Int J Clin Exp Pathol 2017;10(6):7004-7010
www.ijcep.com /ISSN:1936-2625/IJCEP0051053

Original Article

RET mutation and expression in small cell lung cancer

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Received February 17, 2017; Accepted March 20, 2017; Epub June 1, 2017; Published June 15, 2017

Abstract: *RET* is a paradigm of a single gene that causes different types of human neuroendocrine cancers and non-small cell lung cancer, but the role for *RET* in the development of SCLCs was however not widely addressed. In this study, we aimed to study the gene status and expression of *RET* in Chinese small cell lung cancer (SCLC). 88 cases of SCLC samples were collected. All samples were subject to fluorescence *in-situ* hybridization and immunohistochemistry to detect *RET* gene status and protein expression, among which 30 samples were detected by Sanger sequencing for *RET* gene mutations. *RET* positive and high expression was found in 31.8% and 22.7% of this cohort of SCLC samples, among which only 1 case was detected as *RET* gene rearrangement and IHC high expression. No amplification or gene copy number increase was detected in those cases. In addition, no *RET* gene mutation in exons 10, 11 and 13-16 was found in the 30 SCLC patients by Sanger sequencing. We have confirmed *RET* rearrangement in a subpopulation (1.25%) of Chinese SCLC patients, who may benefit from treatment of *RET* tyrosine kinase inhibitor.

Keywords: Small cell lung cancer, *RET*, mutation, gene rearrangement, immunohistochemistry

Introduction

Lung cancer is currently the most common cause of cancer-related death worldwide, with over 1.6 million deaths per year [1]. Small cell lung cancer (SCLC), which comprises about 15%-20% of all lung cancer, is a type of highly malignant neuroendocrine tumor with poor prognosis [2-4]. SCLC occurs almost predominately in heavy smokers, while the underlying mechanism remains largely unknown [5]. Current treatment for SCLC still relies on chemotherapy and radiotherapy as the major therapy methods [6], and personalized treatment for SCLC has not been established yet.

Rearrangement during transfection (RET) gene encodes a receptor tyrosine kinase which is involved in cell proliferation, migration and differentiation through downstream signaling pathways (e.g. RAS/RAF/MEK/ERK, PI3K/AKT) [7]. Germline *RET* gene gain-of-function point mutation was related to hereditary cancer syndromes: multiple endocrine neoplasia type 2A and type 2B (MEN2A and MEN2B), and familial medullary thyroid carcinoma (MTC) [8, 9], while somatic *RET* gene mutation was observed in over half of the sporadic form of the MTC [10]. *RET* gene translocation was observed in papillary thyroid cancer and non-small cell lung cancer, which could also lead to *RET* overexpression by forming fusion transcripts with other genes [11-14].

RET gene translocation was firstly described in non small cell lung adenocarcinoma in 2012, including fusion with *KIF5B*, *ROS1*, or *ALK* genes [15-18], and a frequency of 1% *RET* rearrangements was reported in those studies. Despite those findings, the role for *RET* in the development of SCLCs was not widely addressed. In this study, we aimed to further investigate the prevalence of *RET* gene amplification, mutation and translocation in a cohort of 88 Chinese SCLC samples.

Materials and methods

Patient information

Eighty-eight formalin-fixed paraffin-embedded (FFPE) SCLC tissue samples, including 31 sur-

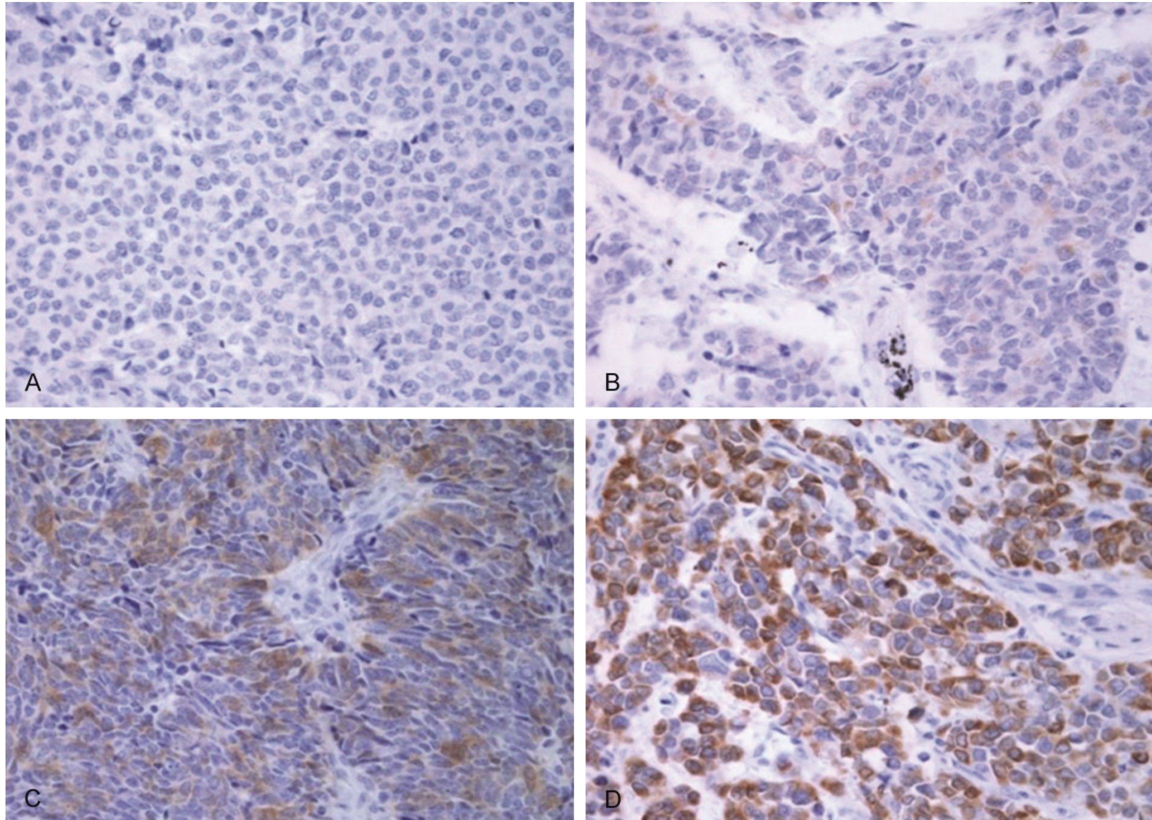

Figure 1. Representative images of RET IHC staining. (A) IHC 0: almost none of the tumor cells had staining; (B) IHC 1+: >10% of the tumor cells had weak staining (C) IHC 2+: >10% tumor cells had moderate staining; (D) IHC 3+: >10% tumor cells had strong staining.

gery samples and 57 biopsy samples, were collected from 88 patients who underwent surgery in Shanghai Renji Hospital within the year 2005 to 2012.

Immunohistochemistry

SCLC tumor tissue samples were collected after the surgery following standard FFPE tissue-processing procedure. Four μm-thick tissue sections were used for IHC study. Sections were firstly incubated at 56°C for one hour, and then deparaffinized in xylene and rehydrated through a graded series of ethanol. Antigen retrieval was performed in PT link (Dako) at 95°C for 15 minutes in target retrieval solution (high pH; Dako, #K8004). Endogenous peroxidase activity was quenched by incubating the sections in hydrogen peroxide (Dako, #S2023) for 10 minutes at room temperature. Sections were then incubated with a rabbit anti-RET monoclonal antibody (1:6000 dilution, clone EPR2871, Epitomics) and RET expression was visualized with EnVision System (Dako, #K4003). Finally, sections were visualized using DAB substrate-chromagen (Dako, #K3468) and washed with deionized water before counterstaining with hematoxylin, followed by dehydration through a graded series of ethanol. Slides were then cleared in xylene and covered in DPX mounting medium. MTC tissue sample was used as positive control for RET immunostaining. SCLC tumor tissue incubated with isotype-matched immunoglobulin fraction instead of primary antibody was used as negative control.

Staining intensity (membrane and cytoplasm) was scored using the following criteria: 0, if absence of staining was observed; 1+, if >10% of the tumor cells had weak staining; 2+, if >10% tumor cells had moderate staining; 3+, if >10% tumor cells had strong staining (see representative images in **Figure 1**). Tumors with RET IHC 1+, 2+, and 3+ were interpreted as positive, while tumors with 0 was interpreted as

Table 1. Primer information for *RET* gene mutation screening

Primer ID	Sequence (5'->3')	Target Tm (°C)	Product length (bp)
RET-ffpet-E10p1-F	GCCTGGGGTGGTCAGGCG	63	229
RET-ffpet-E10p1-R	GTTGAGACCTCTGTGGGGCTGG	63	
RET-ffpet-E11p1-F	AGAGCATACGCAGCCTGTACCCAG	63	211
RET-ffpet-E11p1-R	AGCTGAGGAGATGGGTGGCTTG	63	
RET-ffpet-E11p2-F	CCTTCTGCATCCACTGCTACCACA	63	217
RET-ffpet-E11p2-R	GGGAGGGCAGGGGATCTTCC	63	
RET-ffpet-E13-F	GAAGGGGCTTCCAGGAGCGAT	63	225
RET-ffpet-E13-R	AGGGCTGTATGGAGCCCCCAG	63	
RET-ffpet-E14p1-F	CTCCCACCCCTGGCTCCTG	63	236
RET-ffpet-E14p1-R	ATGAGGTCGCCCATGGTGAGG	63	
RET-ffpet-E14p2-F	GAGTACGCCAAATACGGCTCCCTG	63	228
RET-ffpet-E14p2-R	GGGCTGGCTGGGTGCAGA	63	
RET-ffpet-E15p1-F	TGCTGGTCACACCAGGCTGAG	63	210
RET-ffpet-E15p1-R	ACTGGGCACCTGGCTCCTCT	63	
RET-ffpet-E15p2-F	ACTTGGCAGCCAGAAACATCCTGGT	63	210
RET-ffpet-E15p2-R	TATCTTTCCTAGGCTTCCCAAGGGC	63	
RET-ffpet-E15-Ft1	CCGCTGCTGCCTGGCCATGG	63	212
RET-ffpet-E15-Rt1	GATCCCTGGGAGCCCCGCCT	63	
RET-ffpet-E15-Ft2	CCGCTGCTGCCTGGCCATGG	63	240
RET-ffpet-E15-Rt2	GGCACTGCCTGCCCCATGGT	63	
RET-ffpet-E16-F	CTGGCCTTCTCCTTTACCCCTCCT	63	239
RET-ffpet-E16-R	CTGGCCAAGCTGCACAGACG	63	

Table 2. RET expressions in small cell lung cancer (SCLC)

RET IHC* score	Small cell lung cancer		
	Surgery (31 cases)	Biopsy (57 cases)	All sample (88 cases)
0	19	41	60
1+	3	5	8
2+	3	8	11
3+	6	3	9
Positive rate	38.7% (12/31)	28% (16/57)	31.8% (28/88)

*IHC: immunohistochemistry.

negative. Low expression was defined as IHC 0 or 1+, while high expression was defined as IHC 2+ or 3+.

Fluorescence in situ hybridization (FISH)

RET rearrangement screening was conducted via a dual-probe FISH break-apart test. The *RET* N-terminal FISH probe is a SpectrumRed (ENZO, Cat # 02N34-050) labeled fluorescent DNA probe, which is generated internally from a bacterial artificial clone (BAC) RP11-718J13. The *RET* C-terminal probe is a SpectrumGreen (ENZO, Cat # 02N32-050) labeled fluorescent DNA probe, which is generated internally from BAC RP11-12-4O11.

FISH assays were performed on 4 μm-thick FFPE sections. The SpotLight Tissue pretreatment Kit (Invitrogen, Cat #00-8401) was used for pretreatment according to manufacturer's instructions. Sections and probes were co-denaturated at 79°C for 6 minutes and then hybridized at 37°C for 48 hours (FFPE) or 24 hours (frozen tissues). After a quick post wash off process (0.3% NP40/2× SSC at 75.5°C for 2 minutes, twice in 2× SSC at room temperature for 2 minutes), sections were finally mounted with 0.3 μg/ml DAPI (Vector, Cat #H-1200).

RET gene signals were observed using a BX61 fluorescence microscope (Olympus, Tokyo, Japan) equipped with the appropriate filters allowing visualization of green and red *RET* gene signals, *CEP10* aqua signals and blue nuclei. For gene rearrangement analysis, normal *RET* gene showed co-localization of red and green signals, while rearranged *RET* gene usually shows red and green signal which are apart from each other. Samples were considered as positive of *RET* rearrangement if more than 10% of tumor cells exhibited rearranged *RET* gene. For gene copy number change analysis, samples were defined as *RET* gene amplification if the ratio of *RET* to *CEP10*≥2 or more than 10% of tumor cells showed *RET* gene cluster.

RET mutation detection by Sanger sequencing

PCR was performed in a 25 μl reaction mix containing 1x AmpliTaq Gold 360 Master Mix (Life Technologies, USA), 200 μM of each primer and

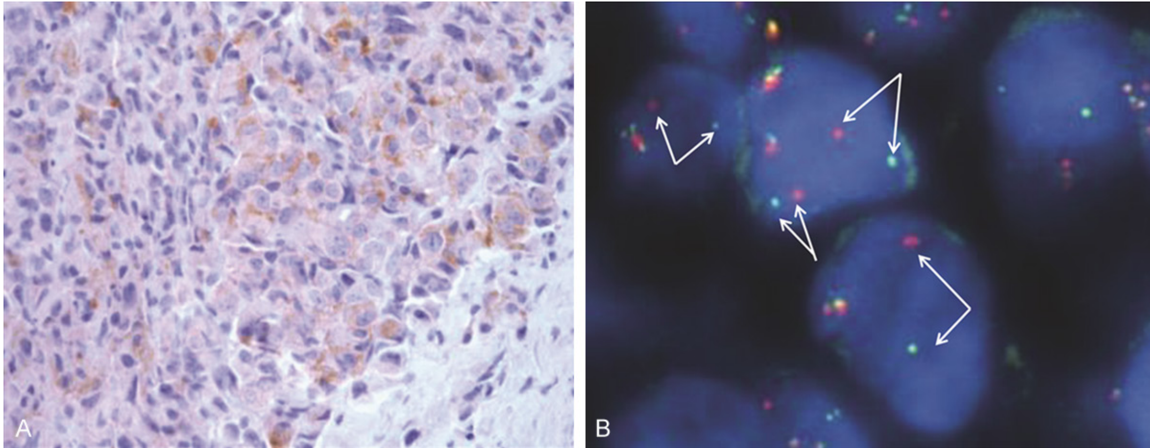

Figure 2. Representative images of the case with both RET high expression and *RET* gene rearrangement. A. RET high expression (2+) was detected by IHC. B. *RET* gene rearrangement on the same case was detected by break-apart FISH. Red signals indicated the upstream of *RET* gene, green signals indicated the downstream of *RET* gene, while the nuclear were counterstained as blue by DAPI. The broken-apart signals of *RET* gene were indicated by white arrowheads.

Table 3. *RET* gene rearrangement and copy number variation in small cell lung cancer (SCLC)

	SCLC (80 cases)	Percentage
Amplification	0	0%
GCN* increase	0	0%
Rearrangement	1	1.25%

*GCN: gene copy number.

20 ng of genomic DNA. Exons 10, 11 and 13-16 of *RET* gene were amplified using primers listed in **Table 1**. The M13 sequence tag was added to the 5' end of each specific primer for universal sequencing. PCR cycling was commenced with a 10-minute incubation at 95°C, followed by 40 cycles of 94°C for 30 seconds, 63°C for 30 seconds, 72°C for 60 seconds, and then a final incubation at 72°C for 10 minutes. The PCR products were purified with ExoSAP-IT reagent (Affymetrix, Cleveland Ohio, USA), and then sequenced in both directions with BigDye Terminator Kit (Life Technologies, USA) and an ABI 3730XL DNA analyzer (Life Technologies, USA) as per the manufacturer's instructions. The sequencing data were analyzed for mutations after assembly and quality calling with SeqScape v2.5 sequence analysis software (Life Technologies, USA).

Results

RET protein expression

Figure 1 illustrated some representative images of RET IHC staining results. In this cohort of SCLC, RET expression (IHC positive) and high expression was detected in 31.8% and 22.7% of all samples. For the surgery samples, RET expression and high expression was 39% and 29.1% and for biopsy samples the numbers were 28% and 19.3% respectively (**Table 2**).

RET gene rearrangement and copy number increase

In the 80 informative SCLC cases, 1 case exhibited *RET* rearrangement (**Figure 2A**) which is accompanied by RET IHC high expression (2+) in the same case (**Figure 2B**).

No *RET* gene amplification or gene copy number increase was detected in those cases (**Table 3**).

RET mutation status detected by Sanger sequencing

Exons 10, 11 and 13-16 of *RET* gene of 30 patients was detected by Sanger sequencing; while no *RET* gene mutation was found.

Discussion

RET is a paradigm of a single gene that causes different types of human neuroendocrine cancers [19-21]. Various germ-line, gain-of-function mutations trigger three dominantly inherited cancer syndromes affecting neuroendocrine tissues: MEN2A, MEN2B, and familial MTC [9, 14, 22, 23]. In addition, somatic mutations in *RET* gene occur in about 50% of sporadic MTC

[8, 24-26]. These malignancies are each characterized by the production of constitutively activated form of the *RET* receptor tyrosine kinase (RTK), resulting in enforced activation of various signaling pathways [27-29].

Previous studies found that the *RET* gene is able to form fusion genes with *CCDC6* or *NCOA4* in papillary thyroid cancer [30]. Most recently, lung adenocarcinomas are reported to harbor fusion gene of *RET* tyrosine kinase domain with *KIF5B* or *CCDC6* genes, resulting in constitutive activation of *RET*, which in turn, stimulates multiple downstream signaling pathways in tumor cells [16, 18, 31]. In our studies, we utilized FISH technology to detect *RET* gene rearrangement and found a 1.1% (1/88) rate of *RET* rearrangement in SCLC patients, which is similar to lung adenocarcinomas (LADCs) [16, 18].

In addition, our result showed a RET high expression rate of 22.7% (20/88) in all SCLC samples (**Table 2**). Expression of RET is normally very low in lung tissues, which may increase significantly in face of *RET* rearrangement or gain-of-function mutation. In our studies, no mutation was detected and only 1 case was detected with *RET* gene rearrangement (accompanied with RET IHC high expression) while the other 19 cases with increased *RET* gene expression did not show *RET* gene rearrangement or gene mutation, indicating an alternative mechanism may have caused RET high expression in those cases, which, however, are unknown and require further investigation.

Recently, Hai-Su Yang et al [32], reported a high prevalence (74/116) of *RET* gene amplification or gains of copy number in a series of lung carcinomas, among which 8 cases were SCLC. In our study, no amplification or gene copy number increase was detected in the 80 informative Chinese SCLC cases, perhaps due to ethnic differences.

In all, our study confirmed a low incidence of *RET* rearrangement (1.1%) occurred in Chinese SCLCs. However, no mutation or amplification was found in this cohort of Chinese SCLC patients. For those patients with *RET* translocations, suppression of *RET* could be a potent therapeutic strategy. Indeed, recent clinical studies have shown that NSCLC patients carrying *RET* gene rearrangements respond favorably to crizotinib therapy [17, 33]. More clinical investigation is needed to further confirm this hypothesis.

Acknowledgements

We thank AstraZeneca Company that fully funded this study.

Disclosure of conflict of interest

None.

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