Analysis of the status of EGFR, ROS1 and MET genes in non-small cell lung adenocarcinoma

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Summary

Purpose: To investigate the status and distribution of epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (MET), and receptor tyrosine kinase (ROS1) genes in patients with non-small cell lung (NSCL) adenocarcinoma.

Methods: The copy number of the MET gene was detected using fluorescence in situ hybridization (FISH). The splice mutation in exon 14 gene was detected by Sanger sequencing. The mutations in EGFR and the fusion of the ROS1 gene were detected using the fluorescence real-time quantitative PCR method (RT-qPCR).

Results: The gene mutation frequency of EGFR was 46.51%. There were 7 types of mutations; exon 19 deletions and exon 21 L858R mutations were most frequent. There were 3 cases of double mutations. The MET gene had in-creased copy numbers in 9.88% of the NSCL adenocarcino-ma] patients; 3.49% of MET mutations in NSCL adenocarcinoma included 3 intron mutations. The ROS1 gene fusion frequency was 1.74%.

Conclusion: The NSCL adenocarcinoma patients who were females, did not have a smoking history, and had high grade of differentiation, had higher EGFR mutation rates. Although the MET gene amplification and ROS1 gene fusion in NSCL adenocarcinoma were low-probability events, detection of the gene status of EGFR, ROS1, and MET will facilitate screening more NSCL adenocarcinoma patients who might benefit from targeted therapy.

Key words: EGFR, MET genes, non-small cell lung adenocarcinoma, ROS1

Introduction

Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancers. Approximately 75% of the patients are in the mid- and late-stages of NSCLC when diagnosed; even with surgical resection, the 5-year survival is < 15% [1]. Recently, with the in-depth studies of cell signaling pathways and expression changes in related genes, some gene changes have been discovered that can promote the progression of NSCL adenocarcinoma, which may lead to the development of targeted site-specific inhibitors [2-4]. These targeted genes may also be significant for predicting appropriate therapy and the prognosis of patients. Clinical studies involving the EGFR gene have shown that EGFR status is an important predictor of the treatment effects of targeted tyrosine kinase inhibitors (TKIs) [5], although other target genes have also been studied in depth. For example, chromosomal rearrangement of the ROS1 gene and the binding of MET with corresponding ligands causes dimerization and phosphorylation of the receptors and amplification, overexpression, and mutation in a variety of tumors, including kidney, colon, and gastric cancers. Especially in NSCLCs, these events can initiate a series of downstream signaling pathways to promote tumor invasion and metastasis. Some gene mutations of EGFR and ROS1 have been used to predict

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treatment effects associated with EGFR-TKI. High levels of MET gene expression may be associated with a poor prognosis [4,6,7]; however, there are no uniform experimental methods and standards for confirming MET overexpression.

The current study aimed to detect the gene status of EGFR, ROS1, and MET in NSCL adenocarcinoma tissues, to summarize the distribution of each gene and the association with clinical pathologic features, and to provide a corresponding theoretical basis for identifying patients suitable for targeted drug treatment for NSCL adenocarcinoma.

Methods

Specimen sources

Experimental samples collected from January-September 2015 were selected as study objects. Samples were chosen according to the following criteria: 1: lung cancer was histopathologically confirmed by 2 pathologists; 2: relevant medical information was complete; 3: patients signed informed consent forms; and 4: patients did not receive radiotherapy or chemotherapy before gene detection. A total of 172 samples were studied, of which 68 surgical resection samples and 104 percutaneous puncture of lung biopsy samples. In addition, 10 cases of normal lung tissues were selected as controls. There were 95 males and 77 females (mean age 59 years; range 26-80). Fifty-four of the patients were non-smokers and 118 smokers. There were 23 cases of highly differentiated adenocarcinoma, 66 cases of moderately differentiated adenocarcinoma, and 83 cases of poorly differentiated adenocarcinoma.

Fluorescence in situ hybridization (FISH)

Paraffin blocks of each sample were selected, sectioned at a thickness of 4-5 μm, and conventionally deparaffinized and hydrated. Samples were loaded on a Thermo Brite automatic FISH instrument (Thermo Brite Elite Leika Biosystems, Richmond Inc, Richmond, USA). The operational procedures were as follows: Each slice was put in distilled water for 25 min at 95°C; pepsin for 20 min at 37°C; 2X SSC for 5 min; and dehydration in an ethanol gradient. Then the slices were removed from the instrument. To each slide, 100 μl of MET probe (Abbott Molecular, Des Plaines, IA, USA) was added. Also, 100 μl of two-color MET and CEP7 probe were added to the slides. The slides were loaded on the automatic FISH hybridization instrument for hybridization at 73°C for 5 min and at 37°C for 16-20 hrs. After washing with 0.3% NP-40 in 0.7% 2×SSC at 67°C for 3 min, the slides were dried at room temperature. Afterwards 15 μl of DAPI were added and the results were evaluated with a fluorescence microscope. The interpretation of the results followed the recommendations of Cappuzzo et al. [7]. For single probe, positive MET amplification was defined as a mean copy number of MET >5/cell. For two-colors, positive MET amplification was defined as a MET:CEP 7 ≥2.

Real-time qPCR

1. EGFR (NM_005228.3) detection: Paraffin blocks of each sample were selected. Three 4-5 μm thickness sections from surgical samples and 10 sections from percutaneous puncture lung biopsy samples were each placed in a 1.5-ml Eppendorf (EP) tube. The DNA was isolated according to the instructions of the FFPE sample DNA isolation reagent kit (Amoy Diagnostics Co., Xiamen, China). After extraction, the DNA purity and concentrations were determined using a UV spectrophotometer. The A_{260}/A_{280} ratio should be between 1.7 and 2.0, and the A_{260}/A_{230} ratio should be >1.7. The concentration of the diluted sample was 5 ng/μl. The specific mutation sites in exons 18, 19, 20, and 21 of the EGFR gene were detected by RT-qPCR using the EGFR gene mutation detection reagent kit (ACCB Biotech, Beijing, China). The reaction conditions were: 95°C for 10 min, 95°C for 15 s, and 60°C for 60 s, for a total of 40 cycles. The data were interpreted according to the following criteria:
1) The hexachlorofluorescein (HEX) and carboxyfluorescein (FAM) of negative control (NC) should be amplified, and the HEX and FAM of positive control (PC) should be amplified (cycle threshold/CT ≤35).
2) The HEX and FAM of the reference gene should be amplified (22≤CT ≤30).
3) The internal control gene, HEX, should be amplified (CT ≤35). If the internal control gene, HEX, was not amplified or if the CT was larger, the FAM signal should be amplified (CT ≤39).
4) For the gene mutations testing, if the FAM signal of the mutation site of the sample was amplified and if the CT was ≤36, the result was positive; if the CT was >39 or if there was no amplification, the result was negative.

2. ROS1(NM_002944.2) detection: Paraffin blocks of each sample were selected. Three 4-5 μm thickness sections from surgical samples and 10 sections from percutaneous puncture lung biopsy samples were placed in individual 1.5-ml EP tubes. Deparaffinization and RNA isolation were performed according to the instructions of the FFPE sample RNA isolation reagent kit (Amoy Diagnostics Co., Xiamen, China). The RNA purity and concentrations were determined using a UV spectrophotometer. The A_{260}/A_{280} ratio should be between 1.9 and 2.1. The human ROS1 gene fusion detection reagent kit (Amoy Diagnostics Co.) was used for reverse transcription reactions. Samples and enzymes were mixed thoroughly and added to 4-strip tubes. The detection of ROS1 fusion genes was performed using RT-qPCR according to the following procedure: The first reaction took place at 95°C for 5 min for 1 cycle. The second reaction took place at 95°C for 25 s, 64°C for 20 s, and 72°C for 20 s, for a total of 15 cycles. The third reaction took place at 93°C for 25 s, 60°C for 35 s, and 72°C for 20 s, for a total of 31 cycles. The data were interpreted according to the following criteria:
1) The HEX and FAM of NC should be amplified, and the HEX in Victoria (VIC) and FAM of all PC should be amplified (CT ≤24).
2) The HEX in VIC should be amplified (CT ≤20).
3) For any tube in samples 1-4 that had a FAM signal am-

plification curve, if the CT value was ≥30, the sample did not contain the ROS1 gene fusion; if the CT value was <30, the sample contained the ROS1 gene fusion.

Nested PCR and Sanger terminal termination method

Target fragments and primers: Exon 14 and the surrounding DNA sequence were as follows, in which the lowercase letters are introns, and the gray background with capitalized letters is the met-14 exon sequence. The PCR primers were as follows:
MET-14-F3: 5'-TTACTGTTCATTTTTAGAAGTTACC-3'
MET-14-R3: 5'-TTAAGAGCCATGTAATTTTGTGTCA-3'
MET-14-F4: 5'-TAAGATTGTCGTCGATTC-3'
MET-14-R4: 5'-TACTTACTTGGCAGAGGT-3'

Nested PCR: primers F3/R3 for 1 round of PCR, F4/R4 for 2 rounds of PCR (1. 94ºC for 5 min, 94ºC for 30s, 58ºC for 45s, 58ºC for 45s, 25 cycles; 94ºC for 20s, 52ºC for 45s, 72ºC for 20s, 10 cycles at 72ºC for 5 min; 94ºC 2 min; 94ºC for 20s, 55ºC for 20s, 72ºC for 20s, 30 cycles; 72ºC for 5 min) and the Sanger terminal termination method. Application Software: SeqMan & Chromas 2, then exon 14 5' intron 50 bp sequence + exon 14 sequence + 3' intron 50 bp sequence (underlined) was selected to verify the authenticity of the mutation according to the results of bidirectional sequencing.

Statistics

SPSS 13.0 statistical software was used for data analysis. The calibrated x^2 test was performed for analysis. The status of each gene was analyzed using Spearman's correlation test. A $p < 0.05$ indicated that the difference was statistically significant.

Results

Analysis of EGFR gene mutation results

Among the 172 cases of NSCL adenocarcinoma patients, 80 cases had detectable EGFR gene mutations. The mutation frequency was 46.51% (80/172). A total of 7 types of mutations were detected, including 1 case with point mutations in exon 18 (exon 18, c. 2155G>A/T, c.2156G>C mutation type [p.G719S/C/A]), 47 cases of exon 19 deletions (exon19, c.2235_2249del15, c.2236_2250del15, c.2237_2251del15, c.2235_2252>AAT, c.2238_2252del15, c.2239_2253del15, c.2240_2254del15, c.2233_2247del15, c.2235_2251>AG, c.2240_2257del18, c.2239_2256del18, c.2238_2255del18, c.2239_2258>CA, c.2236_2253del18, c.2237_2254del18, c.2237_2255>T, c.2235_2252del18, c.2235_2255>AAT, c.2237_2256>TC, c.2238_2248>GC, c.2239_2247del9, c.2239_2248>C, c.2236_2248>CAAC, c.2236_2248>AGAC, c.2235_2248>AATTC, c.2239_2251>C, c.2240_2251del12, c.2238_2252>GCA, c.2239_2252>CA, c.2235_2246del12, c.2235_2251>AATTC), 3 cases of point mutations in exon 20 (exon 20, c.2303G>T mutation type [p.S768I]), 1 case of a point mutation in exon 20 (exon 20, c.2369C>T mutation type [p.T790M]), 2 cases of insertions in exon 20 (exon 20, c.2307_2308ins

GCCAGCGTG, c.2309_2310AC>CCAGCGTGGAT, c.2311_2312insGCGTGGACA, c.2310_2311insGGT, c.2319_2320insAACCCCAC, c.2319_2320insCAC) and 29 cases of exon 21 mutations (exon 21, c.2573T>G, c.2573_2574TG>GT mutation types [p.L858R]). There were 3 cases of double mutations (Figure 1). The differences in gender, smoking status, and differentiation grades between patients with and without EGFR mutations were statistically significant (p<0.05; Table 1).
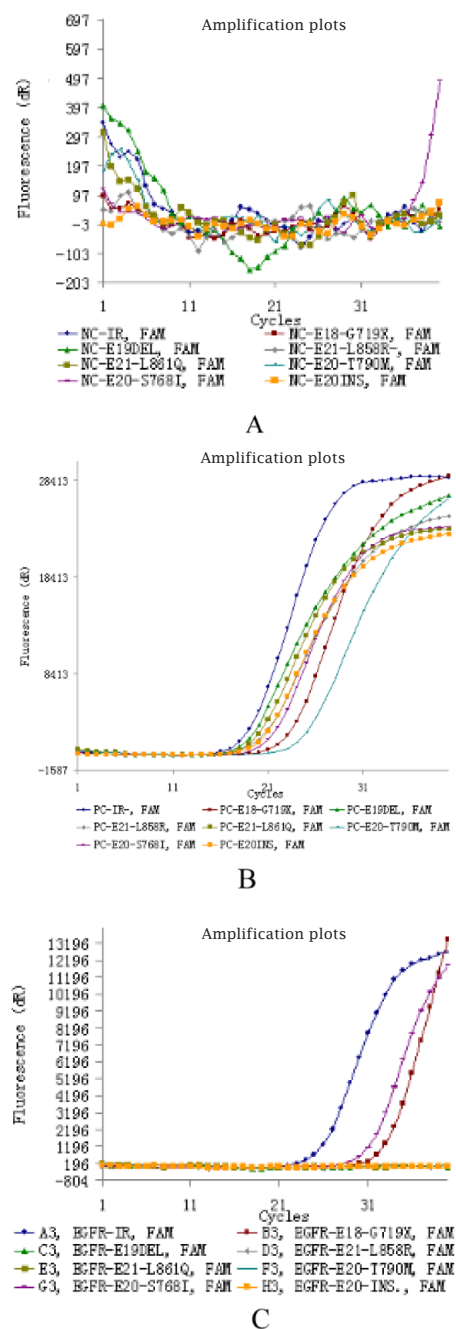

Figure 1. EGFR gene results: **(A)** FAM graph of negative control; **(B)** FAM graph of positive control; **(C)** FAM curve of samples with double mutants: the left curve is the internal reference, the middle curve (CT=32.67) shows an exon18 mutation (c. 2155G>A/T, 2156G>C, p. G719S/C/A), the right curve (CT=34.27) indicates an exon 20 mutation (c.2303G>T, p. S768I).

Analysis of ROS1 gene mutation results

There were 3 cases of the ROS1 gene fusion (Exon-34/SLC34A2 e4, SLC34A2 e14del, CD74 e, SDC4 e4, EZR e10; Figure 2). The fusion frequency was 1.74% (3/172). The differences in age, gender, smoking status, differentiation grade, and specimen type between patients with and without the ROS1 gene fusion were not statistically significant (p>0.05; Table 1).

Analysis of the MET gene amplification results

MET FISH detection was performed on 172 samples; acceptable results were obtained from all of the samples. There were 17 cases of positive MET samples (Figure 3). The positive frequency was 9.88%. The range of the mean MET copy number (total MET signal/50 cells) was 2.0-6.3, and the median was 2.8. There were 12 cases of positive MET samples (Figure 4). The positive frequency was 6.98%.

MET gene amplification was commonly seen in patients who smoked (11/172; 6.4%) and had poor differentiation (10/172; 5.81%). The differences in age, gender, smoking status, differentiation grade, and specimen collection method between the patients with and without MET gene amplification were not significantly different (Table 1).

MET Sanger sequence

There were 6 heterozygous point mutations of the MET gene in NSCL adenocarcinoma, as follows: c.G3028T (p.D1010Y); c.G3028A (p.D1010N); c.T2911C (p.Y971H); c.C3023T (p.P1008L), intron 13-14 c>t, c.G2897A (p.S966N); c.G2958A (p.R986Q), intron 13-50 g>a; and intron 13-11 t>c, intron 14+44 c>t. The differences in age, gender, smoking status, differentiation grade, and specimen collection method between the patients with and without MET gene amplification were not significantly different.

Associations among MET gene amplification, EGFR mutations and ROS1 fusion

Among the 17 cases of patients with MET amplification, 5 (29.41%) had EGFR gene mutations. ROS1 gene fusion was detected in 2 patients with EGFR mutations and MET amplification. MET gene amplification was not associated with EGFR mutations and ROS1 gene fusion (p>0.05; Table 2).

Table 1. Clinicopathological characteristics of non-small-cell lung adenocarcinoma in relation with EGFR, ROS1, and MET genes

Category	n	MET amplification	MET non-amplification	p	ROS1 fusion	ROS1 non-fusion	p	EGFR mutation	EGFR non-mutation	p
Age (years)		17						80	92	
≤59	89	10	79		2	87		36	54	
>59	83	7	76	0.538	1	82	0.602	44	38	0.073
Gender										
Male	95	9	86		2	93		32	63	
Female	77	8	69	0.841	1	76	0.688	48	29	0
Smoking										
Non-smoking	54	6	48		1	53		37	17	
Smoking	118	11	107	0.715	2	116	0.942	43	75	0
Grade of differentiation										
Well and middle	89	7	82		1	88		33	56	
Poor	83	10	73	0.358	2	81	0.52	47	36	0.1
Obtaining specimens by										
Surgery	68	6	62		0	68		28	40	
Biopsy	104	11	93	0.706	3	111	0.177	52	52	0.232

Table 2. MET gene amplification in relation with *EGFR* and *ROS1*

	n	MET mutation	MET non-mutation	r	p
EGFR mutation	80	5	75		
EGFR non-mutation	92	12	80	-0.114	0.138

Figure 3. MET gene amplification detected by FISH with single probe (x1000).
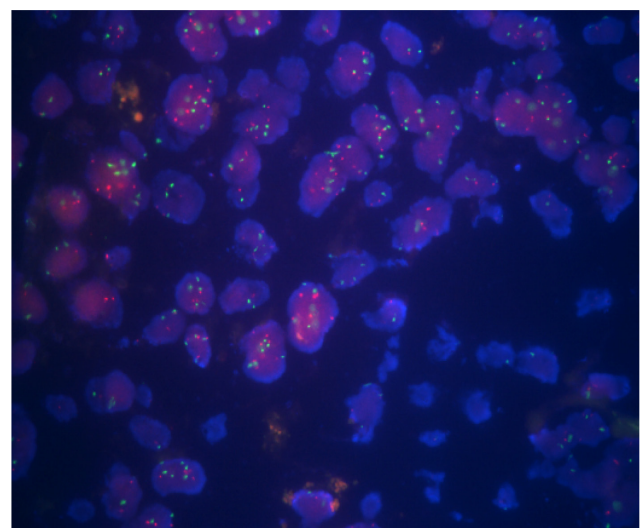
Figure 4. MET gene samplification and CEP 7 detected by FISH with double probes (x1000).
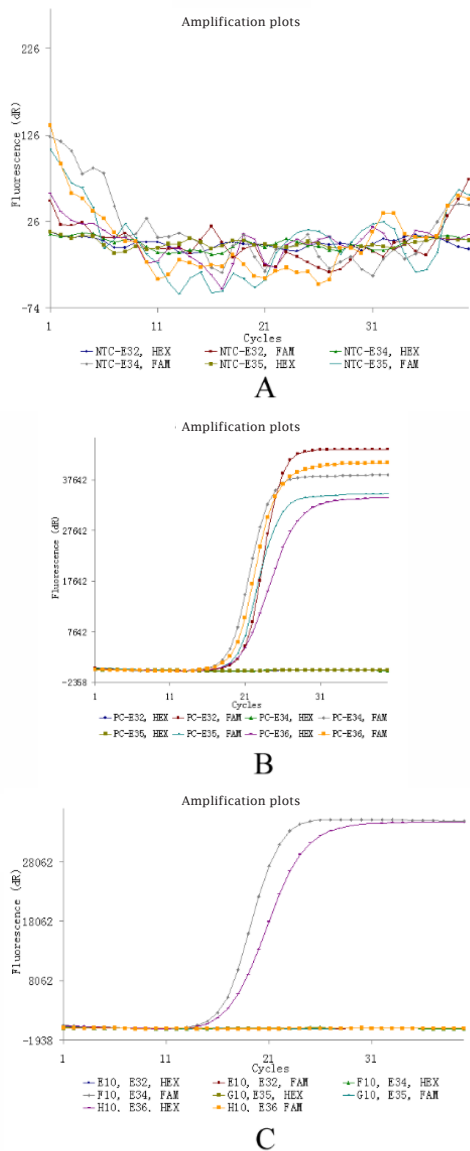
Figure 2. ROS1 gene results: **(A)** FAM and HEX graph of negative control; **(B)** FAM and HEX graph of positive control; **(C)** mutation of the sample: the left curve (CT=16.99) shows a fusion mutation (Exon-34 / SLC34A2 e4, SLC34A2 e14del, CD74 e6, SDC4 e4, EZR e10), and the right curve indicates HEX of the internal reference (CT=18.02).

Discussion

EGFR mutations are generally located between exons 18 and 21 of the coding region for ATP binding by the EGFR tyrosine kinase. In addition, the treatment effects in NSCL adenocarcinoma patients with EGFR mutations have been confirmed by clinical studies. EGFR-TKI drugs have been used as first-line therapeutic drugs for patients with EGFR mutations [8]. This study showed that the EGFR mutation frequency among 172 patients with lung adenocarcinoma was 46.51% (80/172) and EGFR mutations were common in women and non-smokers. These results are consistent with previ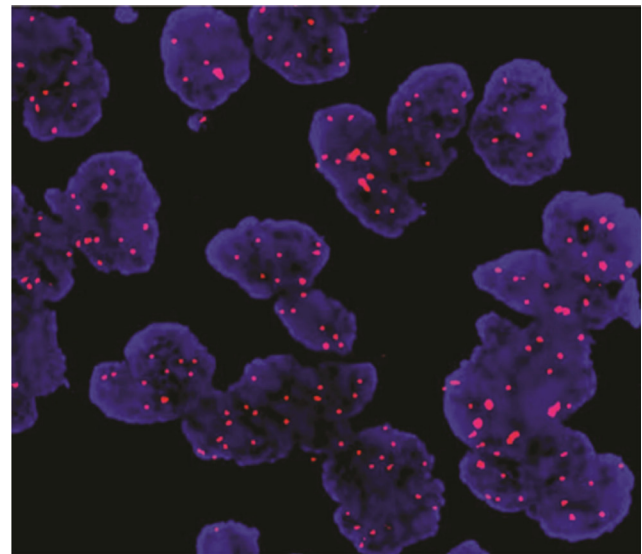ous studies [5,9]. The mutation hotspots in the EFGR gene were mainly exon 19 deletions and exon 21 L858R missense mutations (approximately 95%). Studies have shown that the EGFR mutations are associated with the treatment effects of targeted therapy [10]: the effective rates of EGFR-TKI agents in the treatment of patients with exon 19 deletion mutations or exon 21 point mutations were > 70%, whereas the exon 20 T790M mutation produced gefitinib resistance in patients [11]. Our study detected 3 cases of exon 20 insertional mutations, 1 case of a T790M mutation, and 2 cases of S768I mutations. Previous studies [12,13] have reported different treatment effects of TKI on patients with a S768I mutation. The effects of insertional mutations on the results of treatment with TKI require further observations, and the relevant mechanisms of action are still under study. Our study discovered several

double-mutation cases, including exon 18 and exon 20 S768I, the exon 19 deletion, and exon 21 L858R and T790M. The treatment effects of EGFR-TKI in patients with double mutations are still not clear; an understanding of the relevant clinical efficacy requires further investigation and the study of clinical data.

Abnormal expression of the MET gene and phosphorylation of ligands after binding to HGF [14] together activate a series of intracellular signal transduction pathways, including Ras/Raf/ERK/MAPK and PI3k/Akt [15], to cause the progression of tumor cells into proliferation, invasion, and metastasis. Previous studies [16,17] have shown that MET gene amplification occurs in 4-21% of NSCLC patients and in 4% of adenocarcinoma patients, is associated with a poor prognosis [13,14], and MET gene amplification was involved in the mechanism of acquired drug resistance to EGFR-TKI [18]. The common methods for detection of MET amplification include RT-PCR, FISH and SISH. Studies have shown that the positive MET gene amplification frequencies in NSCL adenocarcinoma detected by RT-qPCR varied, ranging from 5.6-21%, indicating that this method is not the best approach for evaluating MET gene amplification [19,20]. FISH is a better method than RT-PCR for evaluating MET gene amplification because tumor cells can be counted and interference by non-tumor cells can be excluded; however, there is no uniform standard for interpreting MET FISH results. Some studies [21,22] have monitored the statistical results of HER2 FISH in breast cancer, and some studies followed the EGFR FISH in lung cancer. Currently, the commonly used method in clinical studies is the MET mean copy number/50 tumor cells ≥5.0, as proposed by Cappuzzo et al. [7]. This study adopted that method and showed that 9.71% of lung adenocarcinomas contained amplified MET genes by single probe, and for double probe the positive frequency of MET was 6.98%. The detection rate of MET amplification following this standard is relatively low. Our study only used the single-color MET probes to observe the changes in copy number and did not consider the MET/CEP7 ratio and the number of cells with clustered signals.

A series of studies have been conducted involving the association between MET amplification and clinicopathological features; however, there are no consistent conclusions. The study conducted by Okuda et al. [20] showed that 5.6% of male NSCLC patients who smoked cigarettes were prone to MET gene amplification. Another study showed that MET amplification was not associated with gender, smoking history or tissue type [23]. In the current study, we interpreted the data according to the method of Cappuzzo et al. [7] and showed that MET gene amplification was not significantly associated with age, gender, smoking status, differentiation grade, or specimen collection method; however, MET gene amplification is commonly noted in male patients >59 years of age (7/172 [4.07%]) who smoked (11/172; 6.40%) and had poorly differentiated tumors (10/172; 5.81%). We will initiate a study involving the correlation between pathology staging and MET gene amplification in the future.

MET gene amplification in NSCLC will persistently activate the downstream signaling pathways, including PI3K-Akt, and reduce the inhibitory effects of EGFR-TKI on target genes, thus inducing primary or secondary drug resistance [24]. This result suggests that inhibiting EGFR and MET pathways together can overcome acquired drug resistance to EGFR-TKI [25]. Our study showed that the MET gene and the EGFR mutations were not significantly correlated; however, our results also showed that 4 patients with MET gene amplification also had EGFR mutations. In actual clinical treatment, the targeted effect of a single EGFR-TKI drug may be worse than the result of combined drug treatment. The MET gene may act as one of the effective predictive factors for treatment of late-stage NSCL adenocarcinoma.

ROS1 gene is a newly discovered proto-oncogene in NSCLC and codes a receptor tyrosine kinase. When ROS1 gene is fused with other genes, including SLC34A2 and CD74, ROS1 gene will persistently activate the ROS1 tyrosine kinase and the downstream signaling pathways, including JAK/STAT, PI3K/AKT, and RAS/MAPK, to induce the development of tumors. The frequency of ROS1 fusions in NSCLC is 1-2%, mostly in lung adenocarcinoma patients [26-28]. The current study showed that ROS1 gene fusion frequency in lung adenocarcinoma was 1.74%, which was consistent with results in previous reports [29,30]. The majority of ROS1 fusions did not co-exist with the expression of other therapy-targeted genes in NSCLC [28,31,32]. The 3 positive cases in this study did not have EGFR mutations or MET gene amplification. In recent years, it has been shown that ROS1 shares 49% homology with anaplastic lymphoma kinase (ALK); therefore, treatment of NSCLCs that have ROS1 rearrangements using ALK kinase inhibitors is possible [4,32,33]. Whether or not the treatment outcome and prognosis of patients is promising and whether or not there are other clinical effects will require further observations for confirmation.

Many studies have confirmed that mutations

in lung adenocarcinoma driver genes are mutually exclusive; however, some individual case reports have shown the presence of 2 or more gene mutations together [34,35]. The treatment program for patients with more than 2 combined mutations may be different from the treatment program for patients with a single EGFR mutation. Our study did not reveal cases with the presence of EGFR, ROS1 and MET abnormalities together; however, 5 cases of MET amplification occurred in the EGFR mutation cases. Therefore, the effects of targeted therapy require support from further clinical validation. In addition, this study also showed cases with the co-existence of EGFR drug resistance and activation mutations. The targeted therapy effects also require further clinical confirmation.

Acknowledgements

Conflict of interests

The authors declare no conflict of interests.

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