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External Validation of *ALK* and *ROS1* Fusions Detected Using an Oncomine Comprehensive Assay

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Abstract. *Background/Aim: This study aimed to assess the yield of an Oncomine comprehensive assay v3 (OCAv3)-based next-generation sequencing (NGS) analysis for detecting anaplastic lymphoma kinase (ALK) and c-ros oncogene 1 (ROS1) fusions in non-small cell lung cancer (NSCLC). Patients and Methods: NGS data from 85 NSCLC cases were reviewed. ALK and ROS1 fusion status was compared to conventional tests. Results: ALK or ROS1 fusion reads were detected in 17 NSCLC cases. Results in 10 NSCLC cases showed concordance with conventional tests, high-count fusion reads, a lack of mutually exclusive mutations of ALK or ROS1, and frequent signet-ring cell component. Seven NSCLC cases showing discordant results exhibited low to intermediate fusion read counts and mutations mutually exclusive from ALK or ROS1. Conclusion: Cases showing high-count fusion reads in OCAv3-based NGS have a strong possibility of carrying ALK or ROS1 fusion. Cases with low- to intermediate-count fusion reads should be interpreted with caution and may require additional confirmative tests.*

Lung cancer is the most common cause of cancer-related mortality worldwide (1, 2). During the past decade, the identification of the genetic landscape of non-small cell lung cancer (NSCLC) has led to advances in targeted therapies and the prediction of responses to these treatments (3, 4). According to recent clinical practice, all patients with locally advanced or metastatic NSCLC should be tested for important therapeutic targets, such as pathogenic driver mutations (5-14), amplifications (14, 15), and genetic fusions (16-22).

The use of tyrosine kinase inhibitors antagonizing key oncogenic alterations has surprisingly prolonged the survival of distinct subgroups of patients (23, 24). The therapeutic approach for anaplastic lymphoma kinase (*ALK*)-rearranged NSCLC is an evolving paradigm of personalized medicine in oncology (18). The echinoderm microtubule-associated protein-like 4 (*EML4*)–*ALK* rearrangement is the most common *ALK* fusion gene, with an estimated prevalence of 3-5% in NSCLC (25), primarily in adenocarcinomas (26, 27). It increases to ~29% in a subgroup of young patients who have never smoked, have an advanced stage at presentation, and whose tumours have solid architecture and signet-ring cell features (26, 27). The *EML4–ALK* translocation is mutually exclusive from epidermal growth factor receptor (*EGFR*) and Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutations, and tumours with such a translocation expresses a marked response to *ALK* inhibitors such as crizotinib (28, 29). Moreover, another receptor tyrosine kinase, c-ros oncogene 1 (*ROS1*), has also been described in NSCLC, and patients with a *ROS1* rearrangement were reported to respond to ALK inhibitors (30, 31). Therefore, *ALK* and *ROS1* status are important in defining patient eligibility for *ALK*/*ROS1*-directed targeted therapies.

Current diagnostic tests for detecting *ALK*/*ROS1* fusion include fluorescence in situ hybridization (FISH), immunohistochemistry, reverse transcription-quantitative polymerase chain reaction (RT PCR), and next-generation sequencing (NGS) analyses (32). FISH is currently the gold standard for identifying these fusions, and NGS analyses have been of limited use as screening tests or ancillary tools (33). However, to accompany the need for more extensive molecular diagnostics, targeted NGS is becoming a clinically preferred molecular diagnostic method because of its ability

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to detect multiple mutations simultaneously and accurately using small samples in a single test (34). Evidence-based guidelines for the molecular diagnosis and treatment of NSCLC have recently reported that NGS panels are preferred over single genetic testing to identify treatment options other than *ALK*, *EGFR*, and *ROS1* inhibitors and highlight the importance of NGS for genetic investigation (34).

Implementing NGS in clinical laboratories in the context of gene fusion detection will help expand the knowledge base for gene fusion in NSCLC and has the potential to directly influence patient care by detecting therapeutically actionable targets. However, although NGS has been routinely applied as a research tool to discover gene fusions in NSCLC, it is evident that the standardization of fusion calls is an unmet need, as there are no established criteria for the number of fusion scripts that can be classified as positive. The present study aimed to assess the yield of an amplicon-based parallel sequencing (RNA-seq) assay using the Oncomine Comprehensive Assay v3 (OCAv3; Thermo Fisher Scientific, Waltham, MA, USA) test for detection of *ALK* and *ROS1* fusion transcript variants in comparison with conventional methods such as FISH and RT-PCR in NSCLC.

Patients and Methods

Case selection. This study (2021-01-017) was approved by the Institutional Review Board of the Kyung Hee University Hospital (Seoul, Republic of Korea). The Cancer NGS database of Kyung Hee University Hospital (Seoul, Republic of Korea) was searched for cases of patients harbouring either *ALK* or *ROS1* gene arrangements detected using an OCAv3-based RNA fusion assay between January 2019 and November 2020. The entire dataset for the study period consisted of 85 cases NSCLC and 118 of non-pulmonary malignancies. Among the 203 cases, *ALK* or *ROS1* gene rearrangement was detected in 31 cases (NSCLC, n=17; non-pulmonary, n=14). We reviewed the electronic medical records and collected all available clinicopathological data, including age, sex, tumour stage, pathological diagnosis, and previous results of genetic analysis.

Tumour samples used for genetic analysis. The most representative formalin-fixed, paraffin-embedded (FFPE) tissue specimens were selected at the time of pathological diagnosis. Tumour cell-rich areas were selected and microdissected for genetic analysis. All samples contained at least 100 tumour cells. The tumour cellularity and specimen type in each sample were documented. In the case of small biopsy specimens, one haematoxylin-eosin-stained slide was prepared after genetic analysis to confirm that the tumour cells were included in the experiments.

Nucleic acid extraction. FFPE sections (5-μm-thick) were thoroughly deparaffinized in xylene and hydrated using a graded alcohol series in water. The sections were manually microdissected under a dissecting microscope using a scalpel point dipped in ethanol. The scraped material was washed in phosphate-buffered saline and digested overnight in proteinase K at 56˚C in Buffer ATL (Qiagen Inc.,Germantown, CA, USA). DNA and RNA were isolated using the QIAamp DSP DNA FFPE extraction kit according to the manufacturer's instructions. A Qubit® 4.0 Fluorometer (Thermo Fisher Scientific) was used for sample quantitation using highly sensitive and accurate fluorescence-based Qubit® quantitation assays.

NGS and variant analyses. This study used OCAv3 assay (Thermo Fisher Scientific), which is an amplicon-based targeted assay that enables the detection of relevant single-nucleotide variants, amplifications, gene fusions, and indels from 161 unique genes. NGS library preparation for the OCAv3 assay using extracted DNA and RNA was performed using Ion AmpliSeq™ Library Preparation following the IonChef™ System protocol (Thermo Fisher Scientific) (35). Sequencing was performed on an IonTorrent™ S5 XL platform, following the manufacturer's protocols, using positive control cell line mixtures (Horizon Discovery, Cambridge, UK). Genomic data were analysed and alterations were detected using IonReporter™ software (version 5.6; Thermo Fisher Scientific). We also manually reviewed the variant call format file and Integrated Genomic Viewer. Pathogenic variants in coding regions, promoter regions, or splice variants were retained. For a detailed comparison with the conventional method, all available data regarding fusion genes, including fusion partner genes of *ALK* and *ROS1*, exon numbers, and junction spanning read count were documented.

FISH. All samples were tested using *ALK* and *ROS1* FISH assays using a Vysis ALK Break Apart FISH Probe Kit (Abbott Molecular, Abbott Park, IL, USA) and ZytoLight SPEC ROS1 Dual Color Break Apart Probe kit. Tissue sections of 3-μm-thick were prepared for FISH staining. The processes and interpretations of the tests were performed according to the manufacturer's instructions (36). Positive cases were defined as those exhibiting split signals [5'-part (green fluorescence) and 3'-part (red fluorescence) signals were regarded as split when their separation distance was greater than two fluorescence signal diameters] or when an isolated red signal was observed in more than 15% of at least 50 tumour cells.

RT-PCR. The specimens from the patients were tested for *ALK* and *ROS1* status *via* an RT-PCR assay using a Fusion Gene Detection Kit and a ROS1 Fusion Gene Detection Kit (Amoy Diagnostics, Xiamen, PR China) according to the manufacturer's instructions (37). Total RNA was extracted from three to four sections of 3-μm-thick FFPE tissue using a RNeasy FFPE kit (Qiagen Inc.). All assays were performed on an Agilent Mx3000P QPCR instrument (Agilent Technologies, Santa Clara, CA, USA).

Results

Comparison between NGS and conventional tests. Our study consisted of 85 NSCLC cases and 118 cases of non-pulmonary malignancies, including 46 of diffuse glioma, 20 of gastrointestinal and hepatobiliary cancer, 19 of ovarian cancer, 12 of endometrial cancer, 10 of head and neck cancer, four of breast cancer, four of sarcoma, two of neuroendocrine carcinoma, one of kidney cancer, and one of gastrointestinal stromal tumour cases (Figure 1). NGS analysis revealed a small number of *ALK* or *ROS1* fusion reads in 17/85 NSCLC (20%) and 14/118 non-pulmonary malignancy (12%) cases. Eleven out of the 17 NSCLC cases demonstrated findings compatible with both the FISH assay and RT-PCR (65%),

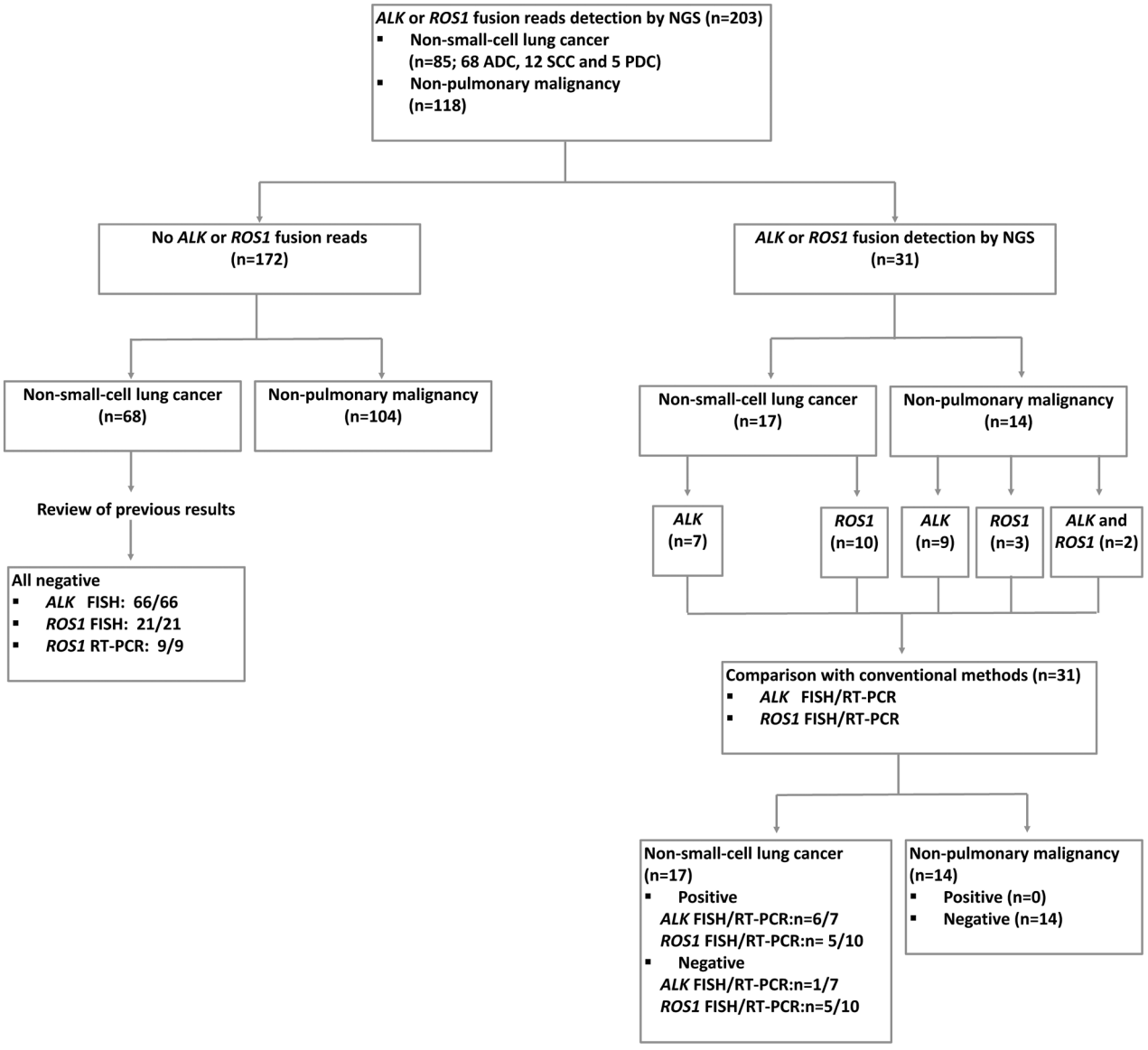

Figure 1. *Flow diagram of the study design and diagnostic workup. ADC: Adenocarcinoma; ALK: anaplastic lymphoma kinase; FISH: fluorescence in situ hybridization; NGS: next-generation sequencing; PDC: poorly differentiated carcinoma; ROS1: c-ros oncogene 1; RT-PCR: reverse transcription-quantitative polymerase chain reaction; SCC: squamous cell carcinoma.*

whereas none of the results for 14 cases of non-pulmonary malignancies matched those of the conventional test. In the 172 cases without *ALK* or *ROS1* fusion reads, prior FISH and RT-PCR results from the 68 NSCLC samples were negative.

Tracking NGS data in cases of patients harbouring ALK fusion reads. Detailed genetic analysis data for seven NSCLC and 11 non-pulmonary malignancy cases harbouring *ALK* fusion reads are shown in Figure 2. Of these, result for six NSCLC cases (no. 1-6) matched those of the conventional tests. The number of *ALK* fusion reads in the six assay-matching cases ranged from 57,064 to 360,764 (median: 186,512), and the proportion of fusion reads to the total read count [(read count/total read count) $\times 10^6$] ranged from 59,396 to 589,262 (median: 197,345). Additional pathogenic variants were found in two out of six cases, consisting of phosphatase and tensin homolog (*PTEN)* and tumor protein 53 (*TP53)* mutations, respectively. The remaining NSCLC case (no. 7) and 11 cases of non-pulmonary malignancies showed unmatched results. The number of *ALK* fusion reads in one NSCLC case showing

Figure 2. *The gene-fusion and mutational landscape according to the results of the conventional fluorescence in situ hybridization (FISH) and reverse transcription-quantitative polymerase chain reaction (RT-PCR) tests of the study cohort. AKT2: AKT serine/threonine kinase 2; ALK: anaplastic lymphoma kinase; ATRX: ATRX chromatin remodeler; BR: breast; CBD: common bile duct; CCND3: cyclin D3; CCNE1: cyclin E1; CD74: CD74 molecule; CNS: central nervous system; CR: colorectum; EGFR: epidermal growth factor receptor; EM: endometrium; EML4: echinoderm microtubule-associated protein-like 4; ERBB2: erb-b2 receptor tyrosine kinase 2; EZR: ezrin; GIST: gastrointestinal stromal tumor; IDH1: isocitrate dehydrogenase 1; LU, lung; MET: MET proto-oncogene, receptor tyrosine kinase; NF1: neurofibromin 1; NFE2L2: nuclear factor erythroid 2-related factor 2; NGS: next-generation sequencing; NOTCH1: notch receptor 1; NP, nasopharynx; OV, ovary; PIK3CA: phosphatidylinositol 3-kinase catalytic alpha polypeptide; PTEN: phosphatase and tensin homolog; RET: ret proto-oncogene; ROS1: c-ros oncogene 1; SLC34A2: solute carrier family 34 member 2; STO, stomach; TERT: telomerase reverse transcriptase; TP53: tumor protein 53. *Same tumor showing both ALK fusion and ROS1 fusion genes at a low read count.*

unmatched results was 18,687 and the proportion of the fusion reads to the total read count was 19,263, which was lower than that of the matched cases. This case had an additional phosphatidylinositol 3-kinase catalytic alpha polypeptide (*PIK3CA)* mutation as well as a cyclin E1 (*CCNE1)* and AKT serine/threonine kinase 2 (*AKT2)* amplification. The number of *ALK* fusion reads in these 11 non-pulmonary malignancies ranged from 110 to 12,005 (median: 784), and the proportion of fusion reads to the total read count ranged from 136 to 25,695 (median: 873). Non-pulmonary malignancies showed at least one additional pathogenic variant, which was a frequently reported mutation in each tumour type. In both the groups with matched and those with unmatched results with conventional tests, *EML4* was the fusion partner. The most common *ALK* breakpoint was exon 13, and there were other breakpoints at exons 6, 17, and 18.

NGS data in cases of patients harbouring ROS1 fusion reads. Detailed genetic analysis data from 10 patients with NSCLC and five with non-pulmonary malignancies harbouring *ROS1* fusion reads are shown in Figure 2. Of these, five NSCLC cases (no. 8-12) exhibited results matched with those of conventional tests. The number of *ROS1* fusion reads in the five matched cases ranged from 15,321 to 221,294 (median: 29,812), and the proportion of fusion reads to the total read count ranged from 25,370 to 386,001 (median: 43,784). No additional pathogenic variants were detected. The remaining five NSCLC cases (no. 13-17) and five cases of non-pulmonary malignancies showed unmatched results. The number of *ROS1* fusion reads in these NSCLC cases showing unmatched results ranged from 369 to 9,160 (median: 420), and the proportion of fusion reads to the total read count ranged from 389 to 15,807 (median: 1,366). Several pathogenic variants were detected concurrently in all five cases. Of note, 4/5 cases showed mutations in genes encoding other receptor tyrosine kinases, such as MET proto-oncogene, receptor tyrosine kinase (*MET)*, ret proto-oncogene (*RET)*, and *EGFR*, which were reported to be mutually exclusive from *ROS1* fusions in NSCLC (38). The number of *ROS1* fusion reads in the five non-pulmonary malignancies ranged from 209 to 1,706 (median: 294), and the proportion of fusion reads to the total read count ranged from 400 to 2,313 (median: 450). These non-pulmonary malignancies showed at least one additional pathogenic variant, which was a frequently reported mutation in each tumour type such as *PIK3CA* mutation in endometrioid carcinoma. In groups with matched and unmatched results with conventional tests, ezrin (*EZR)* and solute carrier family 34 member 2 *(SLC34A2)* were the most common fusion partners (94%, n=14/15); *ROS1* was fused to CD74 molecule *(CD74)* in the remaining case. In these 15 cases, the *ROS1* breakpoint was at exon 10 but there were other breakpoints at exons 13, 4, and 6.

Clinicopathological features observed in NSCLC according to ALK and ROS1 fusion status. Detailed clinicopathological data are shown in Table I. The fusion status in each tumour was classified based on the matched results of the NGS and conventional tests. The mean age of the six patients who were *ALK*+ (no. 1-6) was 70 years (range=61-83 years) and the male-to-female ratio was 1:1. Among these patients, four were never smokers, and the remaining were current or former smokers. The histological type in all six cases was adenocarcinoma, with papillary/micropapillary growth pattern in five out of six and solid growth pattern in one, whilst four out of six also had a signet-ring cell component (67%). Three out of the six *ALK*+ cases had unresectable stage III tumours with mediastinal metastases or stage IV with distant tumour spread at the time of diagnosis; the remaining patients underwent lobectomy for stage I disease. One ALK$^-$ case (no. 7) was an 83-year-old male who had adenocarcinoma with a solid subtype and underwent lobectomy for stage I disease.

All five patients with *ROS1*+ tumours (no. 8-12) were female, with a median age of 58 years (range=37-65 years), never smokers, and had adenocarcinomas. The histological type of these five ROS1+ cases was adenocarcinoma, showing solid (n=3/5) and micropapillary (n=2/5) growth patterns. Tumours in two out of the five cases had signet-ring cell components (40%). Most *ROS1*+ patients (80%, n=4/5) underwent lobectomy for stage I disease. Compared to the *ROS1*+ cases, the mean age of the *ROS1*$^-$ patients was higher (66 years; range=49-74 years) and the male-to-female ratio was 3:2. Among these patients, three had a history of smoking. The histological types of these five *ROS1*$^-$ cases (no. 13-17) were adenocarcinoma in three and squamous cell carcinoma in two. The adenocarcinoma cases had variable histological subtypes, including lepidic, acinar, and papillary patterns without a signet ring cell component. Three *ROS1*$^-$ cases had unresectable stage III-IV tumours at the time of diagnosis; the remaining two patients underwent lobectomy for stage I-II disease.

Discussion

This study investigated the fusion status of *ALK* and *ROS1* using conventional methods (FISH and RT-PCR) to verify the fusion reads detected using the OCAv3-based NGS analysis. *ALK* and *ROS1* fusion reads were detected in a wide range of levels in some cases of NSCLC and other types of tumours. Among them, the results in approximately one-third of the cases of patients harbouring many fusion reads were concordant with the results of the conventional test. There were no cases with false-negative results in the OCAv3 analysis compared to the conventional tests. These findings raise the issue of specificity rather than sensitivity; therefore, the number of fusion reads is important for the interpretation of samples testing positively for *ALK* and *ROS1* fusion *via* the OCAv3-based NGS analysis. As the interpretation of conventional tests also applies the quantitative measurement of fusion genes, the fact that concordance between NGS and conventional tests is primarily affected by the number of fusion reads is reasonable.

The number of fusions reads in our study can be divided into three main groups based on the validation results of the conventional test, with each group showing several distinct findings. The first group comprised those that a had high fusion read count (>20,000), including 10 patients with NSCLC in whom the conventional test was positive in all cases. Mutations in other genes were rarely observed, and even when they were, they were not mutually exclusive from *ALK* or *ROS1*. Histologically, all cases were adenocarcinoma including at least one focal signet-ring cell component, which is a well-known characteristic of *ALK*+ or *ROS1*+ NSCLC (26, 27).

The second group comprised those that had a low fusion read count (<1,000), including three NSCLC cases and 10 non-

Table I. *Summary of anaplastic lymphoma kinase (ALK) and c-ros oncogene 1 (ROS1) status according to clinical features, and analysis using fluorescence in situ hybridization (FISH), reverse transcription-quantitative polymerase chain reaction (RT-PCR) and next-generation sequencing (NGS) in 17 patients with non-small cell lung cancer.*

No.	Age, years/ Gender	Smoking history	Stage	Specimen	Histological type	NGS							
						FISH	RT-PCR	Time to NGS, months	Cellularity, %	Fusion (with exon number)	Fusion read count	Per million: (fusion/total) $\times 10^6$	Other variants
1	80/M	–	IA3	Bx	ADC, Pap and microPap	*ALK*	*ALK*	0	20	*ALK e20–EML4 e18*	360,764	589,262	*PTEN* D252V
2	72/F	–	IIIA	Bx	ADC, microPap with SRC	*ALK*	*ALK*	0	30	*ALK e20–EML4 e13*	358,192	128,710	–
3	61/F	–	IA1	Bx	ADC, Pap with SRC	*ALK*	*ALK*	0	20	*ALK e20–EML4 e13*	236,430	256,348	–
4	78/M	+	1A1	Bx	ADC, microPap	*ALK*	*ALK*	2	40	*ALK e20–EML4 e17*	136,594	138,343	*TP53* trunc
5	69/M	+	IVB	Bx	ADC, solid with SRC	*ALK*	*ALK*	18	20	*ALK e20–EML4 e6*	58,742	59,396	–
6	68/F	–	IVB	Bx	ADC, microPap with SRC	*ALK*	*ALK*	0	80	*ALK e20–EML4 e13*	57,064	263,587	–
7	83/M	–	IA1	Surg	ADC, solid	–	–	92	80	*ALK e20–EML4 e17*	18,687	19,263	*AKT2* amp *CCNE1* amp *PIK3CA* E542K
8	37/F	–	IA1	Surg	ADC, microPap	*ROS1*	*ROS1*	36	90	*ROS1 e32–SLC34A2 e13*	221,294	386,001	–
9	58/F	–	IVB	Bx	ADC solid with SRC	*ROS1*	*ROS1*	12	20	*ROS1 e35–EZR e10*	159,779	191,945	–
10	46/F	–	IVB	Bx	ADC, solid with SRC	*ROS1*	*ROS1*	12	40	*ROS1 e34–CD74 e6*	29,812	43,784	–
11	60/F	–	IIIB	Bx	ADC, solid	*ROS1*	*ROS1*	10	50	*ROS1 e34–EZR e10*	21,629	25,370	–
12	65/F	–	IA2	Surg	ADC, microPap	*ROS1*	*ROS1*	2	50	*ROS1 e34–EZR e10*	15,321	42,816	–
13	74/M	+	IIIA	Surg	ADC, lepidic, acinar	–	–	8	50	*ROS1 e32–SLC34A2 e4*	9,160	15,807	CDK4 amp *TP53* V157F *MET* 14 skipping
14	49/M	+	IIIB	Bx	SCC	–	–	0	80	*ROS1 e34–EZR e10*	1,572	1,492	*RET* C634W *PIK3CA* amp
15	78/F	–	IV	Bx	ADC, acinar	–	–	0	80	*ROS1 e34–EZR e10*	420	1,111	*KIF5B–RET* fusion
16	63/F	–	IA3	Bx	ADC, pap	–	–	0	30	*ROS1 e32–SLC34A2 e4*	397	1,366	*EGFR* L858R *TP53* C176W
17	67/M	+	IIB	Surg	SCC	–	–	1	90	*ROS1 e34–EZR e10*	369	389	*CCND3* amp *TP53* trunc *NFE2L2* D77H

ADC: Adenocarcinoma; *AKT2*: AKT serine/threonine kinase 2; amp: amplification; Bx: biopsy; *CCND3*: cyclin D3; *CCNE1*: cyclin E1; *CD74*: CD74 molecule; *EGFR*: epidermal growth factor receptor; *EML4*: echinoderm microtubule-associated protein-like 4; *EZR*: ezrin; F: female; *KIF5B*, kinesin family member 5B; M: male; *MET*: MET proto-oncogene, receptor tyrosine kinase; *NFE2L2*: nuclear factor erythroid 2-related factor 2; Pap: papillary; *PIK3CA*: phosphatidylinositol 3-kinase catalytic alpha polypeptide; *PTEN*: phosphatase and tensin homolog; *RET*: ret proto-oncogene; Surg: surgically resected; SCC: squamous cell carcinoma; *SLC34A2*: solute carrier family 34 member 2; SRC: signet-ring cell; *TP53*: tumor protein 53; trunc: truncation.

pulmonary malignancies, where none of the cases were positive for *ALK* or *ROS1* in conventional tests. All cases of NSCLC patients harboured founder mutations in either *EGFR* or *PIK3CA*, which were reported to be mutually exclusive from *ALK* and *ROS1* fusions (38, 39). Similarly, all other patients with non-pulmonary malignancies had another concurrent variant known to play a key role in each malignancy. For example, in endometrial cancer, *ALK* fusion co-occurred with *KRAS, PTEN, PIK3CA*, and AT-rich interactive domain-containing protein 1A (*ARID1A)* mutations, which are well-known mutations in endometrial cancer. Histologically, three NSCLC cases in this group with low fusion read consisted of two squamous-cell carcinomas and one adenocarcinoma. None of the three cases had a signet-ring cell component.

The third group comprised those that had an intermediate fusion read count (1,000-20,000), including four NSCLC cases and six cases of non-pulmonary malignancies. Among these, only one case (no. 12) of NSCLC was positive in the conventional tests; all others tested negatively. For the quantitative correction of the fusion read count, we also focused on the proportion of *ALK* and *ROS1* fusion reads to the total mapped fusion reads (reads per million). Interestingly, the corrected value of the one NSCLC case, which did not show any other genetic mutations except for *ROS1,* increased to a high-count level. In contrast, the corrected values did not change substantially in cases 7, 13, and 14, which had other mutations mutually exclusive from *ALK* or *ROS1*. The corrected values of the six non-pulmonary malignancies did not change significantly.

Since this was a validation study of small number of cases at a single institution, there is a limit to estimating the exact cut-off value between each group. However, we observed that well-known histological and molecular characteristics of *ALK* or *ROS1* fusion-related NSCLC cases were observed in the group with a high fusion read count, but not in the low-count group and in most of the intermediate-count group. Low to intermediate fusion read counts were consistently detected in non-pulmonary malignancies that rarely harbour *ALK* or *ROS1* fusions. These findings suggest that the high fusion read count defined by our scale is valuable for predicting true positive cases. It is supported by histological and molecular features of the tumors as well as conventional test results.

In summary, we emphasize that an understanding of the quantitative approach in the interpretation of the results of OCAv3 assay analysis of fusions is required. It is necessary to obtain an approximate cut-off value using the accumulated validation data for every institution and laboratory. Validation in our study indicated that defining only a high fusion read count (>20,000) as a true positive is strongly supported by the epidemiological, histological, and molecular background of *ALK* or *ROS1* fusion-related tumours, as well as conventional test results. In cases with low to intermediate counts, it seems wise to review the histological features, comprehensive genomic profiles, and the proportion of fusion reads to the total mapped fusion reads; if necessary, confirmative conventional tests should be considered.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

All Authors made substantial contributions to the conception and design of the study; acquisition, analysis, and interpretation of the data; drafting of the article; and critical revision of the article for important intellectual content. All Authors provided final approval of the version to be published.

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