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Clinical Characteristics and Molecular Patterns of *RET*-Rearranged Lung Cancer in Chinese Patients

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RET rearrangement has been proven as an oncogenic driver in patients with lung cancer. However, the prevalence, clinical characteristics, molecular features, and therapeutic options in *RET*-rearranged patients remain unclear, especially in Chinese lung cancer patients. We retrospectively collected 6,125 Chinese lung cancer patients who have been profiled using next-generation sequencing (NGS). The clinical demographics and molecular features of *RET* rearrangement-positive patients were analyzed. *RET* rearrangements were identified in 84 patients with a proportion of 1.4% in our cohort. The median age at diagnosis was 58 years, and it mainly occurred in females with adenocarcinoma histology. *KIF5B-RET* was the most frequent fusion type and accounted for 53.8% (57/106) of all *RET* fusions identified, with K15-R12 as the most frequent variant (71.9%). Among 47 RET^{+} patients profiled with larger panels, 72.3% (34/47) harbored concurrent alterations. *TP53* ranked as the most common concurrent alteration, and concomitant *EGFR* oncogenic alterations were identified in seven patients. Moreover, an adenocarcinoma patient harboring concurrent *RET* fusion and *EGFR* L858R responded to combinatorial treatment of cabozantinib and osimertinib, with a progression-free survival of 5 months. Our study improved knowledge of clinical characteristics and molecular features of *RET*-rearranged lung cancers in China. It might be helpful to guide clinicians for more effective personalized diagnostic and therapeutic approaches.

Key words: *RET* rearrangement; Lung cancer; Adenocarcinoma; Clinical characteristics; Concurrent gene alteration

INTRODUCTION

The proto-oncogene gene *RET* encodes a receptor tyrosine kinase that can activate downstream pathways such as MAPK/ERK, PI3K/AKT, and JAK/STAT[1]. RET plays a critical role in cell proliferation, migration, and differentiation[2–5]. *RET* rearrangement was first identified in NIH-3T3 cells transfected with lymphoma DNA in 1985[6], and chromosomal rearrangement could lead to constitutive activation of RET kinase and downstream signaling events, which cause tumorigenesis.

Fusion of *RET* more frequently occurs in radiation-induced papillary thyroid cancer[7,8]. The prevalence of *RET* rearrangement is 0.7%–2% in lung cancer and 1%–2% in non-small cell lung cancer (NSCLC)[9]. To date, several fusion partners have been identified, such as *KIF5B*, *CCDC6*, *NCOA4*, and *TRIM33* in lung cancer, with *KIF5B-RET* fusion accounting for the major proportion[10]. The coiled-coil domain of *RET* partner gene *KIF5B* can activate RET tyrosine kinase domain by ligand-independent homodimerization and autophosphorylation, leading to

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the constitutive activation of downstream pathways and tumorigenesis[11].

Investigation of the prevalence, clinical demographics, and molecular pattern of oncogenic rearrangements may provide comprehensive genomic profiling as well as aiding in the selection of patients for optimal therapies. However, results from studies of *RET* rearrangements in lung cancer are still inconclusive, especially in Chinese patients. Moreover, previous reports about the prevalence and clinical characteristics are conflicting. Lin et al. reported that *RET* rearrangements were more prone to occur in younger age, never-smokers, females with adenocarcinoma in lung cancer[12]. Some studies revealed that there was no statistically significant difference in gender and smoking status, or even drew an opposite conclusion in terms of gender[13,14]. Therefore, we retrospectively analyzed 6,125 Chinese lung cancer patients for *RET* rearrangement using next-generation sequencing and identified 84 *RET* fusion-positive patients. This study demonstrated clinical demographics and molecular features of *RET* rearrangement in Chinese lung cancer patients.

MATERIALS AND METHODS

Patient Information

A total of 6,125 samples (either tissue or plasma) from lung cancer patients were consecutively collected from September 2015 to July 2017 in this retrospective *RET* rearrangement study. There was no preselection with smoking, gender, clinical stage, or age of patients. Eligible patients were histologically diagnosed as lung cancer according to the latest World Health Organization Criteria. Cancer stage was evaluated based on the 7th TNM classification. *RET* rearrangements were identified using next-generation sequencing. Our profiling panels (Burning Rock Biotech, Guangzhou, P.R. China), consisting of 8, 56, 168, or 295 cancer-related genes, were designed and validated for identification of base substitutions, insertions, deletions, copy number variations, and gene fusion. This entire study was approved by the institutional review board of Union Hospital, Tongji Medical College.

Preparation of Tissue DNA and Plasma Cell-Free DNA

Tissue DNA and plasma cell-free DNA were extracted using QIAamp DNA formalin-fixed paraffin-embedded (FFPE) tissue kit (Qiagen, Valencia, CA, USA) and QIAamp Circulating Nucleic Acid kit (Qiagen), respectively, following the manufacturer's instructions.

NGS Library Preparation and Sequencing

DNA shearing was performed using Covaris M220 (Covaris Inc., Woburn, MA, USA) and followed by end repair, phosphorylation, and adaptor ligation. Then 200- to 400-bp fragments were selected by bead (Agencourt AMPure XP Kit; Beckman Coulter, Brea, CA, USA) and hybridized with capture probes baits (SureSelectXT Custom 1kb-499kb; Agilent, Santa Clara, CA, USA). Hybrid selection was performed using magnetic beads (Dynabeads™ MyOne™ Streptavidin T1; Thermo Fisher Scientific, Waltham, MA, USA) and followed by PCR amplification. Bioanalyzer (LabChip GX Touch 24 Nucleic Acid Analyzer; Perkin-Elmer, Waltham, MA, USA) was used to evaluate DNA quality and size by high-sensitivity DNA assay. Indexed samples were sequenced on NextSeq 500 (Illumina, Inc., San Diego, CA, USA).

NGS Data Analysis

All the reads were mapped to the human genome (hg19) with Burrows–Wheeler Aligner (BWA)[15]. Local alignment optimization, mark duplication, and variant calling were performed using Genome Analysis ToolKit (GATK) 3.2[16], picards, and VarScan[17]. Gene rearrangements were called with FACTERA[18], and CNVs were analyzed based on sequencing depth. Variants were filtered using the VarScan fpfilter pipeline, and loci with depth less than 100 were filtered out. At least two and five supporting reads were needed for INDELs, while eight supporting reads were needed to call SNVs, in both plasma and tissue samples. According to the ExAC, 1000 Genomes, dbSNP, ESP6500SI-V2 database, variants with population frequency over 0.1% were grouped as SNP and excluded from further analysis. Remaining variants were annotated with ANNOVAR[19] and SnpEff v3.6[20].

RESULTS

Patient Characteristics

To interrogate *RET* fusion patterns, we retrospectively screened 6,125 NSCLC patients from September 2015 to July 2017 and identified 84 patients harboring *RET* fusion, giving an overall frequency of 1.4%. Thirty-six (42.9%) of them were males and 47 (56.0%) were females. Gender information of the remaining patient (1.2%) was not recorded. Compared to patients in the original cohort ($n=6,125$; 3,411 males, 2,578 females, and 136 unknown), the prevalence of females was significantly higher in the *RET*$^+$ cohort ($n=84$, $p=0.0228$, Pearson's chi-squared test). Median age of patients harboring *RET* fusion was 58 years, ranging from 35 to 81 years. Compared to patient age in the 6,125 original cohort (median age = 61 years), no preference pattern in terms of age was found in the *RET*$^+$ cohort. As to histological subtype, a majority of them (62/84, 73.8%) were diagnosed as lung adenocarcinoma. One patient was diagnosed with lung squamous cell carcinoma, and three patients had a mixture of adenocarcinoma and squamous cell carcinoma. Histological information of the other

Table 1. Summary of Baseline Characteristics of Patients Harboring RET Rearrangement ($N = 84$)

Patient Characteristics	*n* (%)
Gender	
Male	36 (42.9%)
Female	47 (56.0%)
Unknown	1 (1.2%)
Age (years)	
Median	58
Range	35–81
Histological types	
LUAD	62 (72.8%)
LUSC	1 (1.2%)
Mixed LUAD and LUSC	3 (3.6%)
Other lung cancers	18 (21.4%)

LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma.

18 lung cancer patients was not recorded. The detailed patient characteristics are summarized in Table 1.

Overview of RET Fusion Patterns

To interrogate *RET* fusion patterns, we performed ultra-deep targeted sequencing on plasma using panels covering critical exons and introns of lung cancer-related genes. Among them, 32 patients used a panel consisting of 168 lung cancer-related genes, spanning 170 kb of human genome; 10 patients used a 56-gene panel, and the 37 patients used a panel consisting of 7 well-known lung cancer driver genes plus *KRAS*, a well-established prognostic factor. Five patients used a panel consisting of 295 cancer-related genes.

In this cohort, a total of 106 *RET* rearrangements in 84 patients were identified with *RET* rearrangement. Structures of all the *RET* rearrangements with detailed demonstration of breakpoints are presented in Figure 1. The most commonly seen partner was *KIF5B*, and 57 *KIF5B-RET* fusion events were identified with a frequency of 53.8% (57/106) in all *RET* rearrangements and in 67.9% (57/84) patients (Fig. 2). The most frequent variant of *KIF5B-RET* was K15-R12, occurring in 71.9% (41/57) of *KIF5B-RET*$^+$ patients (Fig. 1). This result was in agreement with previous literature, which reported the occurring frequency of about 75%[11,21–23].

The second- and third-ranked fusion partners were *CCDC6* and *NCOA4*, occurring in 17.0% (18/106) and 2.8% (3/106) of all *RET* fusions, and in 21.4% (18/84) and 3.6% (3/84) of patients, respectively. Several rare and novel *RET* fusion partners were identified in our study,

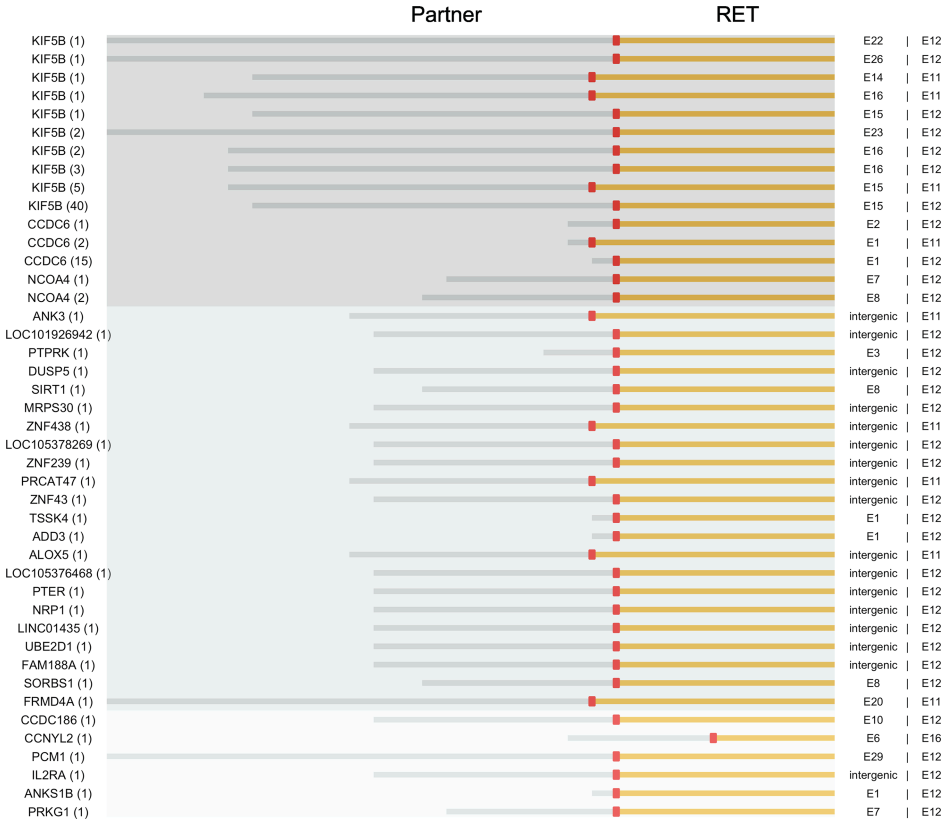

Figure 1. Structure and breakpoints of 106 *RET* fusions detected in 84 lung cancer patients by next-generation sequencing.

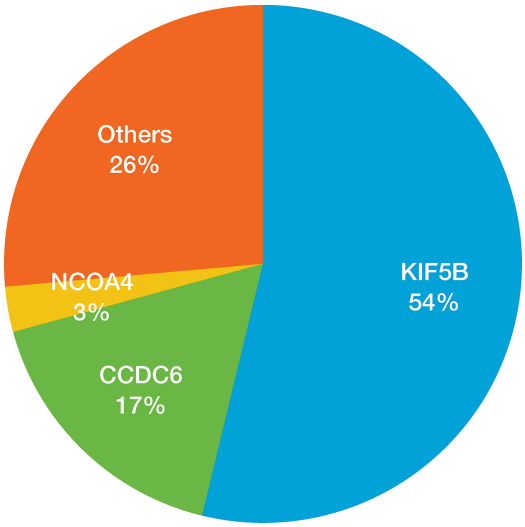

Figure 2. Distribution of various *RET* rearrangement partners identified in the 84 lung cancer patients. Different colors and sizes indicate the occurrence frequency of each *RET* fusion partner in the overall *RET* fusion identified ($n = 106$).

including *TSSK4*, *SORBS1*, *SIRT1*, *PTPRK*, *ADD3-AS1*, *PRKG1*, *IL2RA*, *CCNYL2*, *CCDC186*, and *ANKS1B*. Table 2 lists detailed breakpoints and patient histology of novel *RET* fusions identified in this study, which have not been reported before to the best of our knowledge.

In addition, we observed that multiple *RET* fusions can be detected in individual patients, and 21 patients harbored more than one *RET* fusion. Most studies revealed that for an individual patient harboring multiple fusions, the hotspot partner commonly serves as driver mutation[24].

Concurrent Genomic Alterations in RET Fusion-Positive Patients

Since the discovery of *RET* fusion, there has been no definitive conclusion about the mutual exclusivity between *RET* fusion and other genomic alterations[14,25,26]. We interrogated the mutation spectrum of lung cancer patients with positive *RET* fusions. Patients tested with the eight-gene panel were excluded from this analysis due to the rare chance of harboring dual drivers. Among 47 patients tested with larger panels, 34 of them (72.3%) harbored concurrent mutations, with *TP53* being the most commonly seen concurrent mutation, occurring in 42.5% (20/47) of patients. It was followed by *EGFR* and *MYC*, occurring in 14.9% (7/47) and 10.6% (5/47) of patients, respectively (Fig. 3). The underlying mechanisms of *RET* co-occurrence with other mutations and influence on clinical outcomes are needed to be addressed in further studies.

It has been regarded that actionable driver mutations were commonly mutually exclusive[27–29]. However, the coalteration of *EGFR* and other driver mutations such as *ALK* in a subset of lung cancers has been reported and challenged previous dogma[30–32]. In this cohort, the co-occurrence of *RET* fusion with *EGFR* oncogenic genetic alterations was observed in seven patients, consisting of five exon 19 deletions, two L858R mutations, and two T790M mutations. All seven patients had received previous treatment before the positive detection of *RET* and *EGFR* alterations. One case received previous chemotherapy, and the other six cases received previous EGFR-TKI treatment (including the two T790M$^+$ patient), indicating that *RET* fusion maybe one of the mechanisms that contributes to resistance of EGFR TKI.

Table 2. Novel *RET* Fusion Partners Identified in Chinese Lung Cancer Patients

Fusion	Breakpoints	Histology
TSSK4–RET	Intron1_Intron11	Adenocarcinoma
SORBS1–RET	Intron8_Intron11	Adenocarcinoma
SIRT1–RET	Intron8_Intron11	Adenocarcinoma
PTPRK–RET	Intron3_Intron11	Adenocarcinoma
ADD3–AS1–RET	Intron1_Intron11	Adenocarcinoma
PRKG1–RET	Intron7_Intron11	Adenocarcinoma
IL2RA–RET	Intergenic_Intron11	Adenocarcinoma
CCNYL2–RET	Intron6_Intron15	Not available
CCDC186–RET	Intron10_Intron11	Adenocarcinoma
ANKS1B–RET	Intron1_Intron11	Adenocarcinoma

Interestingly, we found that no *KIF5B-RET* was identified in the seven patients who harbored *EGFR* mutations. The mutual exclusivity of *KIF5B-RET* and *EGFR* alterations suggested that *KIF5B-RET* was a strong driver mutation. For the seven patients harboring concurrent *EGFR* and non-*KIF5B-RET* fusion, the fusion types of *RET* included *CCNYL2-RET* ($n = 1$), *PCM1-RET* ($n = 1$), *CCDC6-RET* ($n = 3$), and *NCOA4-RET* ($n = 2$). Among them, sequencing samples of six patients were plasma. We observed that overall allelic fraction (AF) ratio of first-generation EGFR-TKI sensitizing mutations was higher than non-*KIF5B-RET* in each of the six patients (AF ratio of *EGFR/RET* = 2.4) (Fig. 4), indicating that non-*KIF5B-RET* fusion might function as a potential acquired resistance mechanism to EGFR tyrosine kinase inhibitors. The *RET* rearrangement may exist as a minor clone with *EGFR*-sensitive alterations and expanded while the *EGFR*-sensitive alterations were inhibited by EGFR-TKI.

Clinical Outcomes of an EGFR and RET Fusion Concurrent Patient Treated With Cabozantinib

Several tyrosine kinase inhibitors (TKIs), such as vandetanib, cabozantinib, and sunitinib, have been proven with anti-RET activity. Cabozantinib inhibits a broad range of tyrosine kinases and displayed a 28% ORR, and median PFS and OS of 5.5 months and 9.9 months, respectively, for patients with advanced *RET*-rearranged NSCLC[33].

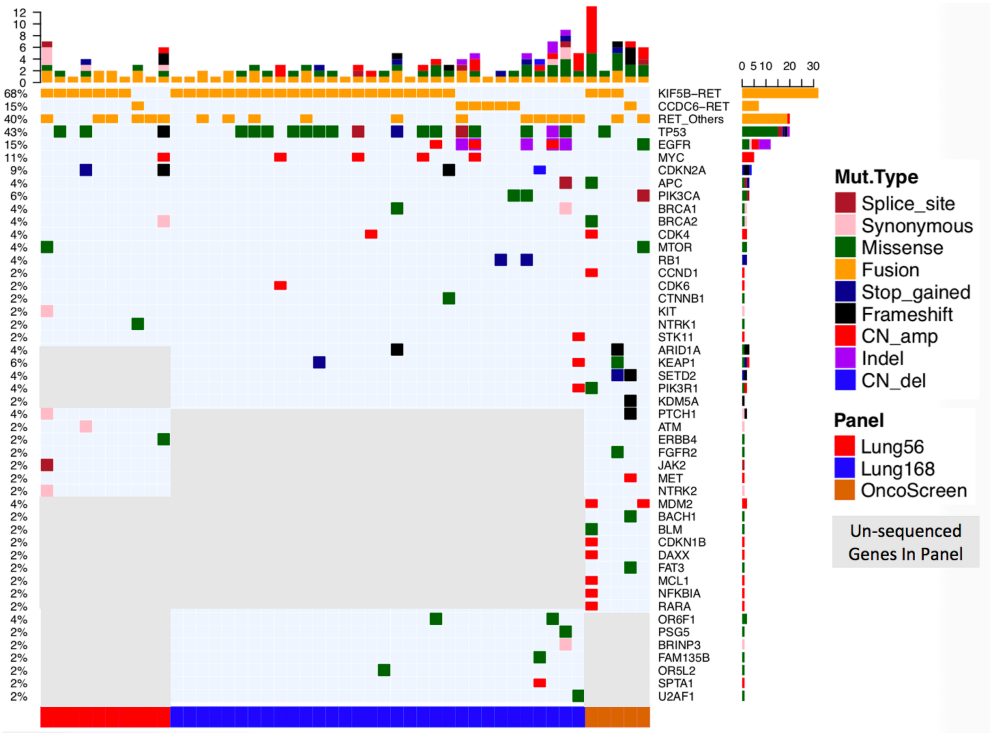

Figure 3. Concurrent genetic alternation analysis demonstrated by oncoPrint. The top bar indicates the number of mutation in each patient. The right-side bar demonstrates the number of patient harboring a specific mutation. Different colors mean different mutation type categories.

In our cohort, clinical outcomes of cabozantinib were available for six *RET*-rearranged patients. The median treatment period of cabozantinib for these patients was 5 months. Among them, one patient was identified harboring concurrent *RET* fusion and *EGFR* mutation. It was a 65-year-old male patient diagnosed with stage IV lung adenocarcinoma with bone metastasis. He was treated with gefitinib and osimertinib as the first- and second-line therapy, and achieved stable disease after the two lines of treatment, with progression-free survival of 8 months and 4 months, respectively. After development of resistance to osimertinib, NGS revealed that this patient had concurrent *CCNYL2-RET* fusion and *EGFR* L858R. Combination of osimertinib and cabozantinib was used to treat this patient after the positive detection of the two concurrent alterations. He achieved stable disease (SD) with a tumor shrinkage of 13% 1 month after treatment initiation. Finally, he experienced disease progression after a PFS of 5 months.

DISCUSSION

In this study, we retrospectively analyzed molecular profiling data of 6,125 Chinese lung cancer patients and identified 84 patients harboring *RET* rearrangements, accounting for 1.4% of this cohort. This ratio was in agreement with a previous study about *RET* fusion[24]. We investigated the distinct clinical characteristics of *RET* fusion patients. Furthermore, we analyzed the fusion partners, demonstrated their molecular pattern, and investigated the mutual exclusivity of *RET* fusion with other concomitant gene alterations.

Several studies have investigated the correlation of *RET* fusion and clinical demographics in lung cancer, and most of them revealed that *RET* rearrangements were more prone to occur in lung adenocarcinomas.[34] In our study, we observed similar results that most of *RET*$^+$ patients were lung adenocarcinomas. However, there were discrepancies among previous studies about other factors such as gender and age. Michels et al. reported that rearrangements of *RET* occurred with a high proportion of men (59% vs. 41%) and median age of 62 years in a European cohort[14]. Another study carried out in Japanese patients revealed that *RET* fusion was not associated with gender ($p=0.524$) but significantly correlated with younger age (57.5 years)[13]. A meta-analysis reported that *RET* fusions were identified at significantly high frequency in younger (<60 years) females[12]. In our Chinese cohort, we observed that *RET* rearrangement had a tendency to occur in females with a median age of 58 years at diagnosis. One explanation is that this discrepancy may have resulted from differences in ethnicity, lifestyles, environmental factors, or molecular heterogeneity. Therefore, studies are needed

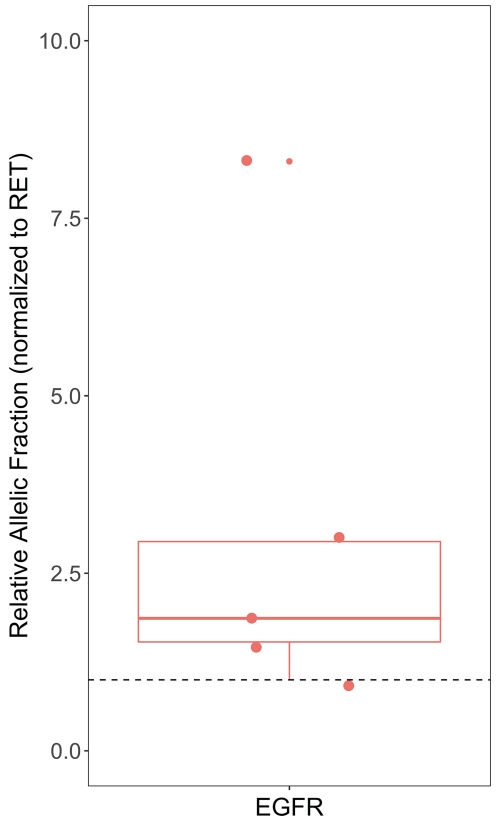

Figure 4. Relative allelic fraction of *EGFR* in seven *RET*-rearranged patients. The *y*-axis indicates *EGFR* relative allelic fraction that was normalized to *RET* fusion in each individual patient.

to further investigate the underlying relationship of *RET* fusion patients and these factors.

KIF5B was the most frequently appeared partner of *RET* fusion and accounted for 53.8% of all the *RET* fusions identified in our study. For *KIF5B-RET*, the most common variant was K15-R12, occurring in 71.9% of all *KIF5B-RET* fusions. The occurring frequencies in this study was similar with previous literature[11,21–23]. However, no consistent conclusion was obtained in terms of clinical outcomes to *RET* inhibitors across different *RET* fusion types[10]. Further studies need to address this issue to provide more guidance for patients at high risks so that optimal treatment strategy may be implemented.

The mutual exclusivity of *RET* fusion and other molecular alterations has been poorly investigated, and no definitive conclusion has been obtained to date. Some studies reported that *RET* fusion was exclusive with other gene alterations. However, Song et al. reported several concomitant genomic alterations, including *EGFR*, *MAP2K1*, *CTNNB1*, and *AKT1*, occurred in 4 of 11 *RET*-rearranged NSCLC patients, with a frequency of 36.4%.[25] Similar co-occurrence frequency of genetic alterations was also found in another study in 10 of 22 *RET*$^+$ patients (45.5%), consisting of eight *TP53*, one *MET* amplification, one *CTNNB11*, and one *EGFR* rare mutation L833F[14]. In our study, we also identified several *RET* fusion co-occurring mutations, and *EGFR* oncogenic driver mutation was identified in seven *RET*$^+$ patients. It was reported that patients harboring both *RET* fusion and *EGFR* mutation were resistant to EGFR TKIs[26,35,36], which suggested that *RET* fusion maybe one of the mechanisms that contributed to resistance of EGFR TKI. This study provided the basis for the hypothesis that an actionable driver mutation could function as an acquired resistant mechanism for another actionable driver alteration.

To date, chemotherapy is still the standard first-line treatment for *RET*-rearranged patients. Several RET inhibitors have been developed, but the overall outcomes to RET inhibitors were inferior to targeted therapies in other lung cancer oncogenic mutations like *ALK* and *ROS1*. Cabozantinib displayed an overall response rate (ORR) of 28% and median PFS of 5.5 months in a phase II clinical trial of RET-rearranged lung cancers ($n=26$)[33]. The ORR to vandetanib was reported as 18% ($n=19$) and to lenvatinib was 16% ($n=25$) in Korean patients. Several reasons are attributed to less sensitivity of RET inhibitors. One reason was that the RET inhibitors could lead to toxicities due to the activity to VEFGR kinase; thus, the clinical uses of these inhibitors were often reduced to 70% at a suboptimal dose[33]. Therefore, more potent and selective RET inhibitors that do not target VEFGR are needed to increase the sensitivity and reduce the off-target toxicity. Moreover, combinatorial treatment strategy is another approach to be taken into account.

There were still some limitations in this study. First, we only analyzed the prevalence of *RET* fusion in terms of gender, age, and histology, and other clinical characteristics like smoking status, tumor size, and metastatic status were not included due to incompleteness of clinical records. Second, owing to the lack of response and survival information, we did not perform survival analysis to interrogate the clinical outcomes across different *RET* variants. Last, but not the least, further validations are needed to support the hypothesis that non-*KIF5B-RET* may serve as an acquired resistance mechanism for another driver mutation.

The development of next-generation sequencing greatly improved the molecular diagnosis of cancer. The knowledge of specific clinical features associated with *RET* fusion can guide patients at high risk for precise diagnosis and treatment strategy. We further propose that studies to be carried out include more clinical feature analysis in *RET* fusion-positive patients and prognostic prediction evaluation among different *RET* fusions variants.

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