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ORIGINAL ARTICLE

Expression and copy number gains of the *RET* gene in 631 early and mid stage non-small cell lung cancer cases

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Copy number variation; early and middle stage; gene rearrangement; non-small cell lung cancer (NSCLC); RET expression.

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Abstract

Background: To identify whether *RET* is a potential target for NSCLC treatment, we examined the status of the *RET* gene in 631 early and mid stage NSCLC cases from south central China.

Methods: *RET* expression was identified by Western blot. *RET*-positive expression samples were verified by immunohistochemistry. *RET* gene mutation, copy number variation, and rearrangement were analyzed by DNA Sanger sequencing, TaqMan copy number assays, and reverse transcription-PCR. *ALK* and *ROS1* expression levels were tested by Western blot and *EGFR* mutation using Sanger sequencing.

Results: The *RET*-positive rate was 2.5% (16/631). *RET*-positive expression was related to poorer tumor differentiation ($P < 0.05$). In the 16 RET-positive samples, only two samples of moderately and poorly differentiated lung adenocarcinomas displayed *RET* rearrangement, both in *RET-KIF5B* fusion partners. Neither *ALK* nor *ROS1* translocation was found. The *EGFR* mutation rate in *RET*-positive samples was significantly lower than in *RET*-negative samples ($P < 0.05$).

Conclusion: *RET*-positive expression in early and mid stage NSCLC cases from south central China is relatively low and is related to poorer tumor differentiation. *RET* gene alterations (copy number gain and rearrangement) exist in all *RET*-positive samples. *RET*-positive expression is a relatively independent factor in NSCLC patients, which indicates that the *RET* gene may be a novel target site for personalized treatment of NSCLC.

Introduction

Non-small cell lung cancer (NSCLC), which accounts for 80–85% of all lung cancer cases, has one of the highest cancer incidences in the world.[1,2] It is usually diagnosed at an advanced stage and has a dismal prognosis.[2] NSCLC is further divided into subtypes based on histology and approximately 30% of cases are squamous cell carcinoma. The remaining 70% are classified as non-squamous NSCLC, which includes adenocarcinomas, large-cell carcinomas, and less well-differentiated tumors.[1,3]

Several key genetic alterations have been found in lung cancer, such as *EGFR* mutations and *ALK* rearrangement.[4–7] The application of EGFR-tyrosine kinase inhibitors (TKIs) has highlighted the importance of targeted therapeutic agents to appropriate patient populations with specific genetic alterations.[8,9] In addition, gene rearrangements, such as *ALK* and *ROS1* have also been identified in NSCLC; tumors with *ALK* and *ROS1* rearrangement are responsive to ALK-TKIs.[10]

Although TKIs are effective for NSCLCs with corresponding gene mutations or rearrangements, the long-term efficacy is not satisfactory because of drug resistance.[4,5] Recently, a new receptor tyrosine kinase gene, *RET*, has been identified in lung cancer, and is rearranged in 1% of lung adenocarcinoma cases.[11] *RET* is a proto-oncogene (l0q 11. 2) located on the long arm of chromosome 10, including

21 exons with a total length of about 60 000 bp.[12] The protein encoded by *RET* is a tyrosine kinase receptor, which binds to the ligand and stimulates intracellular phosphorylation, which in turn activates downstream signals and plays a critical role in proliferation, neuronal navigation, and differentiation.[13,14] In NSCLC, the most common RET fusion pattern is with *KIF5B*.[15,16] The first 15 exons of *KIF5B* containing kinesin motor and coiled-coil domains are rearranged to exons 12–20 of the *RET* gene, which contains the RET kinase domain. This rearrangement produces adverse activation of *RET* with homodimerization underlying the oncogenic potency of the gene fusion product.[15,16] *RET* mutations are present in nearly all hereditary medullary thyroid cancer patients, and approximately 30% with *RET* gene copy number alteration are associated with poor outcomes.[17] Therefore, *RET* copy number alteration is a vital gene alteration in malignant tumors.

In this study, we examined the status of the *RET* gene in 631 early and mid stage NSCLC cases from south central China to identify whether *RET* is a potential target for NSCLC treatment.

Methods

We identified *RET* expression in all samples using Western blot. We then analyzed *RET* gene mutation, copy number variation, and rearrangement in *RET*-positive expression samples using DNA Sanger sequencing, TaqMan copy number assays, and reverse transcription (RT)-PCR. *ALK* and *ROS1* expression was detected by Western blot and *EGFR* mutation by exon sequencing.

Sample and clinical data of non-small cell lung cancer patients

NSCLC samples (n = 631, 466 men, 165 women; age range 21–84) and normal tissues (> 5 cm away from the tumor edge) were consecutively collected from patients by pulmonary lobectomy at the second Xiangya Hospital (Changsha, Hunan, China) from July 2008 to July 2014. All patients signed written consent and the hospital institutional review board approved the study. All patients were from south central China and were classified according to the World Health Organization classification system.

RET protein expression by Western blot

Frozen lung tumors and normal tissues (control) were minced in liquid nitrogen and resuspended in 1 × cell lysis buffer (10X #9803; Cell Signaling Technology, Danvers, MA, USA). Tissue suspension was then sonicated and cleared by centrifugation. RET protein immunoblot analysis was carried out according to the RET antibody (#3223, 1:1000; Cell Signaling Technology) following the manufacturer's standard protocol. A high *RET* expression sample confirmed with *RET* rearrangement by sequencing and a *RET*-positive papillary thyroid carcinoma sample identified by our hospital pathologist were used as positive control samples in immunoblot screening analysis.

Immunohistochemical staining

Immunohistochemical staining for *RET* was characterized by RET antibody (#ab134100, 1:200, monoclonal antibody; Abcam, Cambridge, MA, USA). Five-micrometer tissue sections of *RET* positive and negative lung tumor samples obtained by Western immunoblotting and two *RET*-positive papillary thyroid carcinoma samples identified by our hospital pathologist as control samples were deparaffinized, rehydrated, and subjected to antigen retrieval at high temperature and high pressure. Slides were quenched in 3% H_2O_2 for 10 minutes, washed in diH_2O, and then blocked with tris-buffered saline/0.1% Tween 20/5% goat serum. Slides were incubated overnight at 4°C with RET antibody. Immunohistochemical (IHC) detection was conducted using an UltraSensitive SP IHC Kit (Fuzhou Maixin Biotech, Fuzhou, China). All slides were exposed to an AEC Kit (Fuzhou Maixin Biotech, China) and counterstained with hematoxylin. Images (×20) were acquired using a Leica Light microscope (Leica Microsystems, Wetzlar, Germany) at high magnification and five horizons were randomly selected for immunochemical evaluation of *RET*. *RET* expression levels were scored from 0 to 2+ (0 for no staining, 0.5+ for weak, 1+ for moderate, and 2+ for strong immunoreactivity). The percentages of cells with positive *RET* staining within the cancerous region of a section were scored as follows: 0 for < 5% positive cells, 0.5 + for 5–10%, 1+ for 11–50%, and 2+ for 51–100%.

RET hot-point mutation analysis and reverse transcription-PCR of RET fusion

To analyze *RET* mutation, genomic DNA was extracted from frozen tumor tissues with positive RET protein expression using a Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). Extracted DNA was analyzed by PCR, followed by direct sequencing, as previously described.[18] Further analysis was performed on the exons that were more frequently mutated (exons 8, 10, 11, 13–16).[19] The *RET* fusion variants of *RET*-positive expression samples were determined by RT-PCR using an RNA UltraSense One-step RT-PCR Kit (Life Technologies, Carlsbad, CA, USA) according to the product manual. The *KIF5B* primers used were: forward (5′-ATTAGGTGG

CAACTGTAGAACC-3′) and reverse 5′-CAGGCCCCATACAATTTGAT-3′.

RET gene copy number analysis

Genomic DNA extracted from frozen lung tumors with positive *RET* expression, five samples with negative *RET* expression, and the control samples were analyzed. Three different RET TaqMan Copy Number Assays (Hs00379542-cn, Overlaps Exon18-Intron18; Hs05123164-cn, Intron13; and Hs02375715-cn, Overlaps Exon4-Intron4) were used to detect the *RET* gene copy number variations, respectively, using an ABI7500 Fast Real-Time PCR System TaqMan sequence detector (Life Technologies, USA). TaqMan RNaseP Control Reagent (VIC dye) (Life Technologies, USA) was used as internal control. Multiplex PCR reactions contained: one TaqMan Genotyping Master Mix (Life Technologies, USA), one RNaseP Primer-Probe (VIC dye) Mix, one RET Primer-Probe Mix (FAM dye), and 5 ng template genomic DNA in a total volume of 20 μL. All experiments were conducted in quadruplicate. Data analysis was conducted using CopyCaller version 2.1 (Thermo Fisher Scientific, USA).

Detection of *EGFR* mutation and *ALK* and *ROS1* gene translocation

DNA from *RET*-positive samples and 44 randomly selected *RET*-negative samples were amplified by PCR using primers to exons 18–21 of the *EGFR* gene: EGFRex18F(M13–21) tgtaaaaggagggccagtCCAAATGAGCTGGCAAGTG, EGFRex 18R(M13–24) aacagctatgaccatgTGGAGTTTCCCAAACAC TCAG; EGFRex19F(M13–20) gtaaaacgacggccagtCTCCACA GCCCCAGTGTC; EGFRex19R(M13–48) agcggataacaatttcacacaggaGGCCAGTGCTGTCTCTAAGG; EGFRex20F(M13–21) tgtaaaaggagggccagtCCCTGTGCTAGGTCTTTTGC; EGFRex20R(M13–24) aacagctatgaccatgAAAGGAATGTGTGT GTGCTG; GFRex21F(M13–20) gtaaaacgacggccagtTAAC GTTCGCCAGCCATAAG; and EGFRex21R(M13–48) agcggataacaatttcacacaggaCGAGCTCACCCAGAATGTC.

PCR products were analyzed using bi-direct-sequencing.

ALK and *ROS1* gene translocation analysis of *RET*-positive samples was conducted by ALK and ROS1 protein immunoblot analysis according to the *ALK* (ALK [D5F3] XP Rabbit mAb #3633, 1:2000) and *ROS1* (ROS1 [D4D6] Rabbit mAb #3287, 1:1000) antibodies, following the manufacturer's standard protocol (Cell Signaling Technology, USA).

Statistical analysis

Statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean ± standard deviation. Relationships between *RET* expression and clinicopathologic variables were examined using chi-square tests and correlation analysis. Results were considered statistically significant at $P < 0.05$.

Results

Patient characteristics

The clinicopathologic data of 631 (466 men, 165 women) early and mid stage NSCLC patients were obtained from medical records. The mean age (± standard deviation) of the patients was 57.8 ± 9.57 (range 21–84). The tumor types were: squamous carcinomas (311), adenocarcinomas (287), adenosquamous carcinomas (21), and other NSCLCs (12) (Table 1).

RET expression and immunohistochemical staining

We detected *RET* expression using Western blot (Fig 1). As shown in Table 1, among the 631 early and mid stage

Table 1 Correlation between clinical characteristics and *RET* expression

Variable	*RET* expression		*P*
	Negative (*n* = 615)	Positive (*n* = 16)	
Gender			
Male	454 (73.8%)	12 (75.0%)	0.589
Female	161 (26.2%)	4 (25.0%)	
Age (years)			
≤ 58	301 (48.9%)	7 (43.8%)	0.439
> 58	314 (51.1%)	9 (56.2%)	
Tumor type			
Squamous carcinoma	302 (49.1%)	9 (56.2%)	0.918
Adenocarcinoma	280 (45.5%)	7 (43.8%)	
Adenosquamous carcinoma	21 (3.4%)	0 (0%)	
Other NSCLC	12 (2.0%)	0 (0%)	
Differentiation			
Moderate or poor	450 (73.2%)	15 (93.8%)	0.049*
High	165 (26.8%)	1 (6.2%)	
Stage			
IA	218 (35.4%)	4 (25.0%)	0.065
IB	117 (19.0%)	3 (18.8%)	
IIA	67 (10.9%)	6 (37.5%)	
IIB	213 (34.6%)	3 (18.8%)	
Brinkman index			
≤ 200	202 (32.8%)	5 (31.3%)	0.084
200–400	12 (2.0%)	2 (12.5%)	
400–1000	66 (10.7%)	2 (12.5%)	
≥ 1000	335 (54.5%)	7 (43.7%)	

*P <0.05. NSCLC, non-small cell lung cancer.

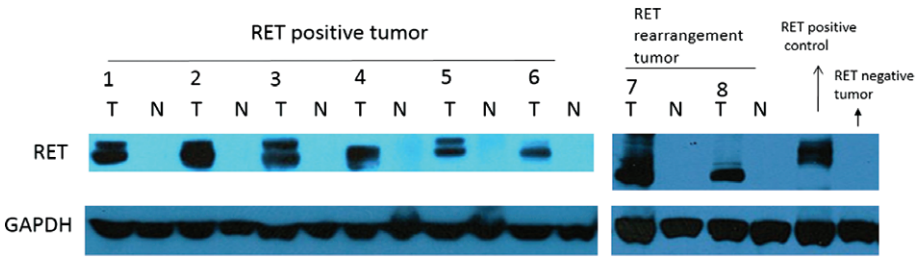

Figure 1 *RET* expression in non-small cell lung cancer samples was detected by Western blot. The figure shows six *RET*-positive expression samples and two *RET*-positive expression samples with *RET* gene rearrangement. The *RET*-positive control is a thyroid cancer sample with *RET*-positive expression. T represents *RET*-positive expression in the tumor; N represents normal tissue from *RET*-positive patients.

NSCLC samples, only 16 displayed positive *RET* expression, at a rate of 2.5% (16/631). The *RET*-positive samples were verified by IHC staining. IHC results showed that nine samples were strong positive (++), five were moderate immunoreactivity positive (+), and the remaining two were weak positive (0.5+) and negative (−) (Fig 2). The results of IHC staining of lung cancer tissues corresponded to the Western blot results. Correlation analysis showed that positive *RET* expression was significantly related to poorer tumor differentiation ($P < 0.05$). There was no correlation between *RET* expression and age, gender, stage, Brinkman index, or histology classification (Table 1).

RET alterations in *RET*-positive samples

After detecting positive *RET* expression in the NSCLC samples, we investigated the potential mechanisms underlying such expression. We first analyzed gene mutation on hot-point exons in *RET*-positive samples using a Genomic DNA Purification Kit (Thermo Fisher Scientific, USA). However, no *RET* gene mutations on hot-point exons 8, 10, 11, or 13–16 were detected in the 16 *RET*-positive samples. In order to explore the specific mechanism of *RET* expression in NSCLC, we detected RET fusion and *RET* copy number variants in the 16 *RET*-positive samples. RT-PCR rearrangement testing showed that only two samples of moderately and poorly differentiated lung adenocarcinomas displayed *RET* rearrangement, both in *RET-KIF5B* fusion partners. The IHC results were strong positive (++). We then investigated *RET* gene copy number alteration in exons 4 and 8 and intron 13 in the remaining 14 samples. All 14 *RET*-positive samples showed *RET* copy number gain compared to the normal tissues and the five *RET*-negative expression samples (Fig 3). These results showed that all *RET*-positive tumor samples presented rearrangement or copy number gain of the *RET* gene.

ALK and *ROS1* expression and *EGFR* mutation in *RET*-positive samples

To identify an association between *RET* expression and *ALK* and *ROS1* translocation and *EGFR* mutation, an analysis of ALK and ROS1 protein expression in the 16 *RET*-positive samples was conducted by immunoblotting. Neither *ALK* nor *ROS1* translocation was found in the 16 RET-positive samples, while one *EGFR* mutation in exon 21 (L858R) was detected. We randomly selected 44 *RET*-negative expression samples for *EGFR* exon

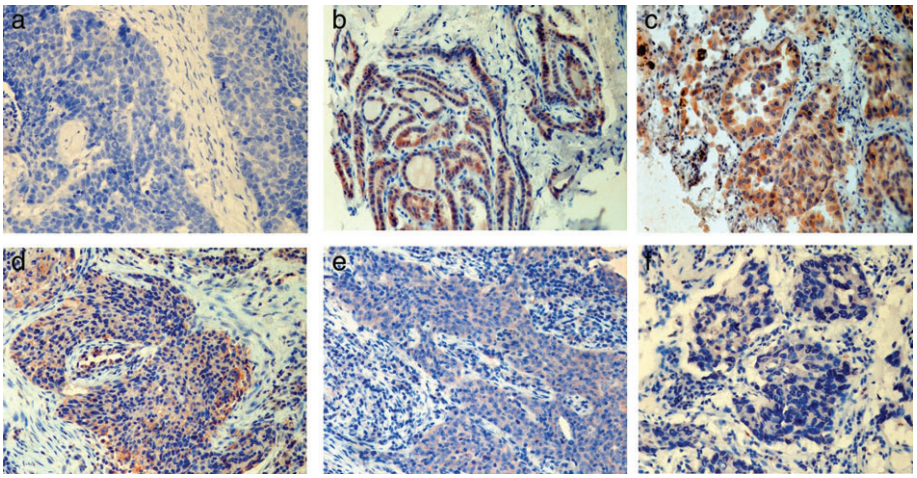

Figure 2 Immunohistochemical results of *RET*-positive expression. (**a**) *RET*-negative expression in non-small cell lung cancer (NSCLC); (**b**) *RET*-positive control in thyroid cancer. (**c**) *RET*-positive expression in NSCLC with *RET* rearrangement (++). (**d–f**) *RET*-positive expression in NSCLC (D–F:++,+,0.5+).

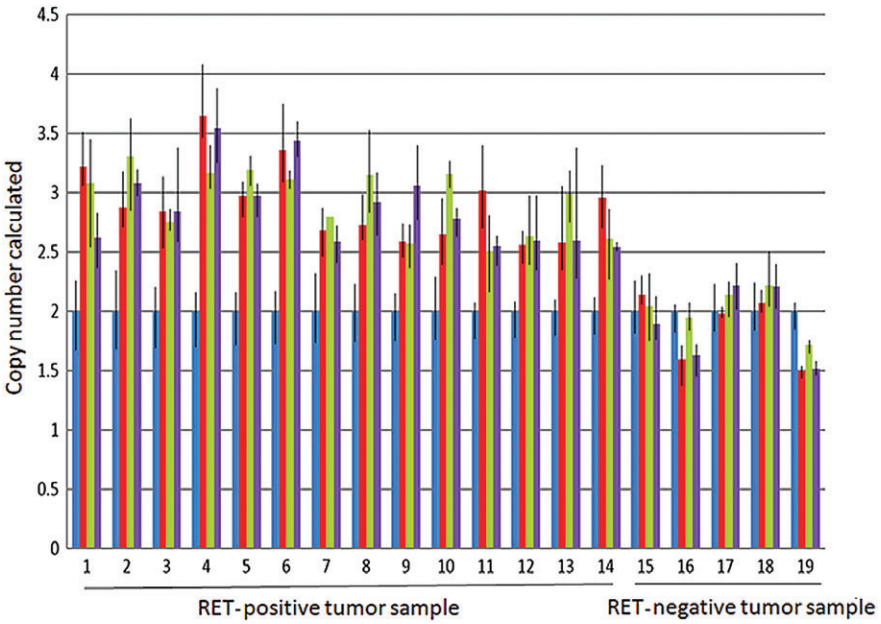

Figure 3 Copy number variation in the *RET*-positive samples. All copy numbers of *RET* exon 4, exon 8, and intron 13 are increased compared to the *RET*-negative samples. ■ Normal, ■ exon 4, ■ exon 8, and ■ instron 13.

sequencing. As Table 2 shows, we found nine samples with *EGFR* exon 21 mutations (L858R) and six with *EGFR* exon 19 deletions. The *EGFR* mutation rate in *RET*-positive samples was significantly lower than in *RET*-negative samples ($P < 0.05$). These results indicate that *ALK* or *ROS1* translocation or *EGFR* mutation rarely occurs in *RET*-positive patients. Therefore, *RET* gene alteration could be a potential target for NSCLC patients without such mutations.

Discussion

After decades of efforts to improve cancer therapy, targeted therapies and personalized medicine have become a new direction for cancer treatment.[6] Targeted therapies can be directed at unique molecular or gene products of cancer cells to produce greater efficacy of cancer treatment with less toxicity. Therefore, it is vital to identify effective tumor markers as the targets for cancer treatment to improve patient survival rates and quality of life in recurrent or advanced-stage malignant tumors.

NSCLC has one of the highest incidences of cancer globally and causes the highest rate of cancer-related death. During the past decades, targeted drugs, such as EGFR and ALK TKIs have been developed and have shown good therapeutic effects.[20,21] Although several genetic mutations have previously been reported, no cancer genome mutation has been observed in a large proportion of NSCLC patients. More than 40% of NSCLCs appear to be driven by unknown genetic events;[22,23] therefore, it is important to identify new biomarkers that can stratify NSCLC patients and acquire a better response to targeted therapy.

The oncogenic effect of *RET* was first identified in papillary thyroid cancer, where diverse kinds of chromosomal translocations and inversions led to the formation of papillary thyroid cancer/*RET* fusion genes.[24] Specific point mutations have also been reported as drivers in *MEN2A* and *MEN2B*.[24] In addition, activated *RET* has been observed in prostate[25] and pancreatic cancers[26] and melanoma.[27] The direct transforming impact of *RET* as a driver is also supported by transgenic mice studies of *RET*, which generated a variety of malignancies.[28,29] A new RET gene fusion with *KIF5B* was first identified in lung cancer in 2012.[15] RET proto-oncogene expression increased with *KIF5B* fusion. *RET* rearrangement at a frequency of 1–2% has been reported.[29,30] Drugs targeted to the *RET* gene inhibit RET kinase, the expression product of *RET*. Taking this into account, we first tested *RET* expression by Western blot in 631 NSCLC samples. Only 16 samples displayed positive *RET* expression at a rate of 2.5% (16/631). A retrospective analysis conducted by Platt *et al.* yielded an *RET* expression rate of 11.6% (40/346) in Asians,[31] which is much higher than our result. Differences in tumor staging may have caused our lower *RET* expression rate. Almost all of the patients in our study were at stages lower than IIB and surgery was indicated, whereas the patients in the

Table 2 *EGFR* mutations in patients with *RET*-positive expression

	RET expression		
EGFR mutation	Positive (n = 16)	Negative (n = 44)	*P*
Wild-type mutation	15	29	0.046
Exon 19	0	6	
Exon 21	1	9	

Platt *et al.* study were at advanced stage (IIIB–IV) and were treated with vandetanib. However, 93.8% (15/16) of the samples in our study were verified positive by IHC staining, indicating that our results are reliable.

No *RET* mutations on hot-point exons 8, 10, 11, or 13–16 were found by direct sequencing. We further examined the gene rearrangement in *RET*-positive samples. RT-PCR showed that only 2 of the 16 samples showed rearrangement with *KIF5B*. Both samples were of moderately or poorly differentiated lung adenocarcinomas and showed strongly positive (++) by IHC. The remaining 14 *RET*-positive samples displayed copy number gain compared to the five *RET*-negative samples. Yang *et al.* reported 1.7% (2/116) *RET* translocation and 64% (74/116) *RET* copy number gain by fluorescence in situ hybridization.[32] The reason for the lower *RET* translocation rate (2/631) and copy number gain in our study may be that we only examined *RET* gene alterations in *RET*-positive samples. The samples with *RET*-negative expression were not examined for gene alterations, and may have contained *RET* alterations. The differences between test methods, geography, and ethnicity may also have contributed to the differing results.

Tumors with *ALK* and *ROS1* rearrangement are responsive to ALK-TKIs; however no *ALK* or *ROS1* expression was found in the 16 samples in our study. Only one *EGFR* mutation in exon 21 (L858R) was detected. Thus, we randomly selected 44 *RET*-negative NSCLC samples for *EGFR* exon sequencing. We found nine samples with *EGFR* exon 21 mutations (L858R) and six with *EGFR* exon 19 deletions. Our *EGFR* mutation result of 26.7% (16/60) with both *RET* negative and positive samples is higher than the 19% (19/99) reported by Yang *et al.*[32] However, only one (6.25%) *EGFR* mutation was found in the *RET*-positive samples. This result indicates that targeted drugs for *ALK*, *ROS1*, or *EGFR* mutations may be not effective for *RET*-positive tumors. Only drugs with activity against *RET* kinase could be effective to these tumors. Recently, Kodama *et al.* demonstrated the antitumor activity of alectinib against *RET*-rearranged NSCLC.[33] Alectinib inhibited *RET* kinase and the growth of RET fusion-positive cells. However, alectinib has low activity against *ROS1* kinase.[34] Although the *RET* expression rate is relatively low in early and mid stage NSCLC samples, *ALK*, *ROS1*, or *EGFR* mutations rarely occur in *RET*-positive patients. Targeted drugs are effective for *RET*-positive NSCLC.

The *RET*-positive expression rate is relatively low at early and mid stage NSCLC in patients from south central China. *RET* gene alterations (copy number gain and rearrangement) exist in all *RET*-positive samples. *RET*-positive expression is a relatively independent factor in NSCLC patients, which indicates that the *RET* gene may be a novel target site for personalized treatment of NSCLC.

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Disclosure

No authors report any conflict of interest.

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