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)
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\[^{[28-30]}\]. 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\[^{[31]}\]. An irrelevant rabbit antiserum served as a negative control. The sections were...
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 \( \leq 3 \) 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 \( \frac{\text{MET}_{\text{tumor}} / \text{Line-1}_{\text{tumor}}}{\text{MET}_{\text{calibrator}} / \text{Line-1}_{\text{calibrator}}} \). 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.

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

| Variable                  | Total (n) | MET protein expression | Cyclin D1 protein expression | MET GCN |
|---------------------------|-----------|------------------------|------------------------------|---------|
|                           | Total (n) | (%)                    | (%)                          | (%)     |
|                           |           | + (%)                  | + (%)                        | < 3 (%) |
|                           |           | P                      | P                            | 3 (%)   |
|                           |           | α                      | α                            | P       |
| Gender                    | 61        | 25(41.0)               | 25(41.0)                     | 50(82.0)|
| Male                      | 47        | 21(44.7)               | 20(42.6)                     | 38(80.9)|
| Female                    | 14        | 4 (28.6)               | 5 (35.7)                     | 12 (85.7)|
| Age (years)               |           |                       |                              |         |
| < 60                      | 29        | 13 (44.8)              | 11 (37.9)                    | 23 (79.3)|
| ≥ 60                      | 32        | 12 (37.5)              | 14 (43.8)                    | 27 (84.4)|
| Smoking history           |           |                       |                              |         |
| Yes                       | 39        | 16 (41.0)              | 15 (38.5)                    | 29 (74.4)|
| No                        | 22        | 9 (39.1)               | 10 (45.5)                    | 21 (95.5)|
| Histology                 |           |                       |                              |         |
| AC                        | 28        | 11 (39.3)              | 10 (35.7)                    | 24 (85.7)|
| SCC                       | 33        | 14 (42.4)              | 15 (45.5)                    | 26 (78.8)|
| Differentiation           |           |                       |                              |         |
| Well differentiated       | 28        | 17 (60.7)              | 13 (46.4)                    | 26 (92.9)|
| Poorly differentiated     | 33        | 8 (24.2)               | 12 (36.4)                    | 24 (75.8)|
| T-status                  |           |                       |                              |         |
| T1-2                      | 48        | 18 (37.5)              | 19 (39.6)                    | 40 (83.3)|
| T3-4                      | 13        | 7 (53.8)               | 6 (46.2)                     | 10 (76.9)|
| Lymph node metastasis     |           |                       |                              |         |
| No                        | 32        | 16 (50.0)              | 15 (46.9)                    | 31 (96.9)|
| Yes                       | 29        | 9 (31.0)               | 10 (34.5)                    | 19 (65.5)|
| TNM stage                 |           |                       |                              |         |
| I + II                    | 45        | 19 (42.2)              | 18 (40.0)                    | 40 (88.9)|
| III + IV                  | 16        | 6 (37.5)               | 7 (43.8)                     | 10 (62.5)|

GCN: gene copy number; AC: adenocarcinoma; SCC: squamous cell carcinoma; TNM: tumor-node-metastasis; NSCLC: non-small cell lung cancer.
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’-GGGTCCAGAGATTCTGGTATG-3’.

Statistical analysis

Statistical analyses were carried out by using SPSS 17.0 (SPSS, Chicago, IL, USA). The chi-squared ($\chi^2$) 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).
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 \( \geq 3 \)). 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 stage cases 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 \( \geq 3 \)) 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 \( \geq 3 \)), 10 cases (10/11, 90.9%) displayed cyclin D1-positive expression.

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

| Variable  | Total (n) | MET GCN | \(< 3\) | \(\geq 3\) | \(r\) | \(P\) |
|-----------|-----------|---------|--------|----------|------|------|
| MET protein |           |         |        |          |      |      |
| -         | 25        | 25      | 0      |          |      |      |
| +         | 36        | 25      | 11     |          |      |      |

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

### 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 |           |                   |        |       |      |      |
| -         | 25        |                   | 16     | 9     |      |      |
| +         | 36        |                   | 9      | 27    |      |      |

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

**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 \( \geq 3 \)/ 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 \( (P = 0.003; \text{Fig. 2A}) \). The median overall survival of patients with high and low MET gene copy number was 11.0 and 30.5 months \( (P < 0.001; \text{Fig. 2B}) \). However, we did not find any association between cyclin D1 protein levels and overall survival \( (P = 0.287; \text{Fig. 2C}) \). 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
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 $\geq 3$/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 $\geq 3$/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 $\geq 3$ 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 $\geq 3$ 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-
Prognostic value of MET and cyclin D1 abnormalities in NSCLC

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 [12,36-39], 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, clinico-pathologic 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,

| Variable | Hazard ratio (95% CI) | P-value |
|----------|-----------------------|---------|
| Female vs male | 1.008 (0.406-2.508) | 0.986 |
| < 60 vs ≥ 60 | 0.946 (0.466-1.920) | 0.879 |
| Smoking history | 0.815 (0.382-1.741) | 0.598 |
| AC vs SCC | 0.764 (0.367-1.589) | 0.471 |
| Poorly versus well differentiated | 2.392 (1.093-5.236) | 0.029 |
| Yes vs No | 2.655 (1.160-6.033) | 0.020 |
| III + IV vs I + II | 7.483 (2.503-22.367) | < 0.001 |
| Positive vs negative | 3.495 (1.511-8.084) | 0.003 |
| Positive vs negative | 1.484 (0.718-3.060) | 0.287 |
| ≥ 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 |

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

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

| Variable | Hazard ratio (95% CI) | P-value |
|----------|-----------------------|---------|
| III + IV vs I + II | 17.027 (4.529 - 64.022) | < 0.001 |
| Positive vs negative | 4.040 (1.618 - 10.087) | 0.003 |
| ≥ 3 vs < 3 | 9.487 (2.510 - 35.848) | 0.001 |

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

Table 6 Multivariate analysis of clinicopathological data for overall survival of NSCLC patients
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. Dziadziouszko 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 $\geq 3$ / 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 $\geq 3$ / 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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