Comprehensive analysis of *RET* and *ROS1* rearrangement in lung adenocarcinoma

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ABSTRACT

The success of crizotinib in *ALK*-positive patients has elicited efforts to find new oncogenic fusions in non-small cell carcinoma. These efforts have led to the discovery of novel oncogenic fusion genes such as *ROS1* and *RET*. However, the molecular and clinicopathologic characteristics associated with *RET* or *ROS1* fusion, compared to *ALK* fusion positive lung cancer, remain unclear. We accordingly analyzed the clinicopathologic characteristics of *RET* and *ROS1*-fusion positive lung adenocarcinomas. We further performed immunohistochemistry and fluorescence in situ hybridization analysis (FISH) in 15 cases of *RET* and 9 cases of *ROS1* fusion tumors by identified NanoString's nCounter™ screening. *RET* fusion positive patients were younger in age, never-smokers, and in early T stage; *ROS1* fusion positive patients had a higher number of never-smokers compared with patients with quintuple-negative (*EGFR*-/*KRAS*-/*ALK*-/*ROS1*-/*RET*-) lung adenocarcinoma. Histologically, *RET* and *ROS1* fusion tumors share the solid signet-ring cell and mucinous cribriform pattern, as previously mentioned in the histology of *ALK* fusion tumors. Therefore, it can be presumed that fusion gene-associated lung adenocarcinomas share similar histologic features. In immunohistochemistry, the majority of 15 *RET* and 9 *ROS1* fusion-positive cases showed positivity of more than moderate intensity and cytoplasmic staining for RET and ROS1 protein, respectively. In FISH, the majority of *RET* and *ROS1* rearrangement showed two signal patterns such as one fusion signal and two separated green and orange signals (1F1G1O) and an isolated 3' green signal pattern(1F1G). Our study has provided not only characteristics of fusion gene-associated histologic features but also a proposal for a future screening strategy which will enable clinicians to select cases needed to be checked for *ROS1* and *RET* rearrangements based on clinico-histologic features.

Key words: *RET*; *ROS1*; *lung cancer;*

INTRODUCTION

Recently, chromosomal rearrangements involving receptor tyrosine kinases (RTKs) have emerged as important oncogenic drivers of non-small cell lung carcinomas (NSCLCs). In 2007, Soda et al[1]. identified a subset of NSCLCs harboring chromosomal translocations involving the *anaplastic lymphoma receptor tyrosine kinase* (*ALK*). *ALK* rearrangements have since been identified in approximately 3-5% of patients with lung cancer [2,3]. *ALK*-positive lung cancer has unique clinicopathologic features and show dramatic clinical response to ALK inhibitors such as crizotinib[2,3]. The success of crizotinib in ALK-positive patients has elicited efforts to find new oncogenic fusions in NSCLCS. These efforts have led to the discovery of novel oncogenic fusions gene such as *ROS1* and *RET*.

ROS1 rearrangements in NSCLC were first identified in the NSCLC cell line (HCC78 cell line) in 2007[4]. *ROS1* rearrangements have been identified in approximately 1-2% of patients with NSCLC[5]. *ROS1* fusion positive tumors define a distinct molecular subtype of NSCLC with unique clinicopathologic features, as well as *ALK*-positive lung cancer. *ROS1* rearrangements were reported in patients with a younger age, no history of smoking, Asian ethnicity, advanced stage, and adenocarcinoma on histology[6]. Unexpectedly, the HCC78 cell line was sensitive to treatment with crizotinib due to homology of the tyrosine kinase domain of *ALK* and *ROS1*[7,8]. Indeed, the use of crizotinib in *ROS1* rearranged NSCLC has exhibited significant clinical activity. Recently, several clinical trials are ongoing on *ROS1* positive patients worldwide [5].

In 2012, Ju et al [9]. reported the first case of a 33 year-old, never-smoker lung adenocarcinoma patient harboring *RET* rearrangement. To date, several cancer genome sequencing studies have discovered *RET* fusions in approximately 1-2% of NSCLC[9-12]. *RET* fusions were the potential therapeutic targets of multi-targeted kinase inhibitors, vandetanib, sunitinib and sorafenib[10-12]. Importantly, *RET* rearrangement is mutually exclusive with

aberrations in *EGFR*, *KRAS*, *ALK*, *HER2*, and *BRAF* in NSCLC [9-12]. Several studies recently reported that *RET* fusion positive tumors represent distinct clinicopathologic features, as well as molecular subset[13,14]. However, the molecular and clinicopathologic characteristics associated with *RET* fusion compared to *ALK* or *ROS1* fusion positive tumors is still unclear. In particular, characteristic morphologic features have not been investigated; and signal patterns of FISH analysis as an effective tool for the detection of *RET* fusions have rarely been described.

Here, we analyzed the clinicopathologic characteristics of *RET* and *ROS1*-fusion positive lung adenocarcinomas along with immunohistochemistry (IHC) and FISH assay.

MATERIALS AND METHODS

Study design and sample selection

We performed simultaneous screening of *ALK*, *ROS1* and *RET* fusions in 295 lung adenocarcinoma specimens by direct, digital transcript profiling using NanoString's nCounter™ technology, as described in a previous study [15]. Additionally, we collected 500 surgically resected lung adenocarcinoma samples from Samsung Medical Center (SMC) with previous full informed consent from the patient and with approval from SMC. A total of 795 cases were screened for *ALK*, *EGFR*, and *KRAS* mutation status. Of them, we screened for the presence of *RET* and *ROS1* fusion transcripts in 94 cases which were negative for *ALK* fusion and also wild type for *EGFR* and *KRAS*. We retrospectively reviewed clinicopathologic data. Histologic subtypes of lung adenocarcinoma were classified according to the new International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) multidisciplinary classification of lung adenocarcinoma. We recorded the predominant histologic pattern (lepidic, acinar, papillary, micropapillary and solid), which can be associated with prognosis. To investigate the association between fusion genes and histologic features, hematoxylin-and-eosin (H&E) slides were reviewed by two pathologists (YLC and SEL).

RET and ROS1 immunohistochemistry

Human tissues obtained were fixed in 10% formalin solution, dehydrated through a graded ethanol series, cleared in xylene and processed for embedding in paraffin wax, according to routine protocols. The sections were incubated in a solution of 0.3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity. The sections were then incubated for 1h at RT with primary antibody solutions : RET antibody (ab134100, Abcam, Cambridge, UK, 1:200 dilution) and ROS1 antibody (#3287, Cell Signaling Technology, Danvers, MA, USA, 1:40

dilution). The detection systems EnVision+ for Rabbit antibodies (K4003, DAKO, Glostrup, Denmark) were applied according to the manufacturers' instructions. Slides were stained with liquid diaminobenzidine tetrahydrochloride (DAB+), a high-sensitivity substrate-chromogen system (K3468, DAKO, Glostrup, Denmark). Counterstaining was performed with Meyer's haematoxylin. The images on the slides were visualized with an Olympus BX40 light microscope.

Fluorescence in Situ Hybridization

RET and *ROS1* FISH tests were performed on FFPE tumor tissues using ZytoLight SPEC ROS1 and RET Dual Color Break Apart Probes according to the manufacturer's instructions (ZytoVision, Bremerhaven, Germany). The SPEC RET Dual Color Break Apart Probe is a mixture of two direct labeled probes hybridizing to the 10q11.21 band. The orange fluorochrome direct labeled probe hybridizes proximal to the *RET* gene, the green fluorochrome direct labeled probe hybridizes distal to the gene. The SPEC ROS1 Dual Color Break Apart Probe contains green-labeled polynucleotides (ZyGreen: excitation at 503 nm and emission at 528 nm, similar to FITC), which target sequences mapping to 6q22.1 proximal to the ROS1 breakpoint cluster, and orange-labeled polynucleotides (ZyOrange: excitation at 547 nm and emission at 572 nm, similar to rhodamine), which target sequences mapping to 6q22.1 distal to the *ROS1* breakpoint cluster. Rearrangement-positive cells were defined as having two rearrangement positive patterns. One was a break-apart pattern with one fusion signal and two separated green and orange signals (1F1G1O). Only signals which were more than one signal diameter apart from each other were counted as breaks. Another was an isolated 3' green signal pattern (1F1G). A case was considered positive for rearrangement if >15% of cells showed split signals or single green signals. Signals for each locus-specific FISH probe were assessed under an Olympus BX51TRF microscope (Olympus,

Tokyo, Japan) equipped with a triple-pass filter (DAPI/Green/ Orange; Vysis, Downers Grove, IL).

RT-PCR and Sequencing

The precise *RET/ROS1* fusion variants were determined by RT-PCR, followed by Sanger sequencing. The RNA UltraSense one-step RT-PCR kit (Life Technologies, Carlsbad, CA) was used to generate RT-PCR products. First-strand cDNA was initially synthesized using gene-specific primers[15]. cDNA was subdivided into different PCR reactions using the appropriate fusion variant primers, and PCR products were separated on a 2% E-Gel SizeSelect agarose gel (Invitrogen, Carlsbad, CA). In reactions producing a PCR product of the expected size, the amplicons were gel purified and sequenced using a 3700 ABI Prism sequencer (Applied Biosystems, Foster City, CA).

Statistical analyses

The χ2-test or Fisher's exact test was used to examine associations between gene fusion status and clinicopathologic parameters. Overall survival (OS) was calculated from the date of diagnosis to the date of death or last follow-up. Recurrence-free survival (RFS) was defined from the day of first surgery until tumor progression, death or end of follow-up. Survival analysis was estimated using the Kaplan–Meier method and compared between two or more groups of patients using the log-rank test. Univariate analysis was performed, and the significance of differences in survival between the groups was determined using the log-rank test. Cumulative survival curves and OS for groups were computed according to the Kaplan–Meier method. Statistical Package for the Social Sciences (SPSS, Chicago, IL) version 18.0 was used for all statistical analyses. All tests were two-sided, with 0.05 serving as the level of significance.

RESULTS

Clinical characteristics

RET and *ROS1* fusions were found in 15 (16.0%) and 9 (9.6%) of 94 *EGFR*-/*KRAS*-/*ALK*- (triple-negative) patients, respectively. The clinical data of the 15 *RET* fusion positive and 9 *ROS1* fusion positive patients compared with 70 patients with quintuple-negative (*EGFR-/KRAS-/ALK-/ROS1-/RET*-) lung adenocarcinoma were summarized in Table 1, 2 and 3. Patients with *RET* fusion positive tumors had an younger (P=0.002) median age of 55 years (range, 22-69 years) compared with patients with quintuple-negative lung adenocarcinoma whose median age was 64 years (range, 37-79 years). *RET* rearrangements were not significantly associated with sex *(*P=0.492*)*. *RET* fusion positive patients had a higher number of never-smokers than quintuple-negative patients (P=0.010). All tumors with *RET* fusion positive tumors were classified into early T stage, such as T1 or T2, but we found no significant differences between nodal distributions among *RET* fusion positive and negative patients. All but one patient received standard lobectomy and lymph node dissection, the remaining one patient to be treated by wedge resection due to the presence of brain metastasis, with evidence of pathologic stage I in 53.3%, stage II in 6.7%, stage III in 33.3%, and stage IV in 6.7%. Preoperative chemotherapy and/or radiotherapy were administered to 2 patients (13.3%), and 7 patients (46.7%) received postoperative adjuvant therapy. The median follow-up duration was 30 months (range, 2-135 months) after the operation. Of 15 patients, recurrence occurred in 4 patients (26.7%), and 4 patients (26.7%) died of the disease.

Patients with *ROS1* fusion positive tumors had a median age of 57 years (range, 43-77 years), and 66.7% of the patients were female, but, there was no statistically significant difference in age and sex. *ROS1* fusion positive patients had a higher number of never-smokers than quintuple-negative patients (P=0.004). *ROS1* rearrangements were not significantly associated with T status, N status, and AJCC stage, compared with quintuple-

negative patients. All patients received standard lobectomy and lymph node dissection, with evidence of pathologic stage I in 55.6%, stage II in 22.2%, stage III in 22.2%, and stage IV in 0%. Preoperative chemotherapy and/or radiotherapy were administered to one patient (11.1%), and 5 patients (55.6%) received postoperative adjuvant therapy. The median follow-up duration was 38 months (range, 9-53 months) after the operation. Of 9 patients, recurrence occurred in 2 patients (22.2%), and 1 patient (11.1%) died of the disease.

Patients harboring *RET/ROS1* fusion have significantly longer RFS than those with quintuple-negative status (P=0.038, P=0.037, respectively). There was no significant difference in OS between the fusion positive and negative patients (P=0.887). Kaplan–Meier survival curves and corresponding P-values are shown in Figure 1.

Histologic characteristics

All 15 *RET*-rearranged tumors showed adenocarcinoma on histology (Table 4). One case (6.7%) was well differentiated, 8 (53.3%) were moderately differentiated, and 6 (40.0%) were poorly differentiated. The predominant growth patterns were acinar in 6 (40.0%) cases, papillary in 3 (20.0%) cases, solid (Figure 2D) in 6 (40.0%) cases. Focal lepidic, solid, and micropapillary component were identified in 1, 2, and 4 cases, respectively. Furthermore, psammomatous calcification was seen in 3 cases. Interestingly, as previously mentioned in the histology of *ALK* and *ROS1*-rearranged lung cancer [16], the solid signet-ring cell pattern (solid growth pattern containing signet-ring cells) (Figure 2A) was at least focally present in 4 (26.7%) cases and the mucinous cribriform pattern (cribriform structure associated with abundant extracellular mucus) (Figure 2B & 2G) was identified at least focally in 4 (26.7%) cases. The solid signet-ring cell pattern was present in 3 of 6 (50.0%) *KIF6B-RET* positive tumors and the mucinous cribriform pattern was present in 4 of 5 (80.0%) *CCDC6-RET* positive tumors.

All 9 *ROS1*-rearranged tumors also showed adenocarcinoma on histology (Table 5). Seven (77.8%) cases were moderately differentiated, and 1 (22.2%) was poorly differentiated. The predominant growth patterns were acinar in 4 (44.4%) cases, papillary in 3 (33.3%) cases, and solid in 2 (22.2%) cases. Focal solid and micropapillary components were identified in 1, and 1 case, respectively. Also, psammomatous calcification was seen in one case. As expected, the solid signet-ring cell pattern and mucinous cribriform pattern (Figure 3A & 3D) were identified at least focally in one (11.1%) case, and 3 (33.3%) cases, respectively. Interestingly, these patterns were present in all 4 cases of *EZR-ROS1* positive tumors.

Identification of fusion partner genes (Figure 4)

RT-PCR followed by DNA sequencing showed that 5 tumors carried fusions of *KIF5B* exon 15 to *RET* exon 12; and 1 carried fusion of *KIF5B* exon 24 to *RET* exon 12; and 5 carried fusions of *CCDC6* exon 1 to *RET* exon 12. One tumor carried fusion of *CUX1* exon 10 to *RET* exon 12, which was recently identified as an additional novel fusion partner gene in a previous study[15]. Four tumors carried *EZR* exon 10-*ROS1* exon 34 fusion; 3 carried *SLC34A*2 exon 13-*ROS1* exon 32 fusions; and 1 carried *CD74* exon 6-*ROS1* exon 34 fusion.

IHC analysis of *RET/ROS1* fusion-positive lung cancer

We performed IHC for RET and ROS1 protein expression in 94 triple negative cases including 24 fusion positive cases (15 *RET*+ and 9 *ROS1*+). IHC data were categorized by the following staining scores: 0=negative, 1=weak, 2=moderate, and 3=strong. Also, staining pattern was evaluated.

All 15 *RET* fusion-positive cases showed RET positive staining but no immunoreactivity for ROS1 protein expression. Of the 15 *RET* fusion-positive cases, 3 were scored as moderate, and 12 as strong. RET localized diffusely to the cytoplasm in all cases; Both cytoplasmic and

membranous patterns were observed in 2 of 15 (13.3%); the granular cytoplasmic pattern (Figure 2B & 2H) were observed in 5 of 15 (33.3%); In one case (6.7%), cytoplasmic and strong perinuclear aggregates pattern (Figure 2E) like previously observed in *ROS1*-rearranged NSCLC. On the other hand, 69 of 79 *RET* fusion-negative cases were completely negative for RET. RET positivity was seen in 10/79 (12.3%) cases but immunoreactivity extent in all of them was focal (10-30%) (Figure 5).

All 9 *ROS1* fusion-positive cases showed ROS1 positive staining but no immunoreactivity for RET protein expression. Of the 9 *ROS1* fusion-positive cases, 2 were scored as moderate, and 7 as strong. ROS1 localized diffusely to the cytoplasm in all cases (Figure 3B & 3E); both cytoplasmic and strong punctate staining was observed in 1 of 9 (11.1%) case. Like RET protein staining, normal adjacent tissue did not stain.

FISH analysis of *RET/ROS1* fusion-positive lung cancer

RET and *ROS1* rearrangements were identified in 14 *RET* positive tumors (93.3%) and 7 *ROS1* positive tumors (77.8%), respectively. The FISH probe did not hybridize in the remaining three cases.

The positive *RET* rearrangement signals (Figure 2C & 2F & 2I) ranged from 20% to 97%. The majority of *RET* rearrangement showed two signal patterns such as one fusion signal and two separated green and orange signals (1F1G1O) and one fusion signal and an isolated 3' green signal pattern (1F1G). The most common rearrangement signal pattern was the 1F1G1O pattern which was observed in 8 cases, and all cases predominantly showed this pattern. All but one case showed a narrow distance between two separated green and orange signals. Notably, in case #9 with *CUX1* of a novel fusion partner of *RET*, the split signal was wide and easily discernible. The tumor cells showed the 1F1G rearrangement signal pattern in 6 cases, and this pattern was predominantly identified in 4 cases. In one case, an isolated

orange signal with a fused signal (1F1O or 2F1O) was identified. The RET copy number was euploidy in the majority of cases, but copy number gain was seen in 7 cases.

The positive *ROS1* rearrangement signals (Figure 3C & 3F) ranged from 50% to 99%. The majority of *ROS1* rearrangement also showed two signal patterns such as one fusion signal and two separated green and orange signals (1F1G1O) and an isolated 3' green signal pattern with one normal fusion signal and one green signal without the corresponding green signal (1F1G). The most common rearrangement signal pattern was the 1F1G1O pattern observed in 4 cases, and 2 out of 4 cases predominantly showed this pattern. Regardless of the fusion partner genes, the distance of two separated green and orange signals was wide and easily discernible in all cases. The tumor cells showed a 1F1G rearrangement signal pattern in 4 cases, and 2 out of 4 cases, this pattern was predominantly identified. *ROS1* copy number gain was seen in 4 cases.

DISCUSSION

RET was mapped to chromosome 10q11.2, where it encodes a receptor tyrosine kinase[16]. Chromosomal rearrangements involving the *RET* proto-oncogene in papillary thyroid cancer were reported in 1990[17]. *CCDC6-RET*[17] and *NCOA4-RET*[18] rearrangements account for the majority of radiation induced and sporadic papillary thyroid cancers[19]. Since the initial report of *RET* rearrangements in NSCLC by Ju et al[9] in 2012, about 100 cases have been described in the literature [5,20,21]. Our study revealed that 16.0% (15 out of 94) of *EGFR-/KRAS-/ALK-(*triple negative) lung adenocarcinomas harbored *RET* rearrangement. This prevalence was higher than that of the entire adenocarcinoma cohort, because we screened the triple negative cohort. *RET* rearrangements are mutually exclusive with other oncogenic alterations such as *EGFR*, *KRAS*, *ALK*, *ERBB2*, and *BRAF*, suggesting that *RET* fusions are independent oncogenic drivers in NSCLC. Although fusion genes are oncogenic drivers, they present in lung cancer at low frequency. Therefore, identifying the enriched population of fusion genes in lung cancer could contribute to future clinical screening.

Current methods for the detection of *ALK*, *ROS1* and *RET* fusions are FISH, IHC, and/or RT-PCR, each assay has its own advantages and disadvantages. However, it is difficult to screen all genomic alterations using these tools in routine clinical practice. Thus, recognizing distinctive clinicopathologic features, especially histologic features, may help find candidates to screen for genomic alterations. Some studies recently reported the clinicopathologic features of *RET* rearrangements [20,2114]. Our study also showed similar results in that patients with *RET* fusion positive tumors were younger in age (median age, 55 years *vs.* 64 years; P=0.002), never-smokers (P=0.010) and early T stage (P< 0.001) compared with patients with *RET* fusion-negative lung adenocarcinoma. However, there was no statistically significant difference in N stage.

Interestingly, as previously reported as a histopathological marker for the presence of *EML4-*

ALK[12,22-24] and as mentioned as histologic features of *ROS1*-rearranged lung cancer [25], the solid signet-ring cell pattern and mucinous cribriform pattern were identified at least focally in *RET* fusion cases. As expected, these patterns were observed in *ROS1* rearranged lung cancer. Interestingly, the mucinous cribriform pattern was present in 4 of 5 (80.0%) *CCDC6-RET* positive tumors and the solid signet-ring cell pattern was present in 3 of 6 (50.0%) *KIF6B-RET* positive tumors. Similar to our results, Takeuchi et al[12] recently described lung cancer harboring a *CCDC6-RET* rearrangement with a mucinous cribriform pattern. Although further study focusing on the relation between these histologic features and the fusion partner genes of *RET* rearrangement, is needed, an important current finding was that fusion gene-associated lung cancer share similar histologic features. When pathologists encounter an adenocarcinoma showing these histologic patterns in routine histopathological diagnosis, it could be helpful to test for the gene fusion. Therefore, recognizing the characteristic histologic features of fusion gene-associated lung cancer is critical.

We detected 15 *RET* fusion transcripts in the previous study and confirmed the fusion status by RT-PCR followed by sequencing. Currently, 5 fusion partners to *RET* (*KIF5B*, *CCDC6*, *TRIM33*, *NCOA4* and *CUX1)* have been identified in NSCLC [14,26,27]. All of these genes are located on chromosome 10 except *TRIM33* and *CUX1*. Translocations can occur within a chromosome (intrachromosomal) or between chromosomes (interchromosomal). *KIF5B* is the most common fusion partner in NSCLC, and our study showed similar results. Therefore, *RET* fusion in NSCLC appear to arise predominantly through intrachromosomal rearrangement. In our study, the majority of *RET* rearrangements showed narrow split with a distance approaching the diameter of 1 to 2 hybridization signals. However, a case of exon 10 of the *CUX1* gene (7q22.1) fused to exon 12 of the *RET* gene (10q11.21) showed a wide split signal. This finding suggests that RET FISH analysis showed differences in interchromosomal and intrachromosomal translocations. The *KIF5B* (10p11.22) and *CCDC6*

(10q21.2) genes are located in the same chromosome from the *RET* gene. In this situation, the simplest mechanism to generate fusion would be interstitial deletion that would result in an FG FISH pattern [25]. However, the FGO pattern was predominantly shown in these intrachromosomal translocation cases. Therefore, it does not seem to be enough to explain the differences in the FGO and FG FISH patterns. Yoshida [25] et al. recently reported no association between *ROS1* fusion partners and FISH signal patterns. We also showed that there were no differences in the FGO and FG FISH patterns and no differences in intra and interchromosomal translocations in ROS1 FISH analysis. Although break-apart FISH is currently the most effective diagnostic tool to detect chromosomal rearrangements, it has not been used routinely in clinical practice due to the high cost and need for technical expertise. Moreover, specific and unknown variants of fusion genes cannot be distinguished by the break-apart FISH assay.

ROS1 IHC is known to be an ineffective tool in the diagnosis of fusion status because *ROS1* mRNA is known to be overexpressed in 20-30% of lung adenocarcinomas [28] regardless of gene rearrangement status[29]. However, a novel ROS1 IHC assay has been developed recently, with no ROS1 staining in both adjacent normal lung tissue and wild-type lung cancer using the D4D6 antibody[30]. Furthermore, additional studies have validated ROS1 IHC using the D4D6 antibody and the results are suggestive that ROS1 IHC may be an effective screening tool [5,31,32]. In our study, all 9 *ROS1* fusion-positive cases showed positivity of more than moderate intensity, for the ROS1 protein. Similarly, RET IHC has been ineffective as a screening tool because some studies showed no significant differences between RET IHC staining patterns among RET-positive and RET-negative specimens[11,14,33]. However, in our study, all 15 *RET* fusion positive cases showed positivity of more than moderate intensity for the RET protein, and the majority showed cytoplasmic staining. On the other hand, in 79 *RET* fusion negative case, 69 cases did not stained at all. Benchmarking to NanoString's

nCounter™ screening results, RET IHC is 100% sensitive and 87.3% specific for the presence of *RET* fusion. Comparing with the ALK IHC which is a reliable screening tool for the identification of ALK rearrangement, ALK IHC assay showed 66-100% sensitivity and 62.5-100% specificity using various antibodies systems [34]. Therefore, we recommend RET and ROS1 IHC as a possible adjunctive diagnostic tool for the detection of *RET* and *ROS1* rearrangements, in formalin fixed paraffin embedded (FFPE).

To the best of our knowledge, this is the largest study to describe detailed histological findings and FISH patterns in *RET* rearrangement lung cancer. Testing the fusion status of the *ROS1* and *RET* gene should be considered in select patients, such as those with adenocarcinoma histology together with aforementioned fusion associated histologic features (solid signet-ring cell pattern and mucinous cribriform pattern).

In conclusion, our study has provided characteristic fusion gene-associated histologic features. We further proposed future screening strategies and enabled clinicians to direct patients to clinical trials targeting such populations.

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FIGURE LEGENDS

Figure 1. *Kaplan-Meier* survival curves with log-rank test of overall survival (OS) and recurrent free survival (RFS) according to *RET/ROS1* fusion status. (A) *RET/ROS1* fusion had significantly longer RFS than those with quintuple-negative status (*P*=0.038, *P*=0.037, respectively). (B) There was no significant difference in OS between the fusion positive and negative patients (*P*=0.887).

Figure 2. Representative *RET*–rearranged lung adenocarcinomas of case #8 (A-C), case #9 (D-F) and case #10 (G-I). (A) Histologic features of lung adenocarcinoma harboring *RET* rearrangement, Solid signet ring cell pattern in H&E (x200). (B) Immunohistochemistry of RET, granular cytoplasmic staining pattern (x400). (C) FISH pattern of case #8. The predominant pattern was 1F2G1O, consisted of one fusion signal and two separated green and one orange signals. (D) Solid pattern in H&E slides (x200). (E) Cytoplasmic and perinuclear aggregate staining pattern in IHC (x400). (F) FISH pattern of case #9. The predominant pattern was 1F1G1O, and the split signal was wide and easily discernible. (G) Mucinous cribriform pattern in H&E slides (x200). (H) Granular cytoplasmic staining pattern in IHC (x400). (I) FISH pattern of case #10. The predominant pattern was 1F1G1O, and the split signal was narrow but easily discernible.

Figure 3. Representative *ROS1*–rearranged lung adenocarcinomas of case #3 (A-C) and case #8 (D-F). (A) Histologic features of lung adenocarcinoma harboring *ROS1* rearrangement, mucinous cribriform pattern in H&E (x200). (B) IHC of ROS1, cytoplasmic pattern (x400). (C) FISH pattern of case #3. The predominant pattern was 2G1O, consisted of two separated green and one orange signals. (D) Mucinous cribriform pattern in H&E (x200). (E)

cytoplasmic pattern in IHC (x400). (F) FISH pattern of case #8. The predominant pattern was 1F1G, consisting of an isolated 3’ green signal pattern with one normal fusion signal and one green signal without the corresponding orange signal.

Figure 4. Identified frequencies of *RET* (A) and *ROS1* (B) fusion partners.

Figure 5. Immunohistochemistry of *RET* fusion negative cases (A) Representative case of completely negative for RET protein. (B) Representative case of focal staining for RET protein.