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RET-rearranged non-small-cell lung carcinoma: a clinicopathological and molecular analysis

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Background: To elucidate clinicopathological characteristics of non-small-cell lung carcinoma (NSCLC) cases carrying *RET* rearrangements causing oncogenic fusions to identify responders to therapy with RET tyrosine kinase inhibitors.

Methods: We investigated 1874 patients with carcinomas, including 1620 adenocarcinomas (ADCs), 203 squamous cell carcinomas (SCCs), 8 large cell carcinomas, and 43 sarcomatoid carcinomas (SACs). Fluorescence *in situ* hybridisation (FISH) and/or reverse transcription–PCR (RT–PCR) were performed to detect *RET* gene rearrangement.

Results: In all, 22 cases (1.2%) showed *RET* rearrangements; all cases were of ADC histology. Of the 22 patients, 19 possessed *KIF5B–RET* fusion genes, whereas 3 possessed *CCDC6–RET* fusion genes. The *RET*-rearranged tumours were significantly more common in younger patients ($P=0.038$) and tended to occur in patients with no history of smoking ($P=0.051$). In addition, *RET* rearrangements were not associated with gender, occupational history (particularly radioactive exposure), tumour size, lymph node status, tumour stage, or patient survival. The predominant growth pattern in *RET*-rearranged ADCs was lepidic in 6 cases, papillary in 9 cases, acinar in 2 cases, micropapillary in 1 case, and solid in 4 cases. Cells with cytoplasmic mucin production were at least focally present in 12 of the 22 (54.5%) *RET*-rearranged ADC cases. Among the 21 analysed *RET*-rearranged tumours, RET immunopositivity was observed in 15 cases (71.4%), and was significantly associated with *RET* rearrangement ($P<0.001$).

Conclusions: The *RET* rearrangements were observed in 1.2% of NSCLCs. All cases of *RET* rearrangement were ADCs. The *RET* rearrangements were more likely to be observed in younger patients. Although cytoplasmic mucin production was at least focally present in 54.5% of *RET*-rearranged ADCs, specific histological features were not detected.

After the discovery of crucial 'driver' oncogenic mutations in the *epidermal growth factor receptor* (*EGFR*) gene, the EGFR tyrosine kinase inhibitor (TKI) was found to improve survival in non-small-cell lung carcinoma (NSCLC) patients possessing an *EGFR* mutation (Lynch *et al*, 2004; Paez *et al*, 2004; Pao *et al*, 2004). A genomic alteration involving the transforming fusion gene joining the *echinoderm microtubule-associated protein-like 4* gene (*EML4*) and the *anaplastic lymphoma kinase* gene (*ALK*) was identified in 3–13% of patients with NSCLC (Soda *et al*, 2007; Koivunen *et al*, 2008; Yoshida *et al*, 2011). A dramatic response has been observed in patients with *ALK* rearrangements under treatment with an ALK TKI crizotinib (PF-02341066) during a recent clinical trial (Kwak *et al*, 2010).

The *rearranged during transfection* (*RET*) proto-oncogene encodes a receptor tyrosine kinase for members of the glial cell line-derived neurotrophic factor family of extracellular signalling molecules (Knowles *et al*, 2006). This proto-oncogene is involved in the growth and differentiation of neural crest-derived tissues

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(Pachnis *et al*, 1993). Chromosomal rearrangements that generate a fusion gene consisting of the juxtaposition of the C-terminal region of the RET protein with the N-terminal portion of another protein can also lead to constitutive activation of the RET kinase. The *RET* gene rearrangements, as represented by papillary thyroid carcinoma (PTC), were most often observed as *coiled-coil domain containing 6* (*CCDC6*)–*RET* (PTC1) (Grieco *et al*, 1990) and *nuclear receptor coactivator 4* (*NCOA4*)–*RET* (PTC3) fusion genes (Santoro *et al*, 1994).

Several investigators, including the authors of the present study, have simultaneously reported on a novel fusion gene comprising parts of the *kinesin family member 5B gene* (*KIF5B*) and the *RET* gene in lung carcinoma (Ju *et al*, 2012; Kohno *et al*, 2012; Lipson *et al*, 2012; Takeuchi *et al*, 2012). Subsequently, other fusion partners of the *RET* genes *CCDC6*, *NCOA4*, and *tripartite motif-containing 33* (*TRIM33*) were identified in NSCLCs (Wang *et al*, 2012; Drilon *et al*, 2013). These fusion transcripts were detected in 0.6–10% of pulmonary adenocarcinomas (ADCs).

Notably, NSCLC cases that are positive for RET fusions have shown responses against existing RET TKIs, including cabozantinib and vandetanib (Drilon *et al*, 2013; Gautschi *et al*, 2013). Therefore, it is important to understand the clinicopathological characteristics of patients with *RET* fusion-positive NSCLCs for improved selection of patients who are likely to benefit from anti-RET therapy. In this study, we analysed *RET* fusions by fluorescence *in situ* hybridisation (FISH) combined with reverse transcription–PCR (RT–PCR) and RNA sequencing data from a large cohort ($n = 1874$) and investigated distinct clinicopathological characteristics of RET fusion-positive cases.

MATERIALS AND METHODS

Case selection. The institutional review board of our hospital approved the study (2010-0077). The specimens used in this study were isolated from 1927 patients who underwent lung resection for ADC, squamous cell carcinoma (SCC), large cell carcinoma (LCC), or sarcomatoid carcinoma (SAC) at the National Cancer Center Hospital (Tokyo, Japan). We collected each patient's age, gender, smoking history, outcome, maximum tumour size (in cm), and pathologic stage (in p-stage). Staging was based on the tumour–necrosis–metastasis (TNM) classification (7th edition; Goldstraw, 2009). Among patients with *RET* rearrangements, we recorded the patients' occupational histories with particular reference to radioactive exposure.

Histological analysis. Histological diagnoses were based on the most recent World Health Organisation classification (Travis *et al*, 2004). Among all ADC cases, the predominant histological patterns were classified based on the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) (Travis *et al*, 2011). In addition, in order to determine whether *RET*-rearranged lung cancer has any of the specific histological features described in prior reports (Wang *et al*, 2012), we performed a detailed histological analysis as follows: (1) the prevalent cytological feature (i.e., Clara/type II pneumocyte, columnar cell, or polygonal cell); (2) the presence of intracytoplasmic mucin production and mucinous cribriform pattern; and (3) the presence of intranuclear inclusion among 22 cases of *RET*-rearranged lung cancer.

Immunohistochemistry. For RET immunohistochemical staining, heat-induced epitope retrieval with Target Retrieval Solution (Dako Corporation, Carpinteria, CA, USA) was performed. The slides were subsequently incubated with primary antibodies against RET (EPR2871; 1 : 250 dilution; Epitomics, Burlingame, CA, USA). Immunoreactions were detected using EnVision-FLEX and LINKER (Dako). Immunopositive cases were defined as those showing cytoplasmic and/or membranous staining in $\geqslant 10\%$ of cells. We then divided the immunopositive cases into membrane staining-only and cytoplasmic-staining cases (irrespective of any membranous staining).

For the *RET*-rearranged NSCLCs, we performed immunohistochemical analysis to exclude metastatic thyroid carcinoma (see Supplementary Table 1).

FISH analysis for RET rearrangements. First, we performed using a dual-colour break-apart probe for the *RET* gene (Supplementary Table 2; Chromosome Science Labo, Inc., Sapporo, Japan). Among *RET* gene break-apart probe-positive cases, we next performed using break-apart probes for both *KIF5B* and *CCDC6*.

A total of 50 non-overlapping tumour cells with hybridisation signals examined for each case were captured using the Metafer Slide Scanning Platform (MetaSystems, Altlussheim, Germany). The signal in each cell was categorised into one of the following seven patterns: (1) fused 3′/5′ only; (2) fused 3′/5′ and both isolated 3′ and 5′ (split); (3) both isolated 3′ and 5′ (split) only; (4) fused 3′/5′ and isolated 5′; (5) fused 3′/5′ and isolated 3′; (6) isolated 5′ only; and (7) isolated 3′ only. A split signal was defined by 5′ and 3′ probes observed at a distance of greater than one-fold the signal size. A FISH-positive case was defined as $\geqslant 20\%$ of tumour cells having any split signals or any isolated 3′ (red) signals. The threshold for the *RET* gene was determined in 27 cases, yielding both FISH and previously reported RNA sequence data (Kohno *et al*, 2012) (Supplementary Figure 1).

RT–PCR analysis. Total RNA (500 ng) was reverse-transcribed onto cDNA using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Complementary DNA (corresponding to 10 ng total RNA) was subjected to multiplex PCR amplification using KAPA Taq DNA Polymerase (KAPA Biosystems, Woburn, MA, USA) and four primers (detection set in Supplementary Table 3). This PCR enabled the detection of all *KIF5B/CCDC6-RET* fusion variants identified to date. The reactions were conducted in a thermal cycler under the following conditions: 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min, with a final extension cycle for 10 min at 72 °C. The housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase was amplified to estimate the efficiency of cDNA synthesis. The PCR products were subjected to agarose gel electrophoresis. When visible bands were detected, the cDNA samples were further subjected to validation PCR (validation set in Supplementary Table 3). When visible bands were detected, the PCR products were subjected to Sanger sequencing in both directions by using the BigDye Terminator kit (Invitrogen) and an ABI 3130xl DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The PCR primers used in the present study are shown in Supplementary Table 3.

We defined the cases with *RET* rearrangement that were RT–PCR-positive or *RET* break-apart probe-positive as well as cases that were *KIF5B* break-apart probe-positive or *CCDC6* break-apart probe-positive in the absence of RT–PCR data.

Analysis of EGFR mutational status and ALK rearrangement. We detected two common *EGFR* mutations (deletions in exon 19 (DEL) and a point mutation at codon 858 in exon 21 (L858R)) by using high-resolution melting analysis (Fukui *et al*, 2008). The *ALK* rearrangement was analysed by immunohistochemistry, RT–PCR, and/or FISH assay (Yoshida *et al*, 2011).

Statistical analysis. Statistical analysis was performed using SPSS Statistics 21 software (IBM Corporation, Somers, NY, USA). Student's *t*-test was used to analyse continuous variables and χ^2 tests were used to analyse categorical variables. Overall survival (OS) curves were calculated using the Kaplan–Meier method. Curves were compared using the log-rank test. Univariate survival analysis was performed using a log-rank test. Statistical significance was set at $P < 0.05$.

RESULTS

Clinicopathological background. Among the 1927 cases investigated, 53 cases were excluded because they lacked RT–PCR data and the FISH analysis failed. Therefore, the final cohort included 1874 cases: 1620 ADCs (56 *in situ*, 41 microinvasive, 366 lepidic-predominant, 179 acinar-predominant, 577 papillary-predominant, 101 micropapillary-predominant, 236 solid-predominant, and 64 invasive mucinous), 203 SCCs, 8 LCCs, and 43 SACs. Among the 1620 ADC cases, 830 cases consisted of consecutively resected cases from 1998 to 2002 (Supplementary Figure 2).

Background clinicopathological data are displayed in Table 1. Because lymph node status was recorded in 1860 cases (99.3%), pathological staging was performed in 1860 cases. The mean follow-up time for all 1874 patients was 62.3 months (range, 0.1–163 months), with 1292 patients still alive at follow-up.

***RET* FISH and RT–PCR.** Among the 1874 cases investigated, 1823 cases yielded break-apart FISH data, 477 cases yielded RT–PCR data, and 456 cases yielded both FISH and RT–PCR data.

Table 1. Clinicopathologic factors of *RET*-rearranged lung carcinomas

	Total	*RET* rearrangement		
	1874	Negative (%)	Positive (%)	*P*-value
Age (year)				
Median	63.1	63.2	57.5	0.038
Range	23–89	23–89	28–78	
Gender				
Female	809	798 (43.1)	11 (50)	0.524
Male	1065	1054 (56.9)	11 (50)	
Smoking				
Never	867	852 (46.1)	15 (68.2)	0.051
Former/current	1007	1000 (53.9)	7 (31.8)	
Tumour size (cm)				
Median	3.0	3.0	2.8	0.598
Range	0.4–17.5	0.4–17.5	1.4–8.0	
N status				
Negative	1377	1362 (74.1)	15 (68.2)	0.624
Positive	483	475 (25.9)	7 (31.8)	
p-Stage				
I + II	1496	1480 (80.5)	16 (72.7)	0.189
III + IV	364	358 (19.5)	6 (27.3)	
Histology				
ADC	1620	1598 (86.3)	22	0.322
SQC	203	203 (11.0)	0	
LCC	8	8 (0.4)	0	
SAC	43	43 (2.3)	0	
***RET* immunostaining**				
Negative		1527 (86.1)	7 (33.3)	<0.001
Positive		247 (13.9)	14 (66.7)	

Abbreviations: ADC = adenocarcinoma; LCC = large cell carcinoma; *RET* = *rearranged during transfection*; SAC = sarcomatoid carcinoma; SQC = squamous cell carcinoma.

Fifty (2.7%) cases were *RET* break-apart probe positive cases (Figure 1). The RT–PCR analysis of 29 of the 50 FISH-positive cases for which RNAs were available verified that 14 (44.8%) cases possessed the *KIF5B–RET* fusion and 2 possessed the *CCDC6–RET* fusion. The most prevalent variant of *KIF5B–RET* was variant K15;R12 (10/14; 71.4%), whereas the other variants were observed in 1 case each (7.1% each; Supplementary Table 4). On the other hand, RT–PCR results were negative for all 406 FISH-negative cases. Based on these RT–PCR data, the split signal sensitivity and specificity was 100% and 44.8%, respectively. The average FISH split signal for RT–PCR-positive and -negative cases was 40.9% (range, 22–72) and 7.4% (range, 0–40), respectively ($P<0.001$). Among the 50 *RET* break-apart probe-positive cases, 13 out of the 40 analysed cases were confirmed to be *KIF5B* break-apart probe-positive cases and 3 out of the 46 analysed cases were confirmed to be *CCDC6* break-apart probe-positive cases. In conjunction with RT–PCR data, 19 cases with *KIF5B–RET* rearrangement and 3 cases with *CCDC6–RET* rearrangement were detected (Supplementary Table 4).

Clinical characteristics of patients with *RET*-rearranged NSCLCs. Based on the aforementioned FISH and RT–PCR data, 22 of the 1874 cases (1.2%) were considered to be *RET* rearrangements. The clinical characteristics of patients with *RET*-rearranged NSCLCs are displayed in Table 1 and Supplementary Table 4. Among 22 cases with *RET* rearrangements, 6 cases were reported previously (case nos. 2, 4, 7, 12, 14, and 15) (Kohno *et al*, 2012). All *RET*-rearranged cases were ADCs. When analysing consecutively resected ADC cases alone, *RET* rearrangements were observed in 7 of 830 ADC cases (0.8%). Of the 22 *RET*-rearranged cases, 19 (86%) possessed *KIF5B–RET* and 3 (14%) had *CCDC6–RET* fusions.

The *RET*-rearranged tumours were significantly more common in younger patients ($P=0.026$) and tended to occur in patients with no history of smoking ($P=0.051$). The *RET* rearrangements were not associated with gender, smoking status, tumour size, tumour stage, or lymph node status. Clinical records revealed that there were no patient histories of occupational exposure to radioactivity (Supplementary Table 5).

Among the 1874 cases examined, *EGFR* mutation was observed in 663 of the 1585 analysed cases (42.7%), and *ALK* rearrangement was observed in 55 of the 1860 analysed cases (3.0%; Table 1). All cases were detected exclusively with additional driver genetic changes (i.e., *RET*, *EGFR*, and *ALK*).

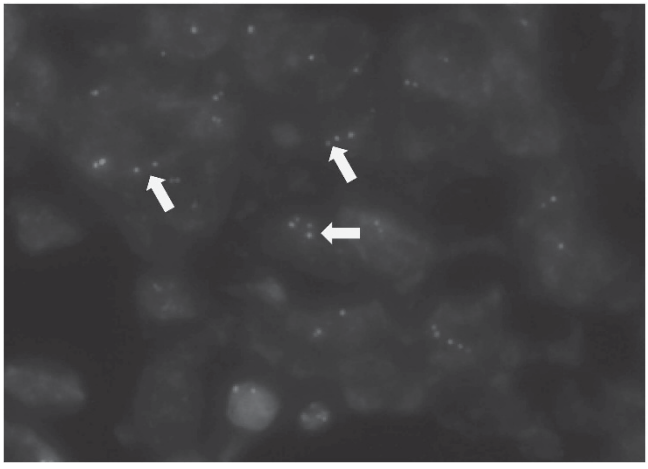


Figure 1. Representative image of fluorescence *in situ* hybridisation using a break-apart probe for *RET*-rearranged carcinoma. White arrows indicate split red–green signals. A full colour version of this figure is available at the *British Journal of Cancer* journal online.

Histological and immunohistochemical characteristics of patients with *RET*-rearranged NSCLCs. Histological findings are summarised in Table 2. The predominant growth pattern was lepidic in 6 cases, papillary in 9 cases, acinar in 2 cases, micropapillary in 1 case, and solid in 4 cases (Figures 2A and B). Focal lepidic, papillary, acinar, micropapillary, and solid patterns were observed in 16, 20, 16, 10, and 7 cases, respectively.

The predominant cell type was Clara/type II in 13 cases, columnar in 4 cases, and polygonal in 5 cases. Cells with cytoplasmic mucin production were at least focally present in 12 of the 22 (54.5%) *RET*-rearranged ADC cases. The presence of signet-ring (Figure 2C) or mucinous cribriform patterns were only observed in 27.3% and 13.6% of cases, respectively. The nuclear inclusion was at least focally present in 9 of the 22 (40.9%) *RET*-rearranged ADC cases (Figure 2D).

RET protein expression by immunohistochemistry. The RET expression was observed in 261 of the 1795 (14.5%) NSCLCs evaluated (Figure 3). The RET-immunopositive cases were significantly associated with histological subtype ($P<0.001$); 255 of the 1543 ADCs (16.5%), 1 of the 201 SCCs (0.5%), none of the 8 LCCs, and 5 of the 43 SACs (11.6%). The RET-immunopositive tumours were more commonly associated with younger patients ($P=0.006$), patients with no history of smoking ($P=0.007$), lymph node metastasis ($P<0.001$), and higher pathological stages ($P=0.001$) compared with RET-immunonegative tumours.

Among *RET*-rearranged cases, RET immunopositivity was observed in 14 of the 21 cases (66.7%) analysed with immunohistochemistry. Although RET immunoreactivity was significantly associated with *RET* rearrangement ($P<0.001$), its test performance was poor with 66.7% sensitivity and 86.1% specificity (Table 1).

Upon classification of the staining pattern of 261 RET-immunopositive cases, we observed that 158 cases (60.5%) displayed cytoplasmic staining, irrespective of membrane staining, and 103 cases (39.5%) displayed only membrane staining. All RET-immunopositive cases with *RET*-rearrangement displayed cytoplasmic staining. Among the 151 cytoplasmic staining cases, the detection rate for *RET*-rearranged cases increased from 5.4 to 9.9%.

Survival analysis. We investigated the existence of an association between patient OS and *RET* gene rearrangement. Follow-up data were available for all 1874 patients for a median of 62.3 months (range, 0.1 − 162.8 months). The *RET* gene rearrangement was not associated with OS in any of the cases analysed, among all cases ($P=0.456$; Figure 4A), among ADC-only cases, ($P=0.611$, Figure 4B), or among consecutively resected ADC cases ($P=251$, Figure 4C).

DISCUSSION

We observed that ~1.2% of NSCLC cases harboured *RET* rearrangements, all of which were ADCs, whereas neither SCCs,

Table 2. Pathological, cytological, and immunohistological features for *RET*-rearranged cases

		Histologic pattern						Cytological features					Immunohistochemical results					
No	Fusion partner	Predominant	LEP	PAP	ACI	MPC	SOL	Cell type	Mucin production	Nuclear inclusion	SRC	M-Crib	RET	Staining pattern	*TTF-1*	Napsin A	*PAX8*	Thyroglobulin
1	KIF5B	MPC	2	3	1	4	0	Type II	+	−	−	−	Pos	C	Pos	Neg	Neg	Neg
2	KIF5B	LEP	8	2	0	0	0	Type II	−	+	−	−	Pos	C	Pos	Pos	Neg	Neg
3	KIF5B	ACI	0	1	6	0	3	Polygonal	+	−	−	−	Pos	C	Pos	Pos	Neg	Neg
4	KIF5B	LEP	5	3	1	1	0	Type II	−	+	−	−	Pos	C	Pos	Pos	Neg	Neg
5	KIF5B	PAP	3	6	0	1	0	Type II	−	+	−	−	Pos	C	Pos	Pos	Neg	Neg
6	KIF5B	PAP	0	4	2	4	0	Columnar	+	−	−	−	Pos	C	Pos	Pos	Neg	Neg
7	KIF5B	ACI	3	2	5	0	0	Type II	+	−	+	−	Pos	C	Pos	Pos	Neg	Neg
8	KIF5B	PAP	1	4	3	0	2	Columnar	−	−	−	−	Neg	−	Pos	Pos	Neg	Neg
9	KIF5B	PAP	1	4	2	3	0	Columnar	+	−	+	+	Neg	−	Pos	Pos	Neg	Neg
10	KIF5B	LEP	8	2	0	0	0	Type II	−	+	−	−	Neg	−	Pos	Pos	Neg	Neg
11	KIF5B	PAP	2	6	1	1	0	Type II	−	+	−	−	Pos	C	Pos	Pos	Neg	Neg
12	KIF5B	PAP	3	6	0	0	0	Type II	−	+	−	−	Pos	C	Pos	Pos	Neg	Neg
13	KIF5B	PAP	4	5	0	0	0	Type II	−	−	−	−	Neg	−	Pos	Pos	Neg	Neg
14	KIF5B	LEP	5	4	0	1	0	Type II	+	+	−	−	Pos	C	Pos	Pos	Neg	Neg
15	KIF5B	PAP	0	4	3	3	0	Type II	+	−	+	+	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
16	KIF5B	SOL	0	2	3	0	5	Polygonal	+	−	−	−	Pos	C	Pos	Pos	Neg	Neg
17	KIF5B	SOL	1	2	1	1	5	Polygonal	+	−	−	−	Pos	C	Pos	Pos	Neg	Neg
18	KIF5B	PAP	2	3	2	2	1	Polygonal	+	−	+	−	Pos	C	Pos	Neg	Neg	Neg
19	KIF5B	LEP	8	0	2	0	0	Type II	−	+	−	−	Neg	−	Pos	Pos	Neg	Neg
20	CCDC6	LEP	5	4	1	0	0	Type II	−	+	−	−	Neg		Pos	Pos	Neg	Neg
21	CCDC6	SOL	0	0	1	0	9	Polygonal	+	−	+	−	Pos	C	Pos	Pos	Neg	Neg
22	CCDC6	SOL	0	1	4	0	5	Columnar	+	−	+	+	Pos	C	Pos	Pos	Neg	Neg

Abbreviations: ACI = acinar; C = cytoplasmic; CCDC6 = coiled-coil domain containing 6; KIF5B = kinesin family member 5B; LEP = lepidic; M = membranous; M-Crib = mucinous cribriform, MPC = micropapillary; #N/A = not assessed; Neg = negative; PAP = papillary; *PAX8* = paired box gene 8; Pos = positive; RET = rearranged during transfection; SOL = solid; SRC = signet-ring cell; *TTF-1* = thyroid transcription factor-1. Case numbers 2, 4, 7, 12, 14, and 15 were reported previously and have been highlighted in bold.

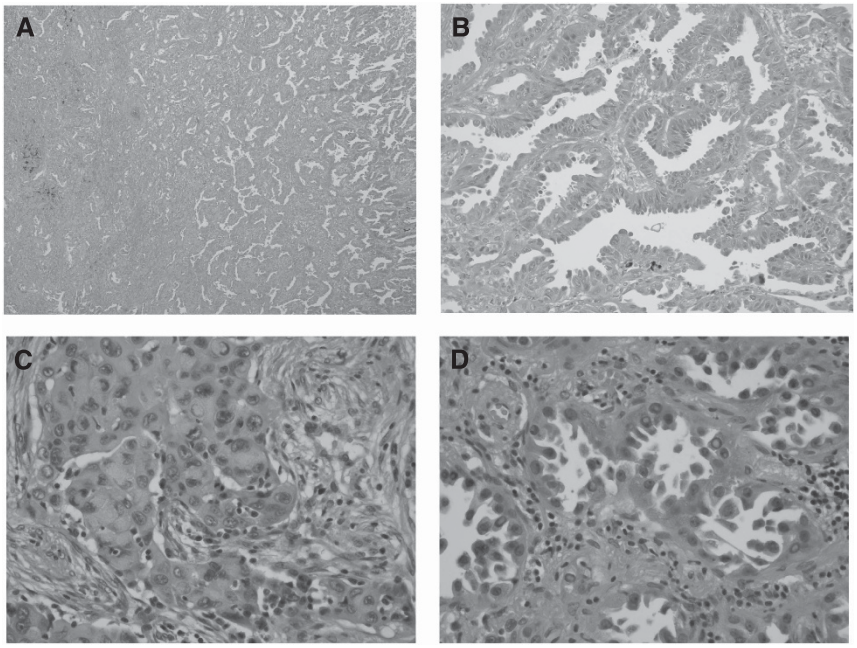


Figure 2. Representative images of *RET*-rearranged adenocarcinoma of the lung. (**A** and **B**) Many *RET*-rearranged adenocarcinomas displayed a papillary growth pattern (**A**: low magnification, and **B**: high magnification). (**C**) Solid signet-ring cell pattern was observed in a minority of *RET*-rearranged adenocarcinoma (original magnification × 200). (**D**) Some tumour cells displayed homogeneously eosinophilic-to-pale inclusions in the nuclei (original magnification × 200).

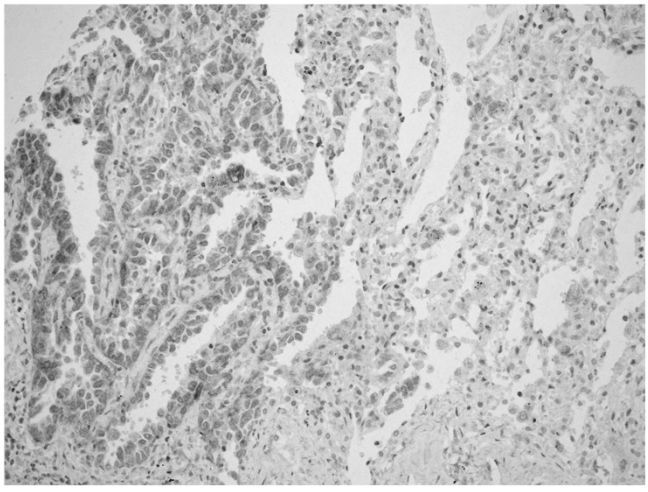


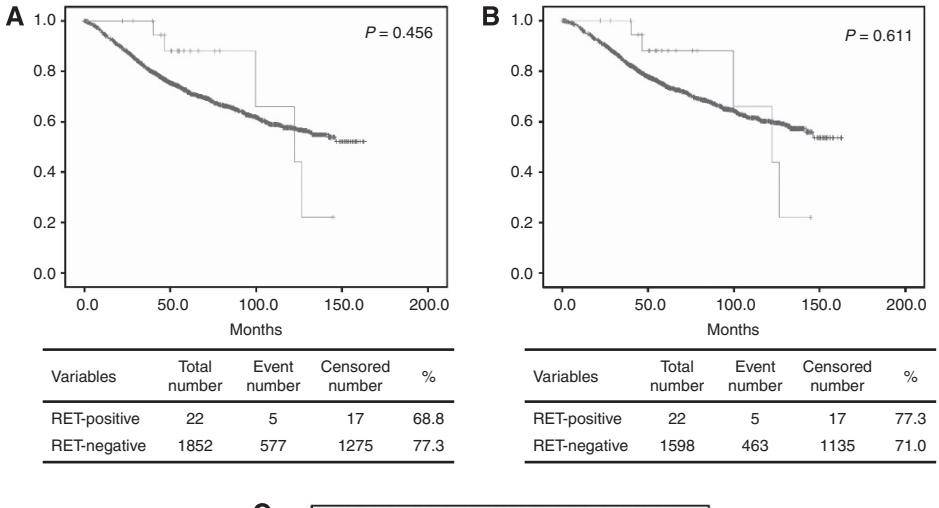
Figure 3. Representative images of RET-immunostaining positivity in a *RET*-rearranged adenocarcinoma. Diffuse, fine granular cytoplasmic staining was observed in the adenocarcinoma component, as shown in the left part of the figure, whereas negative signals were observed in nontumourous areas, as shown in the right part of the figure (original magnification × 400).

SACs, nor LCCs harboured this rearrangement. The prevalence of *RET* rearrangements (1.2%) in our cohort was in line with the range of results reported previously (0.6–10%) (Ju *et al*, 2012; Kohno *et al*, 2012; Lipson *et al*, 2012; Suehara *et al*, 2012; Takeuchi *et al*, 2012; Wang *et al*, 2012). The prevalence rate is considered to be affected by the specific case selection, such as that collected by known gene alterations in the negative cohort. In the subgroup of consecutively resected ADC cases, the frequency of *RET* rearrangements was only 0.9%.

Patients with *RET* rearrangements displayed nearly equivalent gender distributions, were relatively younger in age, and had no history of smoking compared with patients without *RET* rearrangements. The young age of onset and non-smoking history in *RET*-rearranged NSCLCs is reminiscent of the patient characteristics of *ALK*-rearranged NSCLCs (Shaw *et al*, 2009). However, other investigators have observed no statistical differences in age, gender, smoking history, or tumour stage between *RET*-rearranged ADC and wild-type *RET* ADCs (Wang *et al*, 2012). As is well known in PTC, *RET* gene fusions are associated with radiation exposure (Nikiforov and Nikiforova, 2011). However, no exposure to radioactivity was detected in either the current or previously reported *RET*-rearranged NSCLCs (Suehara *et al*, 2012).

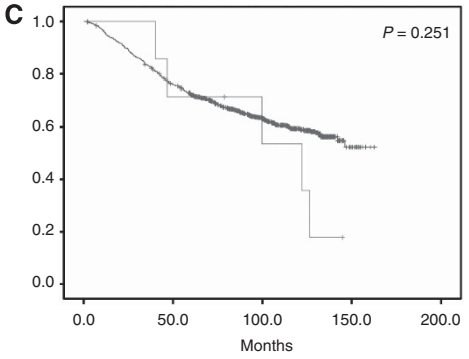
Consistent with previous studies, *RET* rearrangements were more common in ADCs. Wang *et al* (2012) also reported *RET* rearrangements in two cases of adenosquamous carcinomas. There have been no reported *RET* rearrangements in SQC, LCC, or SAC tumours. Based on IASLC/ATS/ERS classification, we and other investigators have reported on the association between papillary growth pattern and *RET* rearrangement (Suehara *et al*, 2012; Yokota *et al*, 2012). Recently, Wang *et al* (2012) reported that a solid pattern was most prevalent in *RET*-rearranged ADCs, with signet-ring cells also frequently observed (36.4%). In this study, although cytoplasmic mucin was present, at least focally, in the majority (59%) of cases, signet-ring cells were observed in only 27% of cases. Of note, the mucinous cribriform pattern – another characteristic morphology associated with *ALK*- and *ROS1*-rearranged lung cancers (Yoshida *et al*, 2011, 2013) – was also infrequently observed (13.6%) in the present cohort.

Determining the gold standard for FISH specificity is challenging, because there may be an unknown fusion partner in FISH-positive cases that could be detectable with RT–PCR or FISH for fusion probes. Therefore, in order to yield a more precise cutoff value for break-apart FISH probes, we used previously reported RNA sequence data as the gold standard. In the present study, FISH analysis with a break-apart probe of the *RET* gene is highly sensitive (100% sensitivity), but unlikely to be sufficient to define *RET*-positive cases because of the potential for false positivity



Variables	Total number	Event number	Censored number	%
RET-positive	22	5	17	68.8
RET-negative	1852	577	1275	77.3

Variables	Total number	Event number	Censored number	%
RET-positive	22	5	17	77.3
RET-negative	1598	463	1135	71.0



Variables	Total number	Event number	Censored number	%
RET-positive	7	5	2	28.6
RET-negative	823	314	509	61.8

Figure 4. Overall survival analysis in *RET*-rearranged lung carcinoma. (**A**) The overall survival curves for patients with *RET*-rearranged (green line) and *RET*-wild-type (blue line) non-small-cell lung carcinomas ($P = 0.9613$). (**B**) The overall survival curves for patients with *RET*-rearranged (green line) and *RET*-wild-type (blue line) adenocarcinoma ($P = 0.9665$). (**C**) The overall survival curves for patients with *RET*-rearranged (green line) and *RET*-wild-type (blue line) consecutively resected adenocarcinoma ($P = 0.547$). A full colour version of this figure is available at the *British Journal of Cancer* journal online.

(58%) based on RT–PCR or break-apart FISH probes for known partners. Compared with the false-positive rate in our study, a slightly lower rate (41%) for *RET* break-apart FISH results has been reported with an alternate probe design (Takeuchi *et al*, 2012). The advantages of using a break-part FISH probe for gene translocation are that it can detect the translocations irrespective of fusion partners. Therefore, the current false-positive rate may be overestimated because it may include an unknown fusion partner for the *RET* gene. However, the possible effect of unknown fusion partners was not likely because the recently reported frequency of novel fusion partners of *RET*-rearranged lung carcinoma (i.e., *NCOA4* and *TRIM33*) was extremely low (Wang *et al*, 2012; Drilon *et al*, 2013). Similar to *EML4–ALK* translocation, there exists intrachromosomal proximity (10.6 Mbp) of *KIF5B* and *RET* genes that complicates the generation of a proper break-apart probe that is easily resolvable by contemporary *in situ* technology.

Although RET immunoreactivity was significantly associated with *RET* rearrangement, its test performance was poor with only 66.7% sensitivity and 86.1% specificity. Other investigators have also reported a slightly lower positivity rate (54%) of RET antibody for *RET*-rearranged NSCLCs (Wang *et al*, 2012). Therefore, we conclude that RET immunohistochemistry possesses limited value in detecting *RET*-rearranged NSCLCs, unlike, for example, immunohistochemistry for human epidermal growth factor 2 status for breast carcinoma (Jacobs *et al*, 1999). Interestingly, cytoplasmic staining was more specific to gene rearrangement than membranous staining, likely because the KIF5B–RET chimeric proteins (except fusion partner of K24;R8) lack a transmembrane domain.

The present survival analysis indicated that *RET* rearrangement was not associated with OS. Even when the analysis was limited to ADCs or cases of consecutively resected ADC, no association was observed, consistent with previous reports (Wang *et al*, 2012; Yokota *et al*, 2012). However, the number of cases that have been investigated has been too small (spanning stages I–III) to draw any definitive conclusions regarding survival of *RET*-rearranged NSCLCs.

In summary, *RET* rearrangements were observed in 1.2% of NSCLC cases. All *RET* rearrangements in NSCLC were observed in ADCs. The *RET* rearrangements were observed in younger patients, patients with no smoking history, and papillary-predominant tumours. Although, cytoplasmic mucin production was at least focally present in 54.5% of *RET*-rearranged ADCs, distinct histological features were not detected. Furthermore, immunohistochemistry for RET protein has limited value to detect *RET*-rearranged NSCLC. Finally, these fusion genes did not coexist with *EGFR* and *ALK* alterations.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conception and design: Koji Tsuta and Takashi Kohno; financial support: Koji Tsuta and Takashi Kohno; administrative support: Koji Tsuta, Hisao Asamura, Takashi Kohno, and Ryoji Kushima; provision of study materials or patients: Koh Furuta and Hisao Asamura; data analysis and interpretation: Koji Tsuta, Yoko Shimada, Takashi Kohn, Koh Furuta, and Ryoji Kushima; manuscript writing: all authors; final approval of manuscript: all authors.

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