Correlation among Genetic Variations of c-MET in Chinese Patient with Non-Small Cell Lung Cancer


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Abstract

Aims: The aim of this study was to determine the relationship between amplification, protein expression and somatic mutation of c-MET in advanced Non-small cell lung cancer. The influence of c-MET abnormalities on clinical outcomes of patients undergoing Crizotinib therapy for treatment of Non-Small Cell Lung Cancer was also evaluated.

Methods: c-MET protein expression, gene copy number (GCN) and somatic mutation for exon 14 were detected by Immunohistochemistry, fluorescent In Situ Hybridization and Denaturing High Performance Liquid Chromatography, respectively, in a large series of 196 NSCLC patients. The correlation of c-MET abnormalities and clinical outcome of targeted therapy was analyzed by McNemar’s test.

Results: c-MET expression was observed in 28.6% (56/196) cases, and among those 13.8% (27/196) was shown to be FISH positive. Only 2.67% patients in this study carried the c-MET mutation. All cases that were c-MET FISH positive were also shown to express c-MET by IHC. However, only half of the cases that were positive for c-MET expression were found to be FISH positive. Among 31 patients with moderate c-MET IHC staining, 11 cases (35.5%) were FISH positive, while 16/25 cases with high IHC staining were also FISH positive. Six patients received Crizotinib as a first-line or second/third-line therapy. Among them, three cases showed ALK protein expression, two patients showed expression of the ROS1 fusion gene, and one was positive for c-MET expression by IHC. The response to Crizotinib in the three patients positive for ALK were all PR, while the c-MET positive patient showed SD and both cases with expression of the ROS1 gene showed PD.

Conclusions: IHC could be a preliminary screening test to facilitate selection of patients with c-MET amplification for ALK inhibitor therapy.

Keywords: Non-small-cell lung cancer; c-MET; Expression; Copy number; Mutation

Abbreviations: NSCLC: Non-Small-Cell Lung Cancer; GCN: Gene Copy Number; IHC: Immunohistochemistry; FISH: Fluorescent In Situ Hybridization; DHPLC: Denaturing High Performance Liquid Chromatography.

Introduction

The c-MET gene encodes a receptor tyrosine kinase and is located on chromosome 7, 17q21-31 [1]. Deregulation of this molecular pathway due to mutation, amplification, overexpression, or activation of c-MET has been observed in a variety of cancers. In primary lung cancer specimens, overexpression of c-MET has been reported in 25–75% of cases [2,3], gene amplification has been observed in 5-22% [2-4], and mutations have been shown to play a role in approximately 5% of tumors [5,6].

Studies of patients with non-small cell lung cancer (NSCLC) treated with tyrosine kinase inhibitors (TKIs) of the epidermal growth factor receptor (EGFR), such as gefitinib or erlotinib, have shown that overexpression of c-MET is responsible for roughly 20% of resistance to EGFR-TKIs [7], which results in activation of the PI3K/Akt pathway. A lung cancer with c-MET amplification also demonstrated high sensitivity to crizotinib, a tyrosine kinase inhibitor targeting the anaplastic lymphoma kinase gene (ALK), suggesting cancers with increased c-MET levels may be sensitive to ALK inhibitors [8]. More recently, ongoing phase I and phase II clinical trials are being carried out with c-MET inhibitors on patients with lung cancer (Cecchi et al. [9]; Liu et al. [10]), and some inhibitors have been found to produce tumor regression [11]. Although many therapies targeting c-MET are part of ongoing clinical trials, there is no general consensus on how c-MET status should be tested in lung cancer tissues or what the relationship is between the results obtained by immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). Therefore, the variability of c-MET status trial results likely reflects variations in the methodology and the interpretation of the test results.
This study was designed to investigate the correlation between c-MET protein expression, gene copy number (GCN) and the presence of mutations using different, complementary methods. We also assessed whether c-MET status determined by these methods was associated with prognosis in a large series of consecutive lung carcinoma cases.

Methods

Patients and tumors

Formalin-fixed and paraffin-embedded (FFPE) samples from 196 NSCLC patients were obtained from the Tissue Bank of Thoracic Medical Oncology Department of Peking Cancer Hospital from 2012 to 2014. This study was approved by the Ethics Committee and written consent was obtained from all patients before inclusion in the study. The median follow-up period of patients was 19.7 (range, 8.7–34.2) months. Patient characteristics are summarized (Table 1).

Table 1: Clinicopathological characteristics of patients according to CMET protein expression and copy number (P > 0.05).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Number</th>
<th>FISH</th>
<th>IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± sd, years)</td>
<td>57.5 ± 5.6</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>97</td>
<td>12 (12.4)</td>
<td>26 (26.8)</td>
</tr>
<tr>
<td>Male</td>
<td>99</td>
<td>15 (15.2)</td>
<td>30 (30.3)</td>
</tr>
<tr>
<td>Smoking History</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>124</td>
<td>16 (12.9)</td>
<td>32 (25.8)</td>
</tr>
<tr>
<td>Former or current</td>
<td>72</td>
<td>11 (15.3)</td>
<td>24 (33.3)</td>
</tr>
<tr>
<td>Histologic type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>180</td>
<td>26 (14.4)</td>
<td>53 (29.4)</td>
</tr>
<tr>
<td>Non-ade</td>
<td>16</td>
<td>1 (6.3)</td>
<td>3 (18.8)</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I+IIa</td>
<td>21</td>
<td>3 (14.3)</td>
<td>7 (33.3)</td>
</tr>
<tr>
<td>IIIb+IV</td>
<td>175</td>
<td>24 (13.7)</td>
<td>49 (28.0)</td>
</tr>
</tbody>
</table>

Immunohistochemistry (IHC)

All slides were processed under identical conditions using standard protocols. Immunohistochemical staining was performed on 4 μm-thick tumor sections. Briefly, the sections were deparaffinized prior to antigen retrieval in a steamer. Monoclonal anti-c-Met antibody MET rabbit monoclonal antibody (clone D1C2, rabbit, Cell Signaling Technology, USA), diluted at 1:150 in Signal Stain antibody diluent (Cell Signaling Technology, USA), was applied to slides for 16h at 4°C. Universal secondary antibody (DAKO) was applied for 15 min. Diaminobenzidine or 3-amino-9-ethylcarbazole were used as chromogens, and slides were counterstained with haematoxylin before mounting according to standard immunohistochemical techniques. The c-MET-score was based on a scale of 0 to 3+: no membrane staining or membrane staining in <10% of tumor cells (score 0), faint/barely perceptible partial membrane staining in >10% of tumor cells (score 1+), moderate staining of the entire membrane in >10% of tumor cells (score 2+), and strong staining of the entire membrane in >10% of tumor cells (score 3+). Scores of 0 and 1+ were considered as negative for c-MET expression, while scores of 2+ and 3+ were regarded as positive.

Fluorescent In-Situ Hybridization (FISH)

Fluorescence in-situ hybridization (FISH) analyses of c-MET gene copy number (GCN) were carried out to validate the results of the immunohistochemical analyses. A commercially available probe cocktail comprised of a green fluorochrome direct labelled CEN 7 probe specific for the alpha satellite centromeric region of chromosome 7 (D7Z1) and a Texas Red fluorochrome direct labelled probe specific for the c-MET gene located at 7q31 was used (Abnova, Taiwan). FISH was performed and analyzed according to the manufacturer's instructions, and 100 cells were analyzed for each sample. In several criteria were used to assess c-MET gene status. A specimen was considered to be displaying c-MET amplification, if one of the following conditions was fulfilled: (i) MET/CEP-7 ratio >2; (ii) ≥ 10% of tumor cells showed ≥ 15 c-MET signals; (iii) tight MET gene clusters. A specimen was considered as showing high polysomy if ≥ 50% of tumor cells contained more than five c-MET signals. Samples that were considered both high polysomy and c-MET amplification were marked as FISH positive.

Results of c-MET FISH and IHC were evaluated by two independent pathologists. Correlation of FISH and IHC results with clinical information was performed after completion of analyses.

c-MET and EGFR mutation analysis

For c-MET mutational analysis, the coding region of tyrosine kinase domain exon 14 was amplified from FFPE tissue DNA and analyzed by denaturing high-performance liquid chromatography (DHPLC) and confirmed by direct sequencing. DHPLC analysis was carried out on Transgenic Wave Nucleic Acid Fragment Analysis System with a DNASep column (Transgenic, Omaha, NE) according to the manufacturer's protocol.

The primer for 5' splice site of exon 14 were

Forward: 5'-TATGTATGTCATAAAACCCCATGAG,
Reverse: 5'-CTTACAGCCCTATCCAAATGAG.
The primer for 3' splice site of exon 14 were

Forward: AAGTGTAAGCCCAACTACAGAA,
Reverse: 5’- GAGGTAATACTTCTTATAGGTTT.

AmoyDxTM EGFR 29 Mutations Detection Kit (Amoy Diagnostics Co, XiaMen, China) was used for EGFR mutation detection.

Statistical Analysis

Frequency tabulation and summary statistics were used to characterize the data distribution. The McNemar test was applied for comparative analysis of MET protein expression and copy number. The associations of categorical variables were analyzed using the chi-square test; however, Fisher’s exact test was used for small sample sizes (expected value <5 in any cell of the contingency table). To test differences between groups with respect to survival, the log-rank test was used. Cox regression model was used for multi-factors analysis. P values <0.05 were considered to be statistically significant. All calculations were performed using SAS statistical software version 15.0 (SAS Institute, Inc., Cary, NC).

Results

c-MET protein expression

c-MET expression can be observed in the cytoplasm of lung cancer cells, and was detected in 56 cases. c-MET-scores, determined by immunohistochemical analysis indicated that 51.5% (101/196), 19.9% (39/196), 15.8% (31/196), and 12.8% (25/196) of the cases were scored as 0, 1+, 2+, and 3+, respectively.

Immunohistochemical staining data was also used to evaluate 28 patients with two-site metachronous specimens, including those that underwent bronchoscopic biopsies twice (n=6), specimens from bronchoscopic biopsy/pulmonary operations (n=3), samples from patients with bronchoscopic biopsy/ other organ metastasis (n=5), specimens from pulmonary operations/bronchoscopic biopsies (n=2), pulmonary operation specimen/other organ metastasis (n=4), and patients with organ metastasis/second organ metastasis (n=8). Positive staining for c-MET was detected in 17.8% (5/28) of first-site specimens and 25.0% (7/28) of re-biopsy samples. There were no significant changes observed between the two-site specimens in the majority of cases (n=24). In total, four cases were positive (score 2+ and 3+) for both specimens, while in the twenty remaining cases, both two-site specimens were negative for c-MET staining (score 0). Further, three cases changed from 0 to 3+ and one case went from strong (score 3+) to moderate (score 1+) staining when the first-site specimen was compared to the re-biopsy.

Evaluation of c-MET gene copy number by FISH

c-MET copy number was found to be positive by FISH in 13.8% cases (27/196), and the FISH patterns are illustrated in Figure 1. Using FISH analysis 86.2%, (169/196) patients were found to be FISH negative, while high polysomy and amplification of the c-MET gene was detected in 9.7% (19/196) and 4.1% (8/196) of patients, respectively. Among eight of those displaying c-MET gene amplification, six had a low levels (gene-to-chromosome ratio ranging between 2.5 and 3.5), and two had high levels of amplification, with approximately 15 and 21 copies.

Figure 1: Relationship between MET protein expression and MET gene copy number in lung adenocarcinoma by IHC and FISH. The left graph shows IHC with moderate (IHC++) and strong (IHC+++). The right graph shows FISH positive and negative specimens that have been divided into two groups based on IHC analysis.

In first-site and rebiopsy specimens, 10.7% (3/28) and 17.9% (5/28) were shown to be FISH positive, respectively. Further, when two-sites specimens were examined, three cases were identified where both tumors were positive, while both specimens were negative in the other 23 patients. In two cases, high polysomy of the MET gene was detected in rebiopsy tumors, but not in first-site tumors where the concordant rate of copy number polysomy or amplification between the first-site tumors and rebiopsy tumors was 92.9% (26/28).

c-MET gene mutation

150 paraffin-embedded tumor samples were available for gene mutation analysis. Four samples were found to harbor mutations. All mutations were localized in the intronic region upstream of the 5’ splice site of exon 14. One of the identified mutations resulted in a 10-base deletion and the other three cases were single base substitution. All the four cases did not carry c-MET expression and copy number amplification.

Association between c-MET protein expression and GCN

In order to determine whether genomic DNA copy number variations contribute to gene expression changes, the correlation between MET gene expression and corresponding DNA copy number changes was determined (Table 2).

<table>
<thead>
<tr>
<th>Item</th>
<th>FISH-</th>
<th>FISH+</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>FISH-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FISH+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Associations between MET protein expression and MET gene copy number.

<table>
<thead>
<tr>
<th>IHC</th>
<th>Negative</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>140</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>169</td>
<td>27</td>
</tr>
</tbody>
</table>

Table 3: Association between individuals positive for MET protein expression and MET gene copy number.

A positive correlation between high protein expression and increased copy number was identified (P<0.001). Of 56 patients shown to be IHC positive, 27 were FISH positive. Conversely, every patient (140) that was shown to be IHC negative was also FISH negative. The sensitivity and specificity of MET IHC analyses were determined to be 100% and 82.8%, respectively. In patients that were found to have no (score=0, n=124) or very faint (score=1+, n=16) MET staining, 22 cases were triploid and 4 were tetraploid for MET, respectively. Among those with moderate MET IHC staining (score=2+, n=31), 11 (35.5%) of the tissues were FISH positive, including 9 (29.0%) with high polysomy and 2 (6.5%) with amplification of the MET gene. Among patients with high IHC staining (score=3+, n=25), 16 (64.0%) were determined to be FISH positive, including 10 (40.0%) with high polysomy and 6 (24.0%) with amplification (Table 3 and Figure 1). There were significant differences between IHC intensity and FISH scoring (p=0.034). Our data suggested that a low number of MET gene copies per cell had not influenced the level of protein expression, whereas cells with increased copy number, including those with high polysomy and amplification, had an impact on protein level.

Table 4: MET protein expression in patients with two-site tumors.

Table 5: MET copy number analysis of specimens isolated from patients with two-site tumors.

Prediction implications of c-MET protein expression and GCN

58 patients were treated with Gefitinib or Erlotinib orally once per day. Treatment was discontinued when the disease progressed or intolerable toxicities appeared.

Neither the overall response rate (ORR) nor progression free survival (PFS, Figure 2) in the different categories of MET protein expression or GCN showed significant differences. In a multivariate analysis where the histologic type, smoking, sex and stage were included as covariates together with mutation, the EGFR mutation was independent predictors of response and PFS (P<0.01).

As a correlative study, MET copy number analysis and the presence of the EGFR mutation were determined in these 58 tumor samples. Among these patients, 44 were found to be EGFR mutants (EGFRmut), and among them, 5 patients were also positive for MET amplification (EGFR mut/METamp). Additionally, 14 cases were shown to be wild-type for EGFR (EGFR wild-type), with one patient also displaying MET amplification (EGFR wild-type/MET amp). To evaluate the response to TKIs, the EGFRI wild-type/METamp patient showed progressive disease (PD), while among five EGFRmut/METamp
patients, one showed PD, 2 showed stable diseases (SD), and 2 displayed partial responses (PR). Notably, the patient (EGFRmut/METamp) who showed PD harbored EGFR T790M/L858R double mutations.

Six patients received Crizotinib as a first-line or second/third-line therapy. Among these cases, ALK protein expression (Ventana IHC assay) was positive in three (ALKpos), MET was positive in one (METpos), and ROS1 was positive in the remaining two (ROS1pos). The response to Crizotinib in the three ALKpos patients were PR, while the single METpos patient showed SD, and both ROS1pos patients both were found to have PD.

Discussion

The need for accurate detection of MET alteration has become much more important, from both a clinical and a molecular standpoint, because the subsets of patients with NSCLC who will benefit from MET inhibition therapy are dependent on this information. MET amplification has also been accepted as one of the mechanisms of acquired resistance to EGFR-TKIs [12]. IHC and FISH are standardized methods for detecting protein expression and copy number in clinical practice, and results from these detection were important for clinical advantage patients screening IHC and FISH have respective advantage and disadvantage in clinical use for MET copy number detection: IHC has inexpensive cost but only half of positive patients were amplification; although FISH is more expensive but it is the golden-standardized method for copy number detection.

Previous studies describe much variation in the frequencies of MET protein overexpression (25%–75%) and copy number variation (3.8-21%) in lung cancer [13-16], largely due to the use of different methods and specimens obtained in primary site or metastatic tumors. In the present study, we performed a direct comparison of MET GCN per cell using FISH with MET protein expression evaluated by IHC in therapy-naive NSCLC. These data suggest that there is a significant correlation between increased GCN and high levels of MET protein expression. In this study, MET protein was found to be overexpressed in 28.6% of cases, with scores of 2+ and 3+ in 15.8% and 12.8%, respectively. MET GCN was positive in 13.8% of patients, with high polysomy (9.7%) and amplification (4.1%) of the MET gene, which showed a significant positive correlation between MET protein expression [17] and GCN. Dziadziuszko et al. [18] performed the representative study on the correlation between MET protein accumulation and gene copy number. Their study included primary tumors from 189 surgically resected NSCLC patients, and showed that MET protein expression was positive in 25% by IHC, and copy number amplification or high polysomy was identified in 12.1% by SISH (Silver in situ hybridization). Our study showed similar results, with the exception that our cohort was comprised of advanced patients and the detection method used to measure copy number was FISH. All patients who displayed amplification (clusters) or high polysomy of the MET gene also were positive for MET protein expression, while only half of the IHC positive patients had amplification or high polysomy. These data suggest that IHC can be a viable.

Alternative screening method, subjected to confirmatory testing by FISH in IHC positive cases, for anti-MET therapy or monitoring of EGFR-TKIs acquired resistance. While patients with no IHC positive staining, indicative of altered MET gene expression are considered FISH negative and therefore do not require reevaluation. However, a portion of lung carcinomas showed disomy while overexpressing the MET protein, suggesting that MET protein expression might also be controlled by mechanisms other than gene copy increase; including hypoxia-induced overexpression [19] and activated ERK/AKT induced MET overexpression through transcriptional mechanisms.

In our cohort, 28 patients had two-site tumors, which were obtained at diagnosis and during target therapy or chemotherapy. Among them, three patients changed from negative to positive of c-MET protein expression, and were all patients that received Tarceva as a first-line treatment. Further, they also received second biopsies to evaluate disease progression, which were confirmed by FISH in two patients that showed c-MET GCN gains involved in resistance to Tarceva.

When EGFR mutation analysis was combined with MET copy number determination, the five patients with double mutations (EGFR mut/METamp) showed PR and SD in four, while only one showed PD and was due to the T790M mutation. Our study suggested that although c-MET amplification preexists in some tumors, EGFR mutation was still strongest predictor for EGFR-TKIs. For the six patients that received Crizotinib, ALK expression was the best factor for predicting response, rather than MET protein expression or ROS1 translocation.

In conclusion, our study provides detailed descriptive analysis of the relationship between MET gene copy number and MET protein expression using different comparisons, demonstrating a good association between these two markers. As MET inhibitors enter the clinical arena in the near future, our results suggest IHC could be as fast and reliable screening method when combined with secondary confirmation by FISH in IHC positive patients.

Conflict of Interest’s Statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled “Correlation Among Genetic Variations of c-MET In Chinese Patient With Non-Small Cell Lung Cancer”.

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