity of staining on 5 randomly selected 20× fields under an optical microscope. The intensity of staining was scored from 0 to 3 (0, without stain; 1, yellow; 2, brown; and 3, tan). The percentage of staining was scored from 0 to 4 (0, negative; 1, 1%-25%; 2, 26%-50%; 3, 51%-75%; and 4, 76%-100%) depending on the percentage of positively stained tumor cells. The overall score was the sum of these two scores. Tumor tissues in each case with a total score ⩽ 3 were considered negative for the expression of the target protein, while those with a final score > 3 were considered positive.

Genomic DNA extraction and Q-PCR for evaluation of *MET* gene copy number

To extract DNA, 5 paraffin-embedded sections (5 μM) were deparaffinized in xylene for 2 hours, and then incubated in 100% and 70% ethanol for 20 minutes to remove xylene. The tissues were collected into microtubes and then digested with 0.5 mg/mL proteinase K and 0.5% sodium dodecyl sulfate (SDS) solution overnight at 37°C. The next day the mixture was extracted with phenol and then precipitated with 70% ethanol in the presence of sodium acetate. The concentration of DNA was quantified by a NanoDropTM spectrometer at A260 absorbance (NanoDrop Technologies, Wilmington, DE, USA).

The *MET* gene copy number was analyzed by Q-PCR in a PRISM 7500 sequence detector (Applied Biosystems, Foster City, CA) with SYBR Premix Ex TaqTM II (Takara, Dalian, China). The standard curve method was used to calculate *MET* gene copy number relative to *Line-1*, an endogenous control used as reported previously[10], and quantification was based on standard curves established from serial dilutions of normal human genomic DNA. The relative *MET* gene copy number was also normalized to the normal human genomic DNA as calibrator and the change of *MET* gene copy number relative to *Line-1* was calculated by formula (MET_{tumor} / $Line\text{-}1_{tumor}$) / ($MET_{calibrator}$ / $Line\text{-}1_{calibrator}$)[10]. Cycle time values in triplicate were averaged for all samples. The conditions of Q-PCR were 95°C for 30 seconds, followed by 40 cycles of 95°C for 10 seconds, 60°C for 20 seconds, 72°C for 20 seconds

and 78°C for 20 seconds. All samples were subjected to a melting analysis to confirm the specificity of the amplicon at the end of PCR. The primer sequences are listed as follows: MET forward, 5´-TCATT–GGTTCCAATCAGCTCA-3´ and MET reverse, 5´-GCCCGAGAGAGGCTAATC-3´; Line-1 forward, 5´-CCGCTCATATGGAAACTG-3´'and Line-1 reverse 5´-GCGTCCCAGAGATTCTGGTATG-3´.

Statistical analysis

Statistical analyses were carried out by using SPSS 17.0 (SPSS, Chicago, IL, USA). The chi-squared (χ^2) test was used to compare categorical variables. Spearman´s correlation test was employed to assess interrelationship of the expression of MET, the expression of cyclin D1 and *MET* gene copy number. Survival curves were calculated by using the Kaplan-Meier method and compared by using the log-rank test. Univariate and multivariate analyses of overall survival were performed by using the log-rank test and Cox proportional hazard regression. A two-sided $P < 0.05$ was considered statistically significant.

RESULTS

Expressions of MET and cyclin D1 by immunohistochemistry and their association with clinicopathological parameters

The positive expression of MET was found in 59.0% (36/61) of NSCLC cases and MET staining was primarily present in the cytoplasm and membrane of tumor cells (***Fig. 1***). MET-positive cases were significantly associated with differentiation ($P = 0.009$), i.e., 75.8% (25/33) of the poorly differentiated NSCLC tissues expressed MET, but only 39.3% (11/28) of the well differentiated did so (***Table 1***). The expression of MET was not associated with gender, age, smoking history, histology, T-status, lymph node metastasis or TNM stage.

The positive expression of cyclin D1 was observed in 59.0% (36/61) of NSCLC cases and cytoplasmic localization was detected by cyclin D1 staining (***Fig. 1***). However, the expression of cyclin D1 was not associated with any clinicopathological parameters, includ–

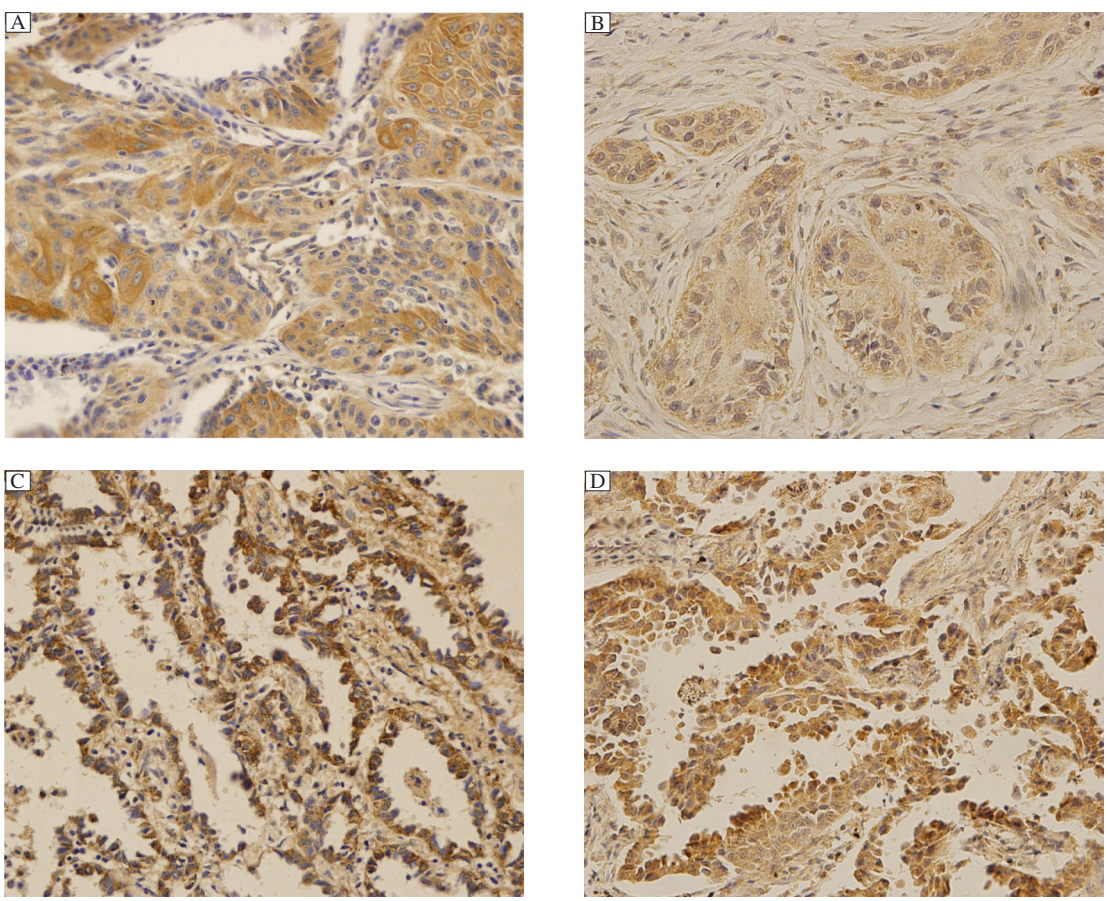

Fig. 1 **Representative images of MET-and cyclin D-positive staining.** A. Positive staining of MET in squamous cell carcinoma. MET shows cytoplasmic and membrane localization. B. Positive staining of cyclin D1 in squamous cell carcinoma. Cyclin D1 is present in the cytoplasm of tumor cells; C. Positive staining of MET in adenocarcinoma. MET is primarily present in the cytoplasm and membrane of tumor cells; D. Positive staining of cyclin D1 in adenocarcinoma. Cyclin D1 staining is primarily present in the cytoplasm of tumor cells (magnification × 400).

Table 2 **Association between *MET* GCN and MET expression in NSCLC tissues**

Variable	Total (*n*)	*MET* GCN			
		< 3	⩾ 3	*r*	*P*
MET protein				0.391	0.002
-	25	25	0		
+	36	25	11		

GCN: gene copy number; NSCLC: non-small cell lung cancer.

ing gender, age, smoking history, histology, differentiation, T-status, lymph node metastasis or TNM stage (***Table 1***).

MET gene copy number by Q-PCR and its association with clinicopathological parameters

Of the 61 NSCLC cases, we observed that 11 (11/61, 18.0%) had an increased *MET* gene copy number when the cut-off was set to 3 (*MET* gene copy number ⩾ 3). Increased *MET* gene copy number was significantly associated with lymph node metastasis ($P = 0.004$), i.e., 34.5% (10/29) of cases with lymph node metastasis had increased *MET* gene copy number, whereas only 3.1% (1/32) of cases without any lymph node metastasis did so. Moreover, the increased *MET* gene copy number was more common in cases with advanced stagecases than in early stage cases (37.5% compared with 11.1%, $P = 0.048$) (***Table 1***). However, increased *MET* gene copy number was not correlated with gender, age, smoking history, histology, differentiation or T-status (***Table 1***).

Interrelationship of the expression of MET, *MET* gene copy number and the expression of cyclin D1

A significant association was observed between the expression of MET and *MET* gene copy number (***Table 2***, Spearman´s $r = 0.391$, $P = 0.002$) in these 61 NSCLCs. All cases (11/11) with increased *MET* gene copy number (*MET* gene copy number ⩾ 3) showed the positive expression of MET. There was a significant association between the expression levels of MET and cyclin D1 (***Table 3***, Spearman´s $r = 0.390$, $P = 0.002$) and the co-expression of MET and cyclin D1 was found in 44.3% (27/61) of NSCLC cases. Of the 36 tumor tissues with MET-positive expression, 27 cases (27/36, 75.0%) displayed cyclin D1-positive expression. There was also a significant association between *MET* gene copy number and the expression of cyclin D1 (***Table 3***, Spearman´s $r = 0.304$, $P = 0.017$). Of the 11 tumor tissues with increased *MET* gene copy number (*MET* gene copy number ⩾ 3), 10 cases (10/11, 90.9%) displayed cyclin D1-positive expression.

Association of combined status of MET /cyclin D1 and *MET* gene copy number/cyclin D1 with clinicopathological parameters

As is shown in ***Table 4***, MET+/ cyclin D1+ phenotype was associated with tumor differentiation ($P = 0.044$) and *MET* gene copy number ⩾ 3/ cyclin D1+ phenotype was found to be significantly correlated with lymph node metastasis ($P = 0.009$).

Survival analysis

At our last follow-up with these patients (June 30, 2011), 49.2% (30/61) of them were still alive. The median overall survival for these patients was 29.0 months, and 1- and 3-year survival probabilities were 85.2% and 21.3%, respectively. The median overall survival of patients with MET-positive expression was 24.0 months, while that of those with the negative expression of MET was 32.5 months ($P = 0.003$; ***Fig. 2A***). The median overall survival of patients with high and low *MET* gene copy number was 11.0 and 30.5 months, respectively ($P < 0.001$; ***Fig. 2B***). However, we did not find any association between cyclin D1 protein levels and overall survival ($P = 0.287$; ***Fig. 2C***). We also investigated the association of the overall survival of patients with the combined expression of MET and cyclin D1, and found that the MET+/ cyclin D1+ phenotype

Table 3 **Association between MET expression/*MET* GCN and cyclin D1 expression in NSCLC tissues**

Variable	Total (*n*)	Cyclin D1 protein			
		-	+	*r*	*P*
MET protein				0.390	0.002
-	25	16	9		
+	36	9	27		
MET GCN				0.304	0.017
< 3	50	24	26		
⩾ 3	11	1	10		

GCN: gene copy number; NSCLC: non-small cell lung cancer.

Table 4 **Association of combined status of MET /Cyclin D1 and *MET* GCN /Cyclin D1 with clinicopathological date from NSCLC patients**

Variables	Total	MET expression / Cyclin D1 expression			*MET* GCN / Cyclin D1 expression		
	(*n*)	MET +/Cyclin D1+ (%)	Others(%)	*P*	*MET* GCN ⩾ 3/Cyclin D1+ (%)	Others(%)	*P*
Total (*n*)	61	27 (44.3)	34 (55.7)		10 (16.4)	51 (83.6)	
Gender							
Male	47	20 (42.6)	27 (57.4)	0.853	8 (17.0)	39 (83.0)	1.000
Female	14	7 (50.0)	7 (50.0)		2 (14.3)	12 (85.7)	
Age (years)							
< 60	29	14 (48.3)	15 (51.7)	0.732	6 (20.7)	23 (79.3)	0.605
⩾ 60	32	13 (40.6)	19 (59.4)		4 (12.5)	28 (87.5)	
Smoking history							
Yes	39	18 (46.2)	21 (53.8)	0.898	9 (23.1)	30 (76.9)	0.129
No	22	9 (40.9)	13 (59.1)		1 (4.5)	21 (95.5)	
Histology							
AC	28	14 (50.0)	14 (50.0)	0.567	3 (10.7)	25 (89.3)	0.449
SCC	33	13 (39.4)	20 (60.6)		7 (21.2)	26 (78.8)	
Differentiation							
Well differentiated	28	8 (28.6)	20 (71.4)	0.044	2 (7.1)	26 (92.9)	0.147
Poorly differentiated	33	19 (57.6)	14 (42.4)		8 (24.2)	25 (75.8)	
T-status							
T1-2	48	22 (45.8)	26 (54.2)	0.873	7 (14.6)	41 (85.4)	0.755
T3-4	13	5 (38.5)	8 (61.5)		3 (23.1)	10 (76.9)	
Lymph node metastasis							
No	32	10 (31.3)	22 (68.7)	0.059	1 (3.1)	31 (96.9)	0.009
Yes	29	17 (58.6)	12 (41.4)		9 (31.0)	20 (69.0)	
TNM Stage							
Ⅰ + Ⅱ	45	20 (44.4)	25(55.6)	1.000	5 (11.1)	40 (88.9)	0.140
Ⅲ + Ⅳ	16	7 (43.8)	9 (56.2)		5 (31.3)	11 (68.7)	

GCN: gene copy number; AC: adenocarcinoma; SCC: squamous cell carcinoma; TNM: tumor-node-metastasis; NSCLC: non-small cell lung cancer.

was associated with shorter overall survival ($P = 0.021$; ***Fig. 2D***). Similarly, when considering overall survival and the combined status of *MET* gene copy number and the expression of cyclin D1, we found that MET gene copy number ⩾ 3/ cyclin D1+ was associated with shorter overall survival ($P < 0.001$; ***Fig. 2E***).

According to the results of the univariate analysis, poor tumor differentiation (HR 2.392, 95% CI = 1.093-5.236, $P = 0.029$), lymph node metastasis (HR 2.655, 95% CI = 1.168-6.033, $P = 0.020$), advanced TNM stage (HR 7.483, 95% CI = 2.503-22.367, $P < 0.001$), the positive expression of MET (HR 3.495, 95% CI = 1.511-8.084, $P = 0.003$), increased *MET* gene copy number (HR 12.018, 95% CI = (3.799-38.018, $P < 0.001$), MET+/ cyclin D1+ (HR 2.586, 95% CI =1.157-5.780, $P = 0.021$) and *MET* gene copy number ⩾ 3/ cyclin D1+ (HR 12.018, 95% CI = (3.799-38.018, $P < 0.001$) were significantly associated with a higher risk of death. Detailed data are shown in ***Table 5***. In the multivariate analysis, stepwise regression procedure was used to remove variables from the model if they were not significantly related to the risk of overall survival. Advanced TNM stage (HR 17.027, 95%CI = 4.529 - 64.022, $P < 0.001$), the positive expression of MET (HR 4.040, 95%CI = 1.618 - 10.087, $P = 0.003$) and increased *MET* gene copy number (HR 9.487, 95%CI = 2.510 - 35.848, $P = 0.001$) increased the risk of death. Detailed data are shown in ***Table 6.***

DISCUSSION

This study is the first to analyze the expression of MET and cyclin D1 proteins and *MET* gene copy number in NSCLC. The positive expressions of MET and cyclin D1 were observed in 59.0% and 59.0% of the study population of NSCLC patients, respectively, and increased *MET* gene copy number was found in 18.0%. MET-positive expression was significantly associated with poor tumor differentiation. Increased *MET* gene copy number was significantly associated with high lymph node metastasis and advanced tumor stage. MET-positive expression and increased *MET* gene copy number led to shorter overall survival and both were adverse prognostic factors for NSCLC patients, whereas the expression of cyclin D1 was not associated with overall survival.

In the current study, positive expression of MET in resected tissue specimens was observed in 59.0%

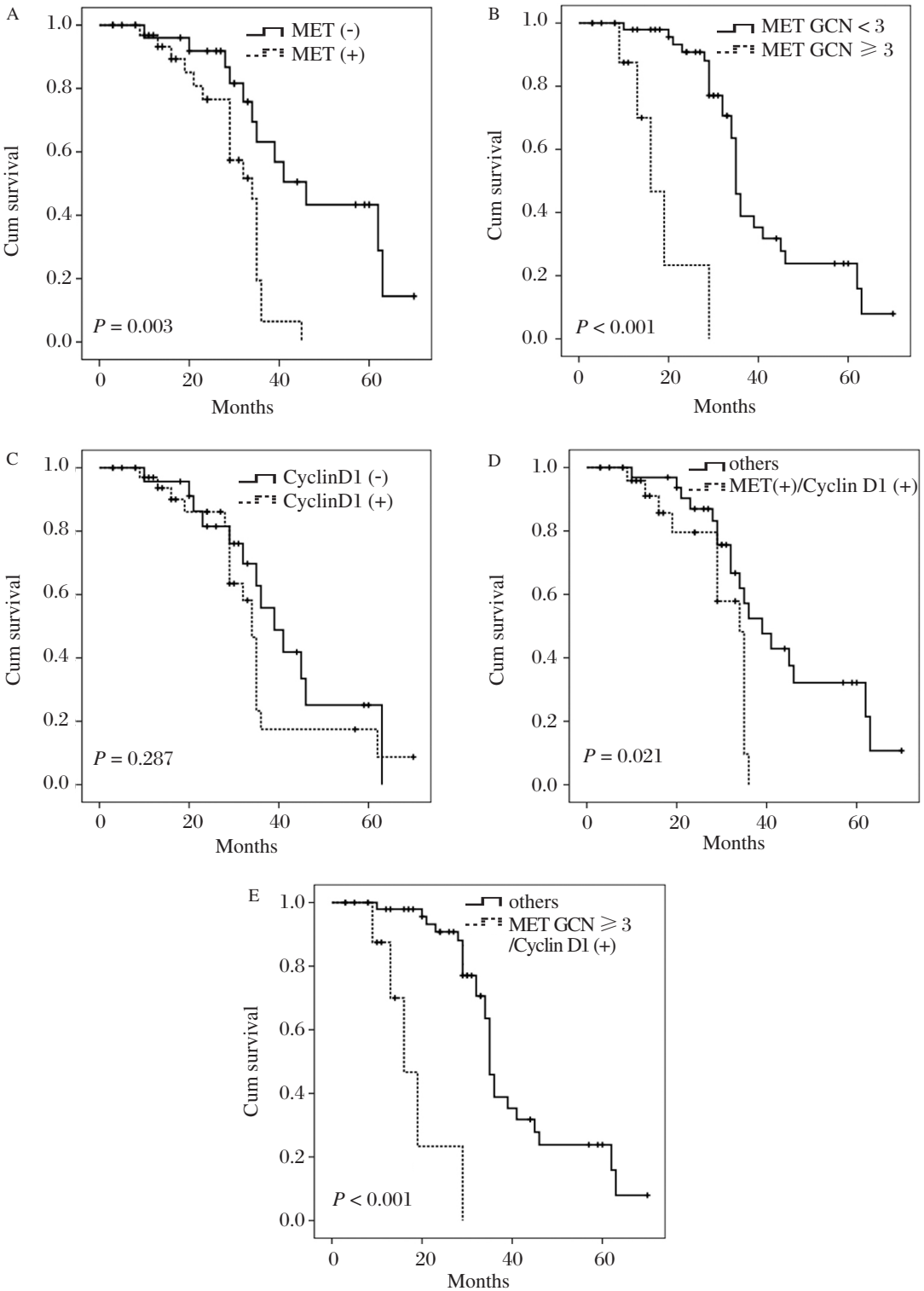

***Fig. 2* Kaplan-Meier survival analysis.** A: Overall survival curves for patients with MET-positive and MET-negative expression ($P = 0.003$). B: Overall survival curves for patients with *MET* gene copy number ⩾ 3 and *MET* gene copy number < 3 ($P < 0.001$). C: Overall survival curves for patients with cyclin D-positive and cyclin D-negative expression ($P = 0.287$). D: Overall survival curves for patients with MET+/ cyclin D1+ and other phenotypes ($P = 0.021$). E: Overall survival curves for patients with *MET* gene copy number ⩾ 3/ cyclin D1+ and other phenotypes ($P < 0.001$).

(36/61) of the NSCLC cases (previously reported to range between 24.0% and 81.0%)[13,35-37] and was associated with poor tumor differentiation. Similar to our results, Tsuta et al.[13] found an association between poor tumor differentiation and MET expression positivity, indicating that MET expression is a differentiation marker for NSCLC. The present survival analysis indicated that MET-positive expression was associ–

Table 5 **Univariate analysis of clinicopathological data for overall survival of NSCLC patients**

Variable	Univariate analysis	
	Hazard ratio (95% CI)	*P*
Gender		
Female vs male	1.008 (0.406-2.508)	0.986
Age (years)		
< 60 vs ⩾ 60	0.946 (0.466-1.920)	0.879
Smoking history		
No vs yes	0.815 (0.382-1.741)	0.598
Histology		
AC vs SCC	0.764 (0.367-1.589)	0.471
Differentiation		
Poorly versus well differentiated	2.392 (1.093-5.236)	0.029
T-status		
T3-4 vs T1-2	1.550(0.624-3.851)	0.345
Lymph node metastasis		
Yes vs No	2.655 (1.168-6.033)	0.020
TNM stage		
Ⅲ + Ⅳ vs Ⅰ + Ⅱ	7.483 (2.503-22.367)	< 0.001
MET expression		
Positive vs negative	3.495 (1.511-8.084)	0.003
Cyclin D1 expression		
Positive vs negative	1.484 (0.718-3.068)	0.287
MET GCN		
⩾ 3 vs < 3	12.018 (3.799-38.018)	< 0.001
MET(+) / Cyclin D1(+) vs Others	2.586 (1.157-5.780)	0.021
MET GCN ⩾ 3/ Cyclin D1(+) vs Others	12.018 (3.799-38.018)	< 0.001

NSCLC: non-small cell lung cancer; AC: adenocarcinoma; SCC: squamous cell carcinoma; TNM: tumor-node-metastasis; GCN: gene copy number.

ated with poor overall survival of NSCLC patients. Our study confirmed data published by others[8,36,38], but the reports of Tsuta et al.[13] and Dziadziuszko et al.[14] showed that expression of MET protein did not associate with survival. The discrepancy may be due to different patient populations, antibodies, methods used or even different scoring systems.

Our current data indicated that increased *MET* gene copy number occurred in 18.0% of the patients, within the range of previously reported *MET* gene amplification rates (1.1-21.0%)[10,28-30,32,39-40]. Similar to previous studies, we found that the increased *MET* gene copy number was significantly associated with lymph node metastasis and advanced tumor stages[12,38-39], which suggested that *MET* gene copy number may contribute to progression of NSCLC. In addition, consistent with other studies[13-14,38], our data showed a significant association between *MET* gene copy number and the protein expression of MET. However, clinicopathologic association with *MET* gene copy number in NSCLC is still controversial. For example, in line with our results, Okuda et al.[10] analyzed *MET* gene copy number in 213 NSCLC tissue specimens using Q-PCR and found no association between *MET* gene copy number and histologic types. Tsuta et al.[13] showed that *MET*-positive bright-field in situ hybridization was more common in adenocarcinoma patients than in squamous cell carcinoma patients. In contrast,

Table 6 **Multivariate analysis of clinicopathological data for overall survival of NSCLC patients**

Variable	Multivariate analysis	
	Hazard ratio (95% CI)	*P*-value
TNM stage		
Ⅲ + Ⅳ vs Ⅰ + Ⅱ	17.027 (4.529 - 64.022)	< 0.001
MET expression		
Positive vs negative	4.040 (1.618 - 10.087)	0.003
MET gene copy number		
⩾ 3 vs < 3	9.487 (2.510 - 35.848)	0.001

TNM: tumor-node-metastasis; NSCLC: non-small cell lung cancer.

Go et al.[11]reported that *MET*-positive FISH was more common in squamous cell carcinoma than in adenocarcinoma of the lung.

Although some studies have confirmed that increased *MET* gene copy number is associated with poor survival[12,38], the prognostic value of *MET* gene copy number in NSCLC is in dispute. We found that increased *MET* gene copy number was significantly associated with shortened median survival and multivariate analysis revealed that it was an adverse prognostic factor. Similarly, Cappuzzo et al.[39] analyzed *MET* gene amplification using FISH in a series of 447 NSCLC tissue samples and provided clear evidence that *MET* amplification was a poor prognostic factor for NSCLC patients. However, Beau-Faller et al.[32] detected *MET* gene copy number in 106 NSCLC patients by using Q-PCR and showed that there was no association between *MET* amplification and overall survival. Dziadziuszko et al.[14] also failed to show any association between *MET* amplification and overall survival of 189 patients. Thus, further study with a larger sample size is warranted to confirm our current data.

Some authors indicated that cyclin D1 had significantly higher positive expression in patients with poorly differentiated carcinoma, in the presence of vascular invasion and visceral pleural invasion[41]. However, we found that the expression of cyclin D1 was not associated with any clinicopathological characteristics. Interestingly, our study showed that the expression of cyclin D1 was significantly associated with the expression of MET and *MET* gene copy number in these NSCLC tissue samples. These data may indicate that the expression of MET could upregulate the expression of cyclin D1 in NSCLC, although molecular mechanism remains to be clarified. MET-associated downstream pathways, such as Ras/MAPKs, may be responsible. When we analyzed the combined expression status of MET /cyclin D1, patients with MET+/ cyclin D1+ phenotype apparently had poor tumor differentiation. *MET* gene copy number $\geqslant$ 3/ cyclin D1+ phenotype was found to be significantly correlated with lymph node metastasis.

The prognostic value of the expression of cyclin D1 remains disputable in previous studies. Some studies observed that patients with positive expression of cyclin D1 had better overall survival and was a favorable prognostic factor[21-23]. In contrast, some studies revealed that cyclin D1-positive expression was associated with a poor survival in NSCLC[24-25]. Finally, other studies could not confirm that the expression of cyclin D1 was a prognostic indicator of NSCLC[26-27]. According to our results, the expression of cyclin D1 lacked prognostic value, whereas the expression of MET and *MET* gene copy number were adverse prognostic factors in patients with NSCLC. Possible reason for the results may be the proposed dual role of cyclin D1 in the regulation of the cell cycle and the presence of other factors affecting the cell cycle. However, MET+/ cyclin D1+ and *MET* gene copy number $\geqslant$ 3/ cyclin D1+ phenotypes had shortened overall survival and both of them were negative prognostic factors, which were found only at the univariate level. These results may indicate that the MET/cyclin D1 signaling pathway might be useful in predicting the prognosis of NSCLCs.

In conclusion, the present study suggested that the overexpression of MET and increased *MET* gene copy number were associated with important clinicopathological parameters in NSCLC and both were shown to be adverse prognostic markers for NSCLC. Furthermore, the expression of cyclin D1 was significantly associated with the expression of MET as well as with *MET* gene copy number. Therefore, the activated MET/cyclin D1 signaling pathway may contribute to carcinogenesis and development of NSCLC and may represent a target for therapy.

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