Modern Pathology (2018) 31, 307–312

Coexistent genetic alterations involving *ALK*, *RET*, *ROS1* or *MET* in 15 cases of lung adenocarcinoma

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In lung cancer, targetable activating alterations in cancer genes, such as *EGFR*, *ALK*, *RET*, *ROS1* and *MET*, are usually mutually exclusive. Rare lung cancer cases with coexistent alterations of *EGFR* and *ALK* or *EGFR* mutations with *RET* or *ROS1* rearrangements have been reported. In this study, we report 15 patients (3 men and 12 women; 14 Caucasians and 1 African American) with ages ranging from 43 to 81 years (median 60 years) with lung adenocarcinoma in which coexistent alterations of two cancer-associated genes, including *ALK, ROS1,* or *RET* rearrangement or *MET* amplification were present. The combination of alterations detected by fluorescence *in situ* hybridization included *ALK* combined with *ROS1* ($n=4$), *ALK* with *MET* ($n=3$), *ALK* with *RET* ($n=1$); *RET* with *MET* ($n=4$), *RET* with *ROS1* ($n=2$), and ROS1 combined with *MET* ($n=1$). The frequencies of involvement were similar for all 4 genes, 53% for both *ALK* and *MET* ($n=8$), 47% for both *RET* and *ROS1* ($n=7$). Activating gene mutations were also detected by next-generation sequencing for *TP53* ($n=6$), *EGFR* ($n=5$), *KRAS* ($n=3$) and *STK11* ($n=2$). Nine patients reported a smoking history (8 heavy and 1 light) and 6 patients were non-smokers. These findings suggest the need for assessing a panel of genes in lung cancer. Since targetable agents are available for each of these activating alterations, treatment with more than one targeted agent may be beneficial for this rare group of patients.
Modern Pathology (2018) **31,** 307–312; doi:10.1038/modpathol.2017.109; published online 15 September 2017

Lung cancer is the most common cause of cancer-related deaths in the United States and many other countries.[1,2] The development of tyrosine kinase inhibitors against epidermal growth factor receptor (*EGFR*) gene mutations (eg, erlotinib) and anaplastic large-cell lymphoma kinase (*ALK*) gene rearrangement (eg, crizotinib) has dramatically improved the outcome of patients with lung cancer.[3–6] Additional novel oncogenic fusion genes with tyrosine kinase functions, such as *RET* and *ROS1* as well as fusions genes combined with *MET* amplification have been identified and tyrosine kinase inhibitors targeting these molecular aberrations, eg, vandetanib against *RET* rearrangement,[7–9] have entered into clinical trials. Historically, activating cancer gene mutations have been considered as mutually exclusive in lung cancer.[10] However, a group of patients bearing co-alterations of *EGFR* and *ALK* has been reported recently,[11–14] and further studies have demonstrated that the addition of *ALK* inhibitor therapy improved the overall survival of these patients.[15] Co-alterations of *EGFR* with genes other than *ALK* (eg, *ROS1*) have been reported in a few cases,[16–18] but co-alterations of *ALK*, *RET*, *ROS1* or *MET* in lung cancer have not been reported to the best of our knowledge. In this study, we report 15 cases of lung adenocarcinoma in which abnormalities of two cancer-associated genes including *ALK*, *RET*, *ROS1* or *MET*. In 5 of these cases activating *EGFR* mutations were also present.

Materials and methods

Patients

We searched the database of the Clinical Cytogenetics Laboratory at The University of Texas MD Anderson Cancer Center for cases of lung cancer assessed by fluorescence *in situ* hybridization (FISH)

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Received 21 March 2017; revised 6 July 2017; accepted 6 July 2017; published online 15 September 2017

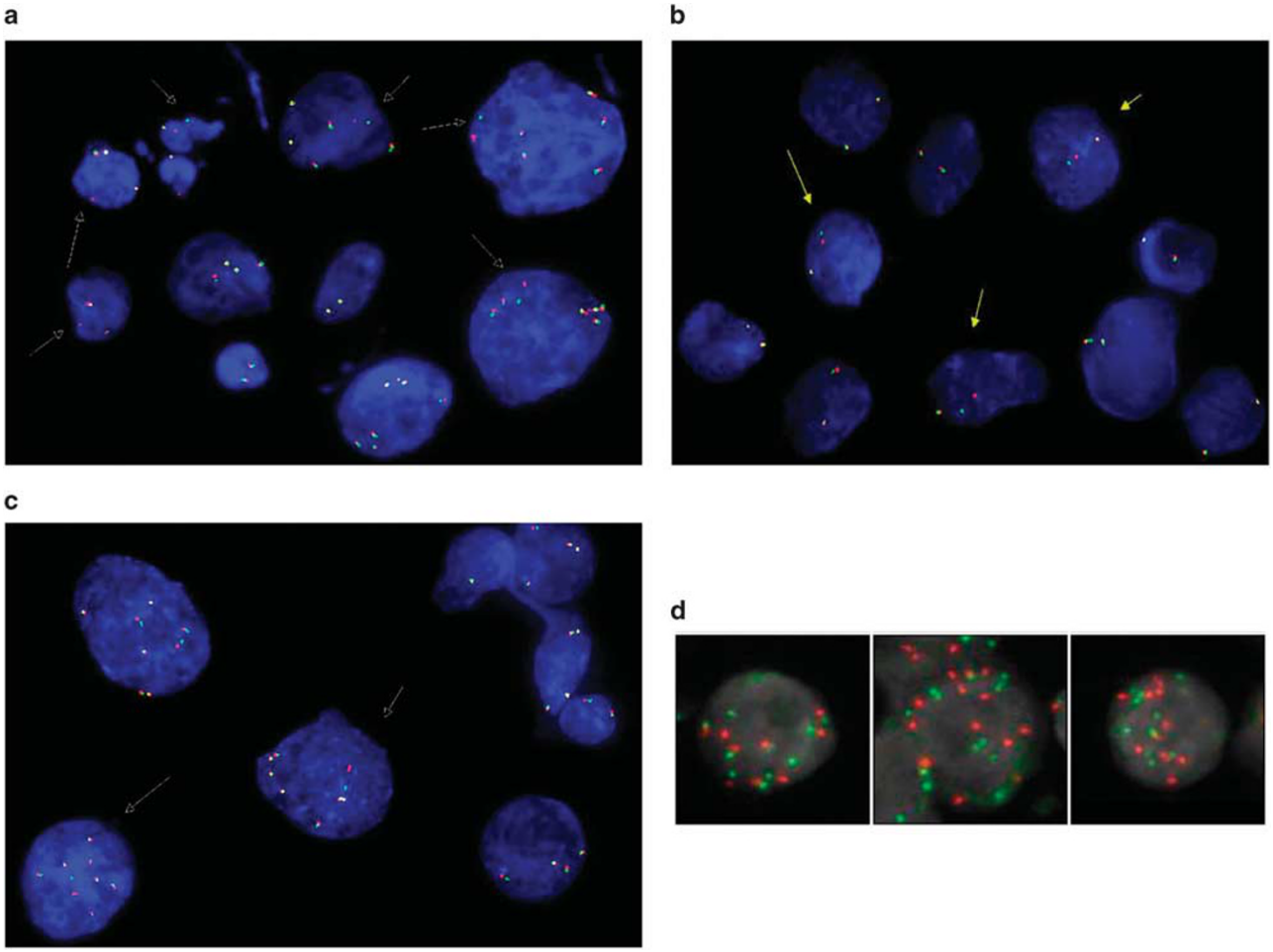

Figure 1 Representative positive signal patterns for *ALK*, *RET*, *ROS1* rearrangement and *MET* amplification. (**a**) Tissue FISH test for *ALK* gene rearrangement (Case 6). Positive cells are labeled with arrows. Two positive signal patterns are present (1R1G1F indicates an *ALK* gene rearrangement; 1R1F indicates an *ALK* gene rearrangement with 5′-ALK deletion (green signal). (**b**) Tissue FISH test for *RET* gene rearrangement (Case 14). Three positive cells with a split signal pattern (1R1G1F) are labeled with arrows. (**c**) Tissue FISH test for *ROS1* gene rearrangement (Case 6). Two positive cells with a split signal plus multiple fusion signal patterns (1R1G6 ~ 7 F) are labeled with arrows. It's also noticed that high percentages of interphase cells exhibit either a single fusion (1F) or multiple fusion (3 ~ 8F) signal patterns. The clinical significance of these cell populations is unknown. (**d**) Tissue FISH test for *MET* gene amplification (Case 6). Three representative cells with > 5 *MET* signals are shown.

for abnormalities of *ALK*, *RET*, *ROS1* or *MET*. Fifteen cases with positive results of more than one of these genes were identified and a thorough chart review was performed. The clinical and laboratory information were collected and reviewed by following institutional guidelines with informed consent in accord with the Declaration of Helsinki.

Fluorescence *In Situ* Hybridization and Scoring

All tissue FISH tests were performed using formalin fixed, paraffin-embedded tissue sections of primary and/or metastatic lung cancer specimen and were validated for these specimens following the American College of Medical Genetics and Genomics guidelines.[7,19] The FDA-approved, LSI *ALK* dual color, breakapart probe was applied for detection of *ALK* rearrangement (Abbott Molecular, Des Plaines, IL). This probe hybridizes to band 2p23 (spectrum green on the centromeric side and spectrum orange on the telomeric side of the *ALK* breakpoint). Fifty nuclei were analyzed routinely, but the analysis was extended to 100 nuclei if results from counting 50 nuclei were indeterminate. The following cutoffs have been established in our laboratory: positive signal pattern in > 25 cells of 50-nuclei analysis or in > 15% of 100-nuclei analysis (Figure 1a).

The Clear-View FISH RET dual color, breakapart probe from CymoGen DX was applied to detect *RET* rearrangement (Biocare Medical, Concord, CA). The probe hybridizes to band 10q11.21 (red on the centromeric side and green on the telomeric side of the *RET* breakpoint). Two hundred interphase nuclei were studied for each case. The cutoff values for a positive *RET* rearrangement established at 95% ($P < 0.05$) confidence level are: > 7.9% for one red, one green, one fusion (1R1G1F) signal pattern (positive for *RET* rearrangement) and > 3.1% for

one green, one fusion (1G1F) signal pattern (positive for *RET* rearrangement with 5′ deletion). A cutoff value of >4.4% was established for one red, one fusion (1R1F) signal pattern, which indicates a positive result for 3′ deletion with unknown clinical significance (Figure 1b).

The XT ROS1 dual color, breakapart probe was used to detect *ROS1* rearrangement detection (MetaSystems Group, Newton, MA). This probe hybridizes to 6q22 (red on the centromeric side and green on the telomeric side of the *ROS1* breakpoint). Two hundred interphase nuclei were studied for each case. The cutoff values for a positive *ROS1* rearrangement established at 95% ($P < 0.05$) confidence level are: >3.5% for either one but not a combination of the following three signal patterns: one red, one green, one fusion (1R1G1F) signal pattern (positive for *ROS1* rearrangement) and one red, one fusion (1R1F) signal pattern (positive for *ROS1* rearrangement with 5′ deletion). A cutoff value of >3.5% was established for one green, one fusion (1G1F) signal pattern indicating a positive result for 3' deletion with unknown clinical significance (Figure 1c).

The CymoGen DX MET/CC7 DNA Probe Kit was used for detection of *MET* amplification (Biocare Medical). The MET probe (red color) is specific for the *MET* locus (7q31) and CC7 probe (green color) is specific for the alpha satellite DNA sequences at the centromeric region of chromosome 7 (7p11.1-q11.1). Sixty interphase cells were studied for each case. These criteria are used for a positive *MET* amplification: 1). MET:CC7 ratio ≥2.0; 2). MET:CC7 ratio <2.0, but average MET signal counts in each cell ≥5; 3). Over 10% of the tumor cells showed ≥20 copies of MET signals or signal clusters (Figure 1d).

This is to point out that signal patterns of 1R1G≥2F were frequently observed in our tissue FISH tests for *ALK*, *RET* and *ROS1* rearrangement (eg, Figures 1a and c). They were considered as positive results with additional gain/amplification of an intact gene, although the clinical significance of gain/amplification of intact *ALK*, *RET* or *ROS1* gene generally remains unknown.

Next-Generation DNA Sequencing for Detection of Gene Mutations

DNA was isolated from tumor tissues and targeted next-generation DNA sequencing was performed using the Ion Torrent PGM with the Ion AmpliSeq Cancer Hotspot Panel as described previously.[20] Most of the cases included in this study were tested with a 50-gene hotspot panel, while the other cases were recently tested with an expanded 128-gene hotspot panel.

Results and discussion

Our laboratory has provided tissue FISH tests for *ALK*, *RET*, *ROS1* rearrangement or *MET* amplification for patients with primary or metastatic lung cancer since February, 2010. At the end of 2016, a total of 5206 cases have been tested, of which 1251 (24%) were tested for all 4 markers, 609 (12%) for 3 markers, 768 (15%) for two markers and 2578 (50%) for one marker, respectively. The rates of a positive result are: 7% (238/3382) for *ALK*, 2% (32/1782) for *ROS1*, 1% (33/2379) for *RET*, and 5% (177/3398) for *MET* respectively, which are very similar to the prevalence of these abnormalities reported from a study of 1139 Chinese lung cancer patients[21] as well as other patient populations.[22] Among these positive cases, 15 (Table 1) cases were identified with concurrent rearrangement of two cancer-associated genes including *ALK*, *RET* and *ROS1* and/or with *MET* amplification. Fourteen of these cases that were tested for all 4 genes whereas case 5 was tested for 3 markers (not *RET*). Therefore, the estimated dual positivity rate is 0.6% (15/2628) if cases with two or more tested markers are included or up to 1.1% (14/1251) if the cases with 4 tested markers are included in our study.

To the best of our knowledge, this is the first report showing concurrent rearrangement or amplification of *ALK*, *RET*, *ROS1* or *MET* in lung cancer cases. As shown in Table 1, the combination of alterations exists in various forms, eg, *ALK* rearrangement combined with *ROS1* ($n=4$, cases 5-8) or *RET* rearrangement ($n=1$, case 4); *RET* rearrangement combined with *ROS1* rearrangement ($n=2$, cases 13 and 14); as well as *MET* amplification combined with *ALK* ($n=3$, cases 1-3), *RET* ($n=4$, cases 9-12) or *ROS1* rearrangement ($n=1$, case 15). The frequency of involvement is similar for all 4 biomarkers: 53% (8/15) for both *ALK* and *MET* and 47% (7/15) for both *RET* and *ROS1* ($n=7$). Interestingly, two cases (cases 9 and 10) exhibited unusual *RET* FISH signal patterns that have not been reported previously. In case 9, a signal pattern of 0R3~20G1~3F was detected indicating a simultaneous deletion of *5′RET* and duplication/amplification of *3′RET*. Lee *et al*[23] has reported a *RET* rearrangement positive case with a predominant signal pattern of 1R2G1F indicating a gain of an extra copy of *3′RET,* but no amplification of *3′RET*. In case 10, the predominant signal pattern is 1R1G3~6F, indicating *RET* rearrangement with gain of multiple extra copies of an intact *RET* gene. The clinical significance of a *RET* amplification in lung cancer remains unknown, but a previous study correlated this finding with coexistence of *EGFR* mutations.[24]

In the past, mutations involving genes with tyrosine kinase function, such as *EGFR*, *KRAS*, *HER2*, *BRAF*, *ALK*, *RET*, *ROS1* and *MET* were viewed as independent, mutually exclusive events.[10] However, in recent years cases of lung cancer with coexistent alterations of *EGFR* and *ALK* or other genes have been reported by others. For example, over 20 cases of lung cancer with simultaneous alterations of *EGFR* and *ALK* or *EGFR* and *ROS1* have been reported.[16–18] Jurmeister *et al* has reported 3 *ALK* rearranged lung cancer cases that also harbored MET protein

Table 1 General information and main laboratory findings in 15 lung adenocarcinoma patients with dual positivity of *ALK*, *RET*, *ROS1* and *MET*

Case #	Age (y)	Sex	Ethnicity	Smoking	Staging of lung adenocarcinoma	History of lung cancer (m) [a]	Tissue FISH tests		Gene mutation results	Therapeutical intervention	Outcomes
							Tissues	Results			
1	46	F	Caucasian	light smoker	Stage IV, T2aN3M1b	3	Left neck mass (metastatic tumor) biopsy	ALK+; MET+	EGFR:p.L745_A750del	Ongoing radiation and EGFR inhibitor	Partial response
2	59	M	Caucasian	1.5 packs/day for 25 years	Stage IV, T2N2M1b	4	Pericardial fluid FNA	ALK+; MET+	KRAS:p.G12V; TP53:p.R248L	Radiation, ongoing ALK inhibitor	Partial response
3	66	M	Caucasian	2 packs/day for 40 years	Stage IV, T3N2M1b	8	Left lung mass biopsy	ALK+; MET+	NA	Radiosurgery to brain and lung	Partial response
4	60	F	Caucasian	no	Stage IIIa, T2aN1M0	36	Left lung lobelectomy	ALK+; RET+	no	Surgery; chemotherapy	Complete response, no recurrence so far
5	65	F	Caucasian	no	Stage IV, T2N2M1b	30	Liver mass biopsy	ALK+; ROS1+	NA	ALK inhibitors (crizotinib, ceritinib); ongoing chemotherapy and radiation	Partial response
6	58	F	Black	40 packs/year for >20 years	Stage I, T1N1M0	11	Right lower paratracheal LN, biopsy	ALK+; ROS1+	STK11:p.G196R; TP53:p.R273H	Radiation; ongoing chemotherapy[b]	Partial response (poor health condition, not a candidate for surgical resection)
7	50	M	Caucasian	no	Stage IV, T2N2M1b	25	Left lung mass biopsy	ALK+; ROS1+	NA	ALK inhibitors; chemotherapy; ongoing PD-1 inhibitors.	Response and recurrence for 3 times; currently partial response
8	77	F	Caucasian	2 packs/day for 15 years	Stage IV, T4N3M1b	16	Right lower paratracheal (4 R) LN biopsy	ALK+; ROS1+	KRAS:p.G12C; NF1:p.A308S, p.G655fs[c]10 and p.S1834fs[c]28; SF3B1:p.K666M	Chemotherapy; ongoing PD-1 inhibitor	Dead due to a heart attack.
9	60	F	Caucasian	2 packs/day for >30 years	Stage IIIa, T1bN1M0	20	Right lung lobelectomy	RET+[c]; MET+	KRAS:p.G12C	Surgery; chemotherapy	Symptoms improved
10	53	F	Caucasian	2 packs/day for 20 years	Stage IV, T4N3M1b	79[d]	Right lung mass biopsy	RET+[c]; MET+	NRAS:p.Q61L; PIK3R1:p.I491M; PTCH1:p.G1343V; TP53:p.[G293A;E294fs[c]6]	Surgery; chemotherapy; VEGF inhibitor; radiation	Dead due to complication
11	61	F	Caucasian	no	Stage IIIA, T2N2M0	24	Right lung lobelectomy	RET+; MET+	No	Surgery; chemoradiation	Symptoms improved
12	43	F	Caucasian	no	Stage IV, T4N3M1b	20	Left lung mass biopsy	RET+; MET+	EGFR:p.E746_A750del; TP53:p.N210fs[c]36	EGFR inhibitors (Rociletinib; afatinib); stereotactic radiosurgery; vandetanib plus everolimus; pembrolizumab; ongoing chemotherapy and RET inhibitor trial	Regression first and then progression for 3 times
13	65	F	Caucasian	0.5 packs/day for 43 years	Stage II, T2aN1M0	36	Right lung mass biopsy	RET+; ROS1+	EGFR:p.L858R; TP53:p.H193R	Surgery; chemoradiation; ongoing EGFR inhibitor	Significant partial response
14	81	F	Caucasian	no	Stage IIb, T3N2M0	10	Left lung mass biopsy	RET+; ROS1+	EGFR:p.L747_A750delinsP; STK11:p.F354L	Radiation; ongoing EGFR inhibitor[b]	Partial response
15	79	F	Caucasian	1 packs/day for 61 years	Stage IV, T2N3M1b	13	Left infraclavicular LN biopsy	ROS1+; MET+	EGFR:p.S768I; KDR:p.P1354S; PTEN:c.[165-2 A>T];[c.165-1G>T]; TP53:p.G244C	Chemotherapy; radiation; EGFR inhibitor	Dead due to complication

Abbreviations: m: months; NA: not tested; no: no mutation detected; y: years.
[a]From diagnosis of lung adenocarcinoma to the detection of *ALK*, *RET*, *ROS1* or *MET* mutation.
[b]No surgery due to poor lung function and general health conditions.
[c]Unusual signal patterns.
[d]Event-free for 46 months after primary lung cancer removal; recurrence for 33 months.

Table 2 Demographic summary and comparison of mutations by clinical staging in 15 lung adenocarcinoma patients

Demography					
M/F	3/12				
Age	43 to 81 year, medium age 60 year				
Ethnicity	14 Caucasians, 1 African American				
Smoking	Yes ($n=9$); No ($n=6$)				
Lung Adenocarcinoma					
History	3 to 79 months, medium 20 months				
Clinical staging	I ($n=1$)	II ($n=2$)	III ($n=3$)	IV ($n=9$)	I+II/III+IV
Mutations					
ALK	1	0	1	6	1/7
ROS1	1	2	0	4	3/4
RET	0	2	3	2	2/5
MET	0	0	2	6	0/8
Total	2	4	6	18	6/24

overexpression, although not *MET* gene amplification.[25] Several research groups have reported that lung cancer patients with alterations of both *EGFR* and *ALK* have distinctive clinical characteristics and they have suggested that adding an ALK inhibitor to therapy can improve overall survival.[11,14–16] All these data therefore show the coexistence of two (or more) driver mutations in lung cancer cases and that these combinations have potential clinical implications in affected patients.[3,5,16] The low frequency of coexistent driver mutations of two (or more) genes including *ALK*, *RET*, *ROS1* and *MET* is likely attributable to two reasons: 1) A low incidence of *ALK*, *RET*, *ROS1* rearrangement as well as *MET* amplification in lung cancer;[21,22] 2) A change in clinical practice for patients with lung cancer has led to more rigorous testing for these genetic abnormalities. In support to the second reason, less than 25% of all lung cancer cases had been tested for all 4 markers simultaneously during the past 6 years at our center.

The demographic data for the 15 patients in this study shows a female predominance (12 women and 3 men) with a median age of 60 years (range, 43 to 81 years). Fourteen patients are Caucasians and one is an African American. Nine patients reported a smoking history (8 heavy and 1 light) and 6 patients were non-smokers. The pathological diagnosis of lung adenocarcinoma was established in these patients from 3 to 79 months (median 20 months) before FISH testing was performed. Three patients had an early-stage lung adenocarcinoma (stage I: case 6; stage II: cases 13 and 14) and 12 patients had advanced-stage lung adenocarcinoma (stage III: cases 4, 9 and 11; stage IV: all the rest cases). Therefore, a dual positivity of these 4 markers can be found in early stage lung adenocarcinoma cases. However, *ALK* rearrangement (7/8) and *MET* amplification (8/8) are mostly found in advanced stage lung adenocarcinoma cases (stage III+stage IV), whereas *ROS1* and *RET* rearrangement are present in both early stage and advanced stage lung adenocarcinoma cases in this study (Table 2). Interestingly, 6 cases (cases 2, 6, 10, 12, 13 and 15) also had coexistent *TP53* mutations; 5 cases (cases 1, 12-15) had *EGFR* mutations; 3 cases (cases 1, 8, 9) had *KRAS* mutations; and 2 cases (cases 6 and 14) had *STK11* mutations detected by next-generation DNA sequencing. Although rare, all these cases demonstrate that co-alterations of tyrosine kinase function genes and tumor suppressor genes can be detected in lung adenocarcinoma cases. This phenomenon may be attributable to recurrence of disease even after an effective target-specific therapy.

All patients were treated according to stages with a combination of surgical resection, radiation therapy and therapeutic agents. Several patients received target-specific therapies, 5 with EGFR inhibitors (cases 1, 12-15), 3 with ALK inhibitors (cases 2, 5, and 7), and 3 with PD-1 inhibitors (cases 7, 8 and 12), but their clinical courses all show a temporarily partial response with local recurrence or new metastasis. One patient (case 12) is currently enrolled in a RET inhibitor trial, whereas no patients have been treated on a trial with ROS1 or MET inhibitor agents, although newly developed target-specific tyrosine kinase inhibitors may be an option during the course of their disease.[4,8,11] At last follow up, 3 patients (cases 1, 9 and 15) died and 12 patients remain alive with partial response to their treatments.

In summary, this is the first report showing concurrent rearrangement or amplification of *ALK*, *RET*, *ROS1* or *MET* in lung cancer cases. Several of the cases in this study also had coexistent *EGFR*, *KRAS* and/or *TP53* mutations. As recommended by the 2015 World Health Organization classification of lung tumors, comprehensive molecular profiling is very important for the workup of lung cancer, and the finding of multiple molecular alterations in lung cancer may have potential clinical implications for these patients.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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