Original Article

The clinical significance of RET gene fusion among Chinese patients with lung cancer

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Background: The incidence of lung cancer is growing fast in China, however, the prognosis remains dismal due to the limited therapeutic approaches. The "ret proto-oncogene mutation" (*RET*) fusions have been proven to be the driver gene in lung cancer development and the therapeutic target of several multi-target tyrosine kinase inhibitors.

Methods: We applied formalin-fixed, paraffin-embedded (FFPE) samples of 39 patients with non-small cell lung cancer (NSCLC) using the Lung Plasma panel covering 168 cancer-associated genes and performed capture-based targeted deep sequencing to identify the *RET* fusion partners and concurrent gene mutation with Miseq. The log-rank test was used to compare the survival difference of patients according to treatment strategies. Statistical analyses and graphs were performed using R language and GraphPad Prism.

Results: Most of the samples were advanced (stage IIIb and IV) lung adenocarcinomas (80.77%). *KIF5B-RET* fusions were identified in 52% of the samples and K15-E12 was the most common variant. 6 (15%) samples harbored concurrent *TP53* mutation and 3 samples were positive with *EGFR* mutation including a mutation in exon 19. Of these patients included, ten received cabozantinib, two received anlotinib, and one received crizotinib. Two (20%; 0–45) samples achieved stable disease and two were progressed in the cabozantinib treated group. Median progression-free survival (PFS) was 4 months (95% CI: 3.2–4.8) and median overall survival (OS) was 25 months (95% CI: 1.5–48.5). Three (11.54%; 0–24) samples achieved partial response in patients without RET inhibitor treatment and 4 (15.38%; 2–29) were stable disease. The median PFS was 11 months (95% CI: 1.2–20.8). There was no significant difference in PFS and OS between groups with or without RET inhibitors treatment.

Conclusion: This study provided insight into the *RET* fusions patients treatment. The survival benefit of current RET inhibitors was limited. More precise and potent RET inhibitors should be developed in the near future.

Keywords: Ret proto-oncogene mutation (RET) fusion; non-small cell lung cancer (NSCLC); cabozantinib; clinical outcome

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Introduction

Lung cancer is the leading cause of cancer-associated death worldwide, consisting of over 50 histomorphology subtypes. Non-small cell lung cancer (NSCLC) is the most common subtype in lung cancer and adenocarcinoma (ADC), along with squamous cell carcinoma (SqCC) accounts for the major histological subtypes. Most of the patients are diagnosed with distant metastasis which made the total resection difficult to achieve. The combination of histology and molecular biomarkers analysis refined the current classification of lung cancer. Epidermal growth factor receptor (*EGFR*) mutations were found in approximately 43–89% of patients with NSCLC. Human epidermal growth factor receptor 2 (*HER2*) exon 20 mutations, analogous to the exon 20 mutations in *EGFR*, was identified as an actionable target in NSCLC development. Multiple novel driver gene mutations including hepatocyte growth factor receptor (*MET*) exon 14 mutations, proto-oncogene tyrosine-protein kinase receptor Ret (*RET*), neurotrophic tyrosine kinase (*NTRK*) fusion, *KRAS* mutations, and neurofibromin 1 (*NF1*) loss were identified and most of them mutated exclusively. Serine/threonine-protein kinase b-raf (*BRAF*), was a newly FDA-approved therapy target (1-3).

The proto-oncogene *RET* was located on chromosomal 10q11.2 and encoded a single-pass transmembrane receptor tyrosine receptor. Previous results highlighted the critical role of the *RET* gene in the development and maintenance of several tissue and cell types (4). *RET* was initially found in NIH-3T3 cells that were transfected with lymphoma DNA and then detected in papillary thyroid cancers. About 1–2% of the patients with lung cancer were positive for the mutation and it occurred more frequently in the non-smoker patients with ADC (5). Kinesin family member 5b (*KIF5B*) and coiled-coil domain containing 6 (*CCDC6*) were the common upstream fusion partners of *RET* fusions in NSCLC. The chromosomal rearrangement could contribute to the constitutive expression of the *RET* fusion protein and activate the survival associated pathways (6).

Targeted therapy has become part of the routine management of patients with positive driver gene mutations recently. Targeted drugs including tyrosine kinase inhibitors (TKI) of *EGFR* mutation, inhibitors of anaplastic lymphoma kinase (*ALK*) rearrangement, anti-angiogenesis agents, antibodies against vascular endothelial growth factor (*VEGF*) were approved for NSCLC treatment (7,8). The targetable fusion proteins in NSCLC were *ALK*, *ROS1*, *NTRK*, and *RET* (9). Several multi-targets RET inhibitors were demonstrated the anti-tumor effect in cell lines and xenograft with *RET* fusion. Clinical trials have proved the efficacy of cabozantinib (10) and vandetanib (11,12) on NSCLC patients with *RET* fusions. Results of a retrospective study from a global multicenter registry in NSCLC patients with *RET* fusions suggested that the anti-tumor activity of RET inhibitors were limited compared to targeted therapy in *EGFR* mutant and *ALK/ROS1*-rearranged patients with lung cancer (5). A retrospective study enrolled 6,125 samples summarized the clinical and molecular features of RET fusions in Chinese patients with NSCLC. However, the therapeutic efficacy of RET inhibitors in these patients remained unknown (13). Therefore, we analyzed 39 NSCLC samples with *RET* fusions using next-generation sequencing and collected the anonymized data. We tried to figure out the molecular features of *RET* fusion in Chinese patients with NSCLC. We present the following article in accordance with the MDAR checklist (available at http://dx.doi.org/10.21037/tcr-20-754).

Methods

Patient information

A total of 39 samples with *RET* fusions were collected from Hunan Provincial Tumor Hospital, Tianjin Cancer Institute, and Chinese Academy of Medical Science Tumor Hospital from 2016 to 2018. The cell origin was identified using histological analysis according to the 4th World Health Organization classification (14). Patients characteristics including gender, age, or clinical stage were collected. The cancer stages were evaluated according to the 8th TNM staging system. the overall survival (OS) data were available in 21 samples and progression-free data were available in 14 samples. The treatment strategies of each patient were obtained from the medical records. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Research Ethics Committee of Cancer Hospital Chinese Academy of Medical Sciences (No. 18-118/1696) and informed consent was taken from all the patients.

Targeted DNA sequencing

DNA of formalin-fixed, paraffin-embedded (FFPE) samples were extracted using QIAamp DNA FFPE tissue kit

(QIAGEN, Valencia, CA) and the DNA concentration was measured by Qubit dsDNA assay. The gDNA quality was assessed to make sure that A260/A280 is within the range of 1.8 to 2.0. For patients with available DNA, targeted DNA sequencing was performed. DNA was profiled using the Lung Plasma panel (Burning Rock Biotech) covering 168 cancer-associated genes. The concentration of the DNA samples was measured with the Qubit dsDNA assay to make sure that genomic DNA was greater than 40 ng. Fragments of 200 to 400-bp sizes were selected with beads (Agencourt AMPure XP kit; Beckman-Coulter, Brea, CA), followed by hybridization with the capture probes baits, hybrid selection with magnetic beads, and PCR amplification. A bioanalyzer high-sensitivity DNA assay was then used to evaluate the quality and size range. Available indexed samples were then sequenced on a Miseq (Illumina, San Diego, CA) with pair-end reads.

Sequencing data analysis

Sequencing data were mapped to the human genome (hg19) using BWA aligner 0.7.10 (15). PCR duplicate reads were removed before base substitution detection. Local alignment optimization and variant calling was performed using GATK v3.2-2 (16). DNA translocation analysis was performed using both Tophat2 and Factera 1.4.3 (17,18). Insert size distribution and library complexity of each sample were computed to assess the level of DNA degradation. Different mutation calling thresholds were applied on samples with different DNA quality to avoid false-positive mutation calls due to DNA damage. SNV and indels identification were annotated using the dbNSFP(v30a), COSMIC (v69), and dbSNP (snp138) database. Variants with a global minor allele frequency greater than 1.0% in 1000Genome Project (Phase3, http://www.1000genomes.org/data) were considered as common SNPs and removed. Integrative Genomics Viewer (Broad Institute, USA) was used to visualize variants aligned against the reference genome to confirm the accuracy of the variant calls by checking for possible strand biases and sequencing errors. Gene-level copy number variation (CNV) was assessed using a statistic after normalizing read depth at each region by total read number and region size and correcting GC-bias using a LOESS algorithm (19).

Statistical analysis

GraphPad Prism 5 was used for graphs and statistical analysis. The data of proportion variables were recorded by frequency or percentage. PFS was defined as the time between RET inhibitor treatment initiation to disease progression evaluated by doctors or death from any cause. OS was the time from disease diagnosis to death from any cause. For those without progression or death event by the end of the study, survival end points were censored at the date of last follow-up. The survival differences were performed using the log-rank test and Kaplan-Meier method was used to estimate the survival curves.

Results

Patients characteristics

To better understand the therapeutic efficacy of RET inhibitors in lung ADC patients harboring *RET* mutations, we collected 39 samples and conducted five-year survival follow-up. Of the 39 subjects included, 19 (48.72%) samples were male and 20 (51.28%) of the patients were female. 76.92% of all samples was under 65 years, ranging from 35 to 82 years with the median age of 59 years old. Most of the samples (36 of 39 patients; 92.31%) were diagnosed with lung adenocarcinoma (LUAD) and one was diagnosed with lung squamous cell carcinoma (LUSC). The tumor stages in our cohort ranged from I to IV and over half of the samples were staged IV. Eight (20.51%) samples were stage III, 3 (7.69%) were stage II and 3 (7.69%) were stage I. The treatment methods of each patient were also recorded. Thirteen patients with *RET* fusions received RET inhibitors. Six (46.15%) of them were females and 7 (53.84%) were males. Most (12 of 13 patients; 92.31%) of them had advanced stage III and IV disease (*Table 1*).

RET fusion overview

A total of 46 *RET* fusions in 39 patients were identified and 24 (52%) patients harbored *KIF5B-RET* fusion. In this cohort, the most common variant (21 of 24 variants, 87.5%) of *KIF5B-RET* fusion was *KIF5B* exon 15 fused to *RET* exon 12 (K15-R12) variant. The other three were K15-R13, K16-R12, and K23-R12 respectively (*Figure 1*). *CCDC6* was the second common partner of *RET* fusion as 8 (20.51%) patients were positive for this variant. Several new and rare *RET* fusions occurred in one patient including that *RET* fused with *ZNF438*, *ZNF43*, *STK33*, *REEP3*, *MRPS30*, *MIR7854*, *MARCH8*, *LOC401312*, *LINC01435*, *LINC00841*, *KIAA1217*, *ERC1*, *CBWD6* and *ARHGAP12*

Table 1 Patient characteristics

Patient characteristics	Total	RET inhibitors		KIF5B-RET	
		Treated (n=13)	Non-treated (n=26)	Positive (n=23)	Negative (n=16)
Gender, n (%)					
Male	19 (48.72)	6 (46.15)	13 (50.00)	10 (43.48)	9 (56.25)
Female	20 (51.28)	7 (53.84)	13 (50.00)	13 (56.52)	7 (43.75)
Age (years), n (%)					
<65	30 (76.92)	10 (76.92)	20 (76.92)	16 (69.57)	14 (87.50)
≥65	9 (23.08)	3 (23.08)	6 (23.08)	7 (30.43)	2 (12.50)
Histological types, n (%)					
Lung adenocarcinoma (LUAD)	36 (92.31)	11 (84.61)	25 (96.15)	21 (91.30)	15 (93.75)
Other lung cancers	3 (7.69)	2 (15.38)	1 (3.84)	2 (8.70)	1 (6.25)
Tumor stages, n (%)					
Ia	2 (5.13)	0	2 (7.69)	1 (4.35)	1 (6.25)
IIb	1 (2.56)	0	1 (3.85)	1 (4.35)	0
IIa	1 (2.56)	1 (7.69)	0	1 (4.35)	0
IIb	2 (5.13)	1 (7.69)	1 (3.85)	1 (4.35)	1 (6.25)
IIIa	4 (10.25)	3 (23.08)	1 (3.85)	1 (4.35)	3 (18.75)
IIIb	3 (7.69)	0	3 (11.54)	3 (13.04)	0
IV	25 (64.10)	9 (69.23)	16 (61.54)	15 (65.22)	10 (62.50)

(*Figure 2*) (*Table S1*). Of eight samples who harbored two *RET* fusions, five were positive for *KIF5B-RET* fusion and three were positive for *CCDC6-RET* fusions.

Concurrent genomic alterations in patients with positive RET-fusion

A total of 8 samples in this cohort were positive with at least two variants in this panel. *TP53* was the most common concurrent gene mutation, 6 (15%) samples in this cohort harbored *TP53* mutation along with the *RET* fusions. Three samples harbored three variants which all belonged to the *RET* fusion and *TP53* concurrent mutation group. And the third variant occurred in *GRIN2A*, *NAV3*, and *SOX9*, respectively. Regarding the *RET* fusions that occurred with *TP53* mutation, concurrent mutations in *ERC1*, *CCDC6*, and *KIF5B* were all observed in our cohort. Besides, we observed the concurrent mutations in *ERBB2* (1/39) and *NTRK1* (1/39). *ERBB2* mutation and *KIF5B-RET* fusion were only noted in a patient with pulmonary sarcomatoid carcinoma (PSC). *NTRK1* mutation was found to cooccur with *CCDC6-RET* and *LINC01435-RET* fusions. Three samples accounting for 8% of all the cases harbored *EGFR* mutation and one sample was positive with exon 19 *EGFR* mutation (*Figure 3*).

Clinical outcomes of patients receiving RET inhibitors treatment

Cabozantinib was administered in nine patients, among whom one was treated with erlotinib and cabozantinib and three patients were received Anlotinib or Crizotinib. Of those who were available for the response to Cabozantinib, 2 patients (20%; 0–45) with respective *KIF5B-RET* and *ERC1-RET* fusions experienced disease progression, 2 (20%; 0–45) patients with *KIF5B-RET* fusions achieved stable disease (SD) and 1 (10%; 0–29) patient was not evaluable. However, no patient achieved partial or complete response (CR). Twenty-six patients with *RET* fusions were not administered RET inhibitors.

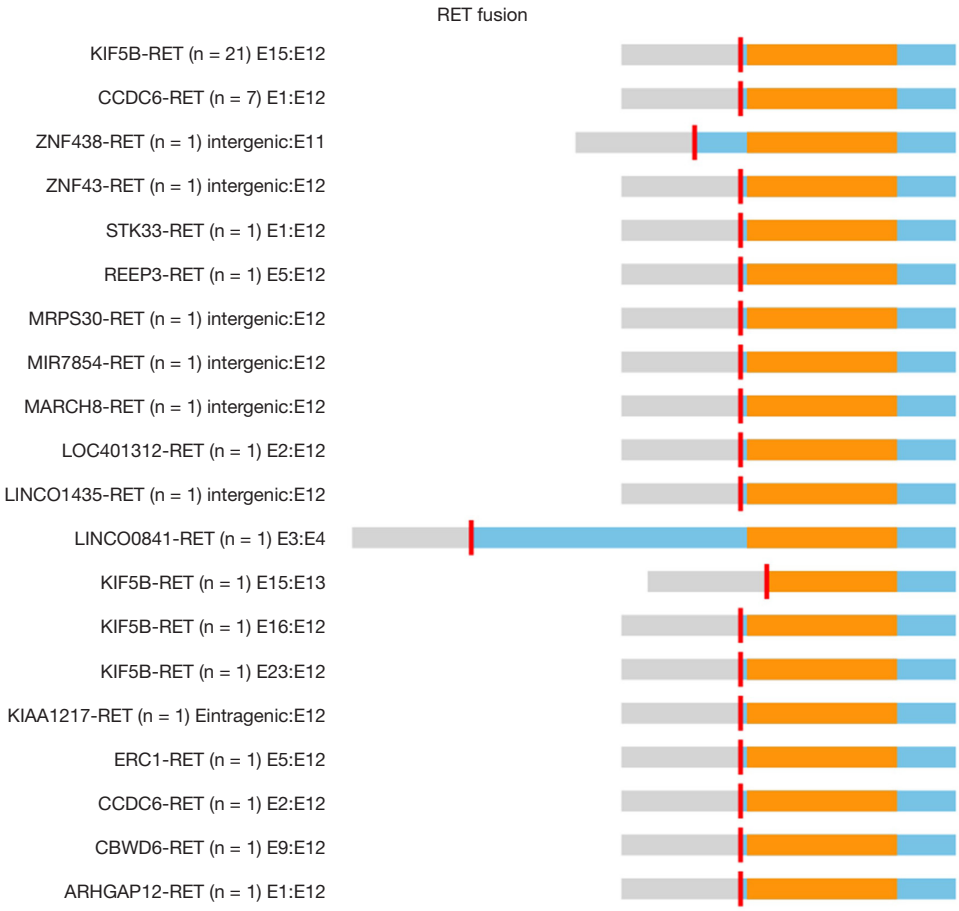

Figure 1 The structure and breakpoints of RET fusions.

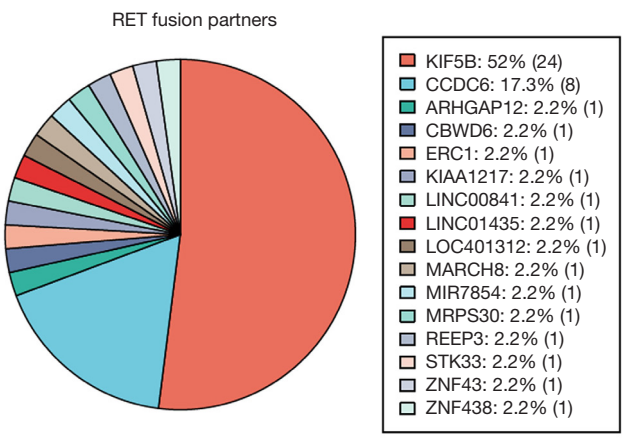

Figure 2 The RET rearrangement partners detected in 39 lung adenocarcinoma (LUAD) patients using next-generation sequencing. The frequency of each RET fusion partner were plotted by different color and size. The exact number was shown on the right side.

Among these patients, 3 patients achieved partial response (11.54%; 0–24) including 2 with *KIF5B-RET* fusion and 1 with *CCDC6-RET* fusion. Stable disease was noted in 4 patients (15.38%; 2–29), 2 subjects harbored *KIF5B-RET* fusions, 1 had two *RET* fusion partners including *ARHGAP12* and *KIF5B* and the other was positive for *CCDC6-RET* fusion. The PFS and OS data were available for 6 patients and 5 patients respectively in the group who received Cabozantinib. The median PFS and OS were 4 months (95% CI, 3.2–4.8) and 25 months (95% CI, 1.48–48.52). With regard to the patients who did not receive RET inhibitors, the PFS and OS data were available for 8 patients and 16 patients. The median PFS was 11 months (95% CI, 1.16–20.84) (*Table 2*). Survival comparison between patients who received and not received RET inhibitors indicated that there was no significant difference between the two groups in terms of PFS (*Figure 4A*) and OS (*Figure 4B*).

Figure 3 The concurrent gene mutations of RET rearrangement in 39 lung adenocarcinoma (LUAD) patients.

Discussion

Several *in vitro* and *in vivo* studies have identified the potential significance of *RET* as a promising targetable driver gene in NSCLC. However, the efficacy of RET inhibitors in Chinese patients remained unclear (9). A previous large-scale study in lung cancer patients suggested that *RET* fusions occurred in about 1–2% among East Asian patients (20). A similar mutation rate was observed in a recent study including 6,125 Chinese lung cancer patients in which 1.4% of the samples were positive with *RET* fusions (13). Taking the rare mutation rate of *RET* fusions into consideration, it was hard to generate meaningful clinical data in a clinical study with small sample size. We collected 39 samples with a least one *RET* fusion detected using NGS. In our study, most of the samples were diagnosed with LUAD and the median age of the patients at diagnosis was 59 years. The results were consistent with most studies in which *RET* fusion prone to occur in patients with LUAD at younger age. Another meta-analysis including 84 patients with *RET* fusions showed a higher mutation frequency of *RET* fusion in younger, non-smoking female patients especially in Asian area (21). However, there was some discrepancy in the association between gender and *RET* fusion. A study in the Japanese cohort showed no difference in the frequency of *RET* fusion between male and female patients (22). Another study in the European cohort reported a higher frequency of the male patients than the female patients (23). Our study involved 19 (48.72%) female patients and 20 (51.28%) male patients. The discrepancy may come from the difference in ethnicity, environment and sample size. Most of the samples we collected were diagnosed with advanced lung cancer as 84.61% of the patients were stage IIIb and IV, which highlighted the potential role of *RET* mutation in advanced NSCLC treatment.

In lung tissues, KIF5B was dominantly expressed and functioned to activate the ALK/RET tyrosine kinase and downstream oncogenic effector. In NSCLC, *KIF5B* was the fusion partner of *ALK*, protein tyrosine kinase (*PTK*), and *RET* (24). *KIF5B-RET* fusion accounted for 2% of patients with NSCLC. Takashi Kohno et. al firstly identified *KIF5B-RET* fusion in patients with lung cancer from Japan and the United States using whole-transcriptome sequencing. They found an increase of RET expression in cells with *KIF5B-RET* fusion (25). The most common *KIF5B-RET* variant was K15-R12 which accounted for 87.5% of all the *KIF5B-RET* mutations in our study. The prevalence of K15-R12 was higher than the predicted frequency of 75% in previous reports (9,13). *CCDC6* was the second common fusion partner of RET, 8 patients were positive with *CCDC6-RET*

Table 2 Clinical outcome with different therapy management

Drug	Complete response (%; 95% CI)	Partial response (%; 95% CI)	Stable disease (%; 95% CI)	Disease progression (%; 95% CI)	Not evaluable (%; 95% CI)	Missing data (%; 95% CI)	Median PFS (95% CI)	Median OS (95% CI)
Cabozantinib (n=10)	0	0	2 (20%; 0–45)	2 (20%; 0–45)	1 (10%; 0–29)	5 (50%; 19–81)	4 (3.2–4.8)	25 (1.48–48.52)
Anlotinib (n=2)	0	0	0	0	0	2		
Crizotinib (n=1)	0	0	0	0	0	1		
RET inhibitor not treated (n=26)	0	3 (11.54%; 0–24)	4 (15.38%; 2–29)	0	3 (11.54%; 0–24)	16 (61.54%; 43–80)	11 (1.16–20.84)	Not reached

fusion in our study. Besides fusing to *RET*, *CCDC6* was also the fusion partner of *PDGFR*, *ROS1*, and *KITLG* in NSCLC samples. CCDC6 was necessary for the activation of RET and played a critical role in sustaining the DNA damage checkpoints. The defective of CCDC6 conferred the resistance to cis-platinum and sensitized cancer cells to small molecular inhibitors of repair enzymes (26,27). Several new or rare fusion partners of *RET* including *ZNF438*, *ZNF43*, *STK33*, *REEP3*, *MRPS30*, *MIR7854*, *MARCH8*, *LOC401312*, *LINC01435*, *LINC00841*, *KIAA1217*, *ERC1*, *CBWD6*, and *ARHGAP12* was also identified in our study. NGS was a more comprehensive platform to detect the new fusion partners and concurrent gene mutation comparing to reverse transcription PCR (RT-PCR) and break-apart fluorescence *in situ* hybridization (FISH) (28). In our study, we applied the samples using the Lung Plasma panel and capture-targeted deep sequencing to better identify the RET fusion partners and concurrent gene mutations.

As an actionable driver mutation, *RET* fusions were considered to mutate exclusively with other actionable driver genes (29). However, we found concurrent *TP53* and *EGFR* mutations in eight and three *RET* fusion positive patients, respectively. A 49-year-old female patient in our study was diagnosed with advanced (stage IV) LUAD, and received Bevacizumab combined with pemetrexed and AZD9291 (an EGFR inhibitor) as first-line and second-line treatment. After she was resistant to AZD9291, results of NGS suggested that the patient harbored *ERC1-RET* fusion and *EGFR* exon 19 mutation. Therefore, the patient was treated with Cabozantinib afterward. The efficacy was limited as the tumor progressed after 1 month. *RET* fusion might involve in the resistance of tumor cells with *EGFR* mutation to TKI therapy (13). The multi-target TKI Cabozantinib has been identified as a potent RET inhibitor. A phase II single-arm trial (NCT01639508) including 25 advanced NSCLC patients with *RET* rearrangement from the USA achieved 28% overall response and the median PFS and OS were 5.5 and 9.9 months respectively (30). Given that the overall response in this study was defined as the confirmed complete response or partial response, the overall response of Cabozantinib was 0. Although 39 patients was collected at the initiation of this study, Cabozantinib was administered in ten patients and only five samples in our study were available for the drug activity evaluation. Unfortunately, no significant survival benefit of patients receiving Cabozantinib was noted. Currently, most of the RET inhibitors were multi-targeted and some researches showed that the type of *RET* fusion might

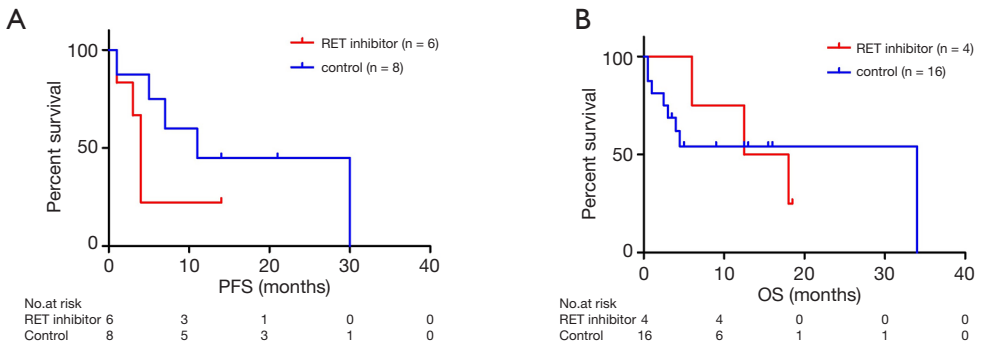

Figure 4 Survival comparison of patients who received and not received RET inhibitor (A) Kaplan-Meier curves of progression-free survival (PFS) (B) Kaplan-Meier curves of overall survival (OS).

influence drug response (12). More precise and potent RET inhibitors may yield better clinical outcomes.

From the objective view, limitations were inevitably existed in our study. Firstly, the sample size of survival analysis was relatively small and the censoring rate was high which might contribute to the discrepancy between our results study and previous reports. Secondly, our study was designed as a retrospective analysis and some bias could not be avoided. In conclusion, *RET* fusion was a promising target in NSCLC, further randomized controlled clinical trials were warranted to validate the conclusion in the future.

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Footnote

Reporting Checklist: The authors have completed the MDAR checklist. Available at http://dx.doi.org/10.21037/tcr-20-754

Data Sharing Statement: Available at http://dx.doi.org/10.21037/tcr-20-754

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Conflicts of Interest : All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr-20-754). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Research Ethics Committee of Cancer Hospital Chinese Academy of Medical Sciences (No.18-118/1696) and informed consent was taken from all the patients.

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Supplementary

Table S1 overview of RET fusion variants

RET fusion	Variant	N
KIF5B-RET	Exon 15–12	21
	Exon 15–13	1
	Exon 16–12	1
	Exon 23–12	1
CCDC6-RET	Exon 1–12	7
	Exon 2–12	1
ZNF438-RET	Intergenic region-exon 11	1
ZNF43-RET	Intergenic region-exon 12	1
STK33-RET	Exon 1–12	1
REEP3-RET	Exon 5–12	1
MRPS30-RET	Intergenic region-exon 12	1
MIR7854-RET	Intergenic region-exon 12	1
MARCH8-RET	Intergenic region-exon 12	1
LOC401312-RET	Exon 2–12	1
LINC01435-RET	Intergenic region-exon 12	1
LINC00841-RET	Intergenic region-exon 12	1
KIAA1217-RET	Intragenic region-exon12	1
ERC1-RET	Exon 5–12	1
CBWD6-RET	Exon 9–12	1
ARHGAP12-RET	Exon 1–12	1