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ORIGINAL ARTICLE

Comparison of the *c-MET* gene amplification between primary tumor and metastatic lymph nodes in non-small cell lung cancer

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Abstract

Background: *c-MET* has recently been identified as a promising novel target in non-small cell lung cancer (NSCLC). We detected the consistency of *c-MET* gene amplification in metastatic lymph nodes and tumor tissues of NSCLC patients and discuss the clinical application value of *c-MET* gene amplification in metastatic lymph nodes.

Methods: Real-time fluorescent quantitative PCR was used to test tumor tissues in 368 NSCLC patients and 178 paired metastatic lymph node samples. The amplification consistency in metastatic lymph nodes and tissue samples were compared and the correlation between *c-MET* gene amplification and the clinical characteristics of patients was analyzed.

Results: The *c-MET* gene amplification rate was 8.97% (33/368) in tumor tissues. Of the 178 paired cases, *c-MET* gene amplification was positive in 7.95% (15/178) of cancerous tissues and 18.54% (33/178) of metastatic lymph nodes. *c-MET* gene amplification was detected more frequently in metastatic lymph nodes than in primary cancerous tissue. When metastatic lymph nodes were used as surrogate samples of primary cancerous tissues, the sensitivity was 86.67% (13/15) and the specificity was 87.69% (143/163).

Conclusions: Screening for *c-MET* gene amplification in lymph node metastases could determine which patients are eligible for tyrosine kinase inhibitor therapy. Lymph node metastasis can predict *c-MET* gene amplification in a primary tumor and guide the clinical use of *c-MET* gene targeted drugs.

Introduction

Lung cancer is the most common cause of cancer-related mortality worldwide. Non-small cell lung cancer (NSCLC) accounts for about 80% of all lung cancers. It is believed that some genetic alterations are required before lung cancers become clinically evident.[1,2] NSCLC has been classified as either oncogenic-driver or wild type. Several molecular alterations, such as epidermal growth factor receptor (*EGFR*) mutations[3] or anaplastic lymphoma kinase (*ALK*) and ROS proto-oncogene 1 (*ROS1*) rearrangements[4,5] predict high sensitivity to specific targeted therapies. These developments have greatly improved patient outcome and quality of life.

The *c-MET* proto-oncogene is located on chromosome band 7q31 and encodes a heterodimeric transmembrane receptor with tyrosine kinase activity (RTK).[6,7] Activation of *c-MET* leads to a wide range of biological activities, including angiogenesis; cellular motility, growth, and invasion; reduced apoptosis; epithelial to mesenchymal

transition; and metastatic potential.[8,9] MET protein overexpression and high *c-MET* gene copy numbers are considered negative prognostic factors in NSCLC[10,11] and are associated with resistance to EGFR-targeted agents.[12] *c-MET* amplification is detected in approximately 4–5% of patients with NSCLC, mostly in young patients with adenocarcinoma who are non-smokers or light smokers.[13] *MET* has recently been identified as a promising novel target in NSCLC, therefore some c-MET inhibitors have been developed.[14,15] The efficacy of crizotinib in patients with *c-MET* amplification highlights the importance of screening for this genetic alteration in patients with advanced NSCLC,[16] considering that nearly two-thirds of patients with NSCLC present with locally advanced or metastatic disease at diagnosis[17] and that metastatic lymph nodes are easier to obtain for biopsy at earlier stages of disease. The primary aim of this study was to evaluate *c-MET* amplification status in advanced NSCLC patients and compare the consistency of *c-MET* amplification analyses in metastatic lymph nodes and tumor tissues.

Methods

Sample collection and DNA extraction

Patients with pathologically confirmed NSCLC were recruited between November 2004 and October 2014. Patients from three institutions in China – the Zhejiang Cancer Center, Fujian Cancer Hospital, and Zhejiang Rongjun Hospital – were enrolled. The diagnosis of NSCLC was based on histological findings of the tumor tissue, and the histological type was determined according to World Health Organization criteria.[18] Stages were identified according the 7th edition of the Tumor, Node, Metastasis (TNM) Classification of Lung Cancer.[19] The ethics committees of all three institutions reviewed and approved the study. All patients signed informed consent to participate in this study and gave permission for the use of their lymph nodes and tumor tissues. Patients did not receive any neoadjuvant treatment. Surgery and biopsy specimens from 368 cases were tested. Cancerous tissues with matched metastatic lymph node samples from 178 NSCLC patients were used to investigate the consistency of *c-MET* amplification. These samples were obtained during the same operation. Five samples of normal lung tissue were used as the control group. The inclusion criteria were: (i) - formalin-fixed and paraffin-embedded tumor tissues from stage III–IV NSCLC patients had been stored in a freezer at −80°C until analysis; (ii) the matched metastatic lymph nodes were at least N2 level or higher; (iii) none of the patients received any anti-tumor treatment, such as radiotherapy, chemotherapy or targeted therapy; (iv) patients had not been diagnosed with other malignancies before enrollment in the study; and (v) none of the patients had uncontrolled comorbid conditions, particularly hepatic disease. A total of 368 cases of advanced NSCLC were enrolled (193 men, 175 women), with a median age of 59 (range 29–84). There were 120 cases of squamous cell cancer and 248 of adenocarcinoma.

Four to eight slices of 4 μm thick paraffin tissue were taken and dewaxed. Genomic DNA was isolated using a proteinase-K digestion and phenol/chloroform extraction procedure using the QIAamp DNA FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was stored at −20°C until used.[20]

Real-time fluorescent quantitative PCR was used to detect the *c-MET* gene amplification in tumor tissues and metastatic lymph node samples. *c-MET* levels were evaluated using a method previously described.[21] Methylenetetrahydrofolate reductase (*MTHFR*) was selected as the control gene. The primers used were as follows: *MET*-sense: 5′-CCATCCAGTGTCTCCAGAAGTG-3′; *MET*-anti-sense: 5′-TTCCCAGTGATAACCAGTGTGTAG-3′; *MTHFR*-sense: 5′-CCATCTTCCTGCTGCTGTAACTG-3′; *MTHFR*-anti-sense: 5′-GCCTTCTCTGCC AACTGTCC-3′. Genomic DNA (20 ng) was amplified for 45 cycles (10 seconds at 94°C, 40 seconds at 58°C) in a Stratagene MX3000P Real-time PCR system (Agilent Technologies, Santa Clara, CA, USA), using the QuantiTect SYBR-Green PCR kit (Qiagen, Valencia, CA, USA) and 400 nm primers. We chose a ratio of *MET*: MTHFR > 1.5 to define *c-MET* amplification.[21] The *c-MET* gene amplifications were tested in metastatic lymph nodes and tissue samples and the consistency between these was compared. The potential association between *c-MET* gene amplification and clinical parameters was statistically evaluated.

Statistical and database analysis

A chi-square test was used to compare the association of *c-MET* gene amplification in metastatic lymph nodes and tumor tissues. Correlations between *c-MET* gene amplification and patients' clinical characteristics were analyzed using Fisher's exact test. $P < 0.05$ was considered statistically significant. $\kappa > 0.75$ represented excellent consistency, $0.4 \leq \kappa \leq 0.75$ good consistency, and $\kappa < 0.4$ a difference in consistency. All statistical analyses were performed using SSPS version 19.0 (IBM Corp., Armonk, NY, USA).

Results

c-MET gene amplification in metastatic lymph nodes and tumor tissues

Three hundred and sixty-eight cases of NSCLC were enrolled in this study. The *c-MET* gene amplification rate

was 8.97% (33/368) in cancerous tissues. In the 178 paired cases, *c-MET* gene amplification was positive in 7.95% (15/178) of cancerous tissues and 18.54% (33/178) of metastatic lymph nodes; 13 patients had positive *c-MET* gene amplification in both tissue and node samples. In the 33 cases of metastatic lymph nodes with positive *c-MET* gene amplification, the corresponding primary cancerous tissues were negative in 20 cases. In the 15 cases of primary cancerous tissues with positive *c-MET* gene amplification, the corresponding metastatic lymph nodes were negative in two cases (Table 1). Positive *c-MET* gene amplification was detected in metastatic lymph nodes more frequently than in primary cancerous tissues ($\chi 2 = 45.536$; $P < 0.001$). Consistency was measured using Cohen's Kappa coefficient (Kappa = 0.482; $P < 0.001$) (Table 2). When metastatic lymph nodes were used as surrogate samples of primary cancerous tissues, the sensitivity was 86.67% (13/15), and the specificity was 87.69% (143/163).

c-MET gene amplification and clinical characteristics

In 368 cancerous tissue samples, active *c-MET* gene amplification was detected in 33 samples, at an amplification rate of 8.97%. *c-MET* amplification status in primary cancerous tissue had no significant relationship with patient age, gender, pathological type, or smoking status ($P > 0.05$) (Table 1). In the 178 primary and metastatic lymph node paired cases, there was no association between *c-MET* amplification in the primary site and patients' basic characteristics (Table 3). However, a higher activated *c-MET* gene amplification rate indicated a significant association between metastatic lymph nodes and male smokers ($P < 0.001$) (Table 4). In patients with N2 stage or higher, the amplification rate was 28.41% (25/88) in men and 28.57% (28/98) in smokers; the positive amplification rate in male smokers was 29.51% (18/61).

Table 2 *c-MET* amplification in advanced primary NSCLC tissues and matched metastatic lymph node samples

		P			
M	Cases (n = 187)	+	–	P value	κ value
+	33	13	20	<0.001	0.482
–	145	2	143		

M, metastatic lymph node; NSCLC, non-small cell lung cancer; P, primary cancerous tissue.

Discussion

Lung cancer is the leading cause of cancer-related death globally. Chemotherapy has been the primary treatment for advanced lung cancer for several decades. The emergence of EGFR-tyrosine kinase inhibitors (EGFR-TKIs) has led to personalized therapy in recent years, taking into account patient-specific characteristics, such as the harboring of active *EGFR* mutations or other positive driver genes. The hepatocyte growth factor/c-MET signaling pathway has been recognized as a negative prognostic factor and potential target,[10] thus several anti-MET compounds are under evaluation in clinical trials.[22] EGFR-TKIs can provide better efficacy and quality of life to suitable patients. *c-MET* amplification plays a limited role in primary resistance to TKIs; however, it is one of the most relevant factors of EGFR-TKI acquired resistance.[21] The molecular targeted drug crizotinib[23] and other compounds, such as MET monoclonal antibodies[24,25] and kinase inhibitors,[26,27] have been US FDA approved for use in *ALK* and *ROS1* positive NSCLC, however *c-MET* gene amplification was not included as a potential prognostic factor. Thus, discovering the mechanism of *c-MET* gene amplification is urgent, as the ability to obtain comprehensive molecular profiling in clinical practice using different DNA purified skills to detect driver oncogenes could assist in early diagnosis and enhance treatment.

Table 1 Correlation of *c-MET* amplification in advanced primary NSCLC tissue and basic patient characteristics

Characteristics	Cases (n = 368)	*c-MET* amplification	*c-MET* non-amplification	%	χ^2 and P values
Gender					
Male	193	21	172	10.88	$\chi^2 = 1.820$
Female	175	12	163	6.86	$P = 0.177$
Age (year)					
≥60	213	15	198	7.04	$\chi^2 = 2.296$
<60	155	18	137	11.61	$P = 0.130$
Smoking status					
Smoker	228	25	203	10.96	$\chi^2 = 2.929$
Non-smoker	140	8	132	5.71	$P = 0.087$
Pathological type					
Squamous	120	6	114	5.00	$\chi^2 = 3.433$
Adenocarcinoma	248	27	221	10.89	$P = 0.064$

NSCLC, non-small cell lung cancer.

Table 3 Correlation of *c-MET* amplification in primary and metastasis tissue paired with advanced NSCLC primary cancerous tissue and patients' basic characteristics

Characteristics	Cases (n = 187)	c-MET amplification	c-MET non-amplification	%	χ^2 and P valve
Gender					
Male	88	7	81	7.95	χ^2 = 0.050
Female	90	8	82	8.89	P = 0.822
Age (year)					
≥60	103	8	95	7.77	χ^2 = 0.138
<60	75	7	68	9.33	P = 0.710
Smoking status					
Smoker	98	11	87	11.22	χ^2 = 1.478
Non-smoker	80	4	76	5.00	P = 0.224
Pathological type					
Squamous cancer	63	3	60	4.76	χ^2 = 1.042
Adenocarcinoma	115	12	103	10.43	P = 0.307

NSCLC, non-small cell lung cancer.

In our study, *c-MET* gene amplification was detected in 368 cases of advanced primary NSCLC by reverse transcription-PCR, with a *c-MET* gene amplification rate of 8.97%. This result is inconsistent with the results of a study conducted by Bean *et al.*[21] however, differences in race, sample size, and ratios of adenocarcinoma and squamous cell cancer, as well as the conditions and experimental methods may explain this discrepancy. Our results indicated that, although the positive *c-MET* gene amplification rate was not as high as the *EGFR* mutation rate, targeted therapy may benefit overall survival in advanced NSCLC by almost 10%. Because amplification leads to EGFR-TKI primary and secondary resistance, it is of important clinical value and should be evaluated along with *EGFR* and *ALK* in standard gene detection practice. The positive *c-MET* gene amplification rate increases after tumor metastasis or administration of EGFR-TKIs, therefore future studies should include cases of metastasis and patients treated with EGFR-TKIs in order to achieve consistent conclusions.

Our results showed that positive *c-MET* gene amplification was significantly higher in lymph node metastasis than in the primary tumor, consistent with Han *et al.*'s results.[28] MET protein is a product of the *c-MET* gene and promotes tumor invasion and metastasis.[13] Our study results prove that the MET protein promotes tumor invasion and metastasis at a DNA level and that MET protein overexpression occurs at gene level. However, the positive rate of MET protein overexpression was significantly higher than that of *c-MET* gene amplification.[29] In addition to gene level, there may be other regulatory mechanisms in tumor invasion and metastasis, which would allow for the earlier detection of NSCLC and for further individualized treatment. The relationship between these needs to be further demonstrated.

Good consistency was shown for *c-MET* gene amplification in metastatic lymph nodes, with sensitivity and specificity of 86.67% and 87.69%, respectively. *c-MET* gene amplification in primary and metastatic lymph nodes was consistent (κ = 0.482, P < 0.001). Lymph node metastasis

Table 4 Correlation of *c-MET* amplification in primary and metastatic tissues paired with advanced NSCLC metastatic lymph node samples and basic patient characteristics

Characteristics	Cases (n = 187)	*c-MET* amplification	*c-MET* non-amplification	%	χ^2 and P values
Gender					
Male	88	25	63	28.41	χ^2 = 11.226
Female	90	8	82	8.89	P < 0.001
Age (year)					
≥60	103	15	88	14.56	χ^2 = 2.559
<60	75	18	57	24.00	P = 0.110
Smoking status					
Smoker	98	28	70	28.57	χ^2 = 14.531
Non-smoker	80	5	75	6.25	P < 0.001
Pathological type					
Squamous cancer	63	7	56	11.11	χ^2 = 3.563
Adenocarcinoma	115	26	89	22.61	P = 0.059

NSCLC, non-small cell lung cancer.

can be used as a surrogate to predict amplification of the *c-MET* gene in primary tumors when it is difficult to obtain primary tissue. Moreover, samples from primary or metastatic lesions can be used as long as positive amplification of the *c-MET* gene is detected, at which time targeted therapy is strongly recommended. In patients with greater than N2 lymph node metastasis, *c-MET* gene amplification was higher than in primary cancer tissue ($\chi^2 = 45.536$; $P < 0.001$). The detection of metastatic lymph node gene amplification in these patients could improve the sensitivity rate, and expand the indications for targeted therapy, perhaps better than detection in primary tumors.

In male smoking patients with lymph node metastasis above stage N2, the positive *c-MET* gene amplification rate was as high as 29.51%. This result indicates that these patients may benefit from *c-MET* gene amplification assay and the administration of related targeted therapies. However, when using primary tumor samples, no association between *c-MET* gene amplification and clinical characteristics was observed. These results were consistent with studies by Han *et al.*[28] and Beau-Faller *et al.*[30] In patients with greater than stage N2 lymph node metastasis, although the amplification rate in adenocarcinoma was substantially higher than in squamous cell carcinoma, the difference was not statistically significant. This may be because a trend could not be formed with the small sample size.

Our study showed that gene mutation status is not consistent between lymph node metastasis and primary tumors. Because of the heterogeneity of tumors, there is often a large difference. It has been reported that some patients with negative *EGFR* mutation status are still able to benefit from EGFR-TKI treatment, and the reason for this may lie in the heterogeneity of tumors.[31] Oncologists often pay more attention to mutation in primary tumors, preferring primary tumor biopsy for gene detection, but ignore metastatic gene mutation when both tissues could be obtained, resulting in EGFR gene mutation negative results in primary tumors but positive in metastatic patients, thereby missing an opportunity to apply targeted treatment.[32–34] The therapeutic effect of EGFR-TKIs is not consistent in primary tumors and metastases, which may also be explained by tumor heterogeneity. The results of our study indicate that close attention should be paid to metastatic tissue mutation during *c-MET* targeted therapy to avoid missing a diagnosis or an opportunity for therapeutic effect. This requires oncologists to evaluate each NSCLC driver gene by its primary and metastatic lesions whether there are differences in the future, in order to define the significance of driver gene detection in lymph node metastasis.

In conclusion, the identification of *c-MET* gene amplification is crucial for therapy selection and should be included as a part of routine detection. Lymph node metastasis samples can be used as alternative lesions to detect *c-MET* gene amplification when it is difficult to obtain sufficient samples from primary tumor tissue. The highest *c-MET* gene amplification rate was observed in male smokers with stage N2 or greater lymph node metastasis. Heterogeneity of gene mutations in primary and metastatic lymph nodes should be considered in molecular targeted therapy. Further investigations with larger sample sizes to validate our results are warranted.

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Disclosure

No authors report any conflict of interest.

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