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PD-L1 expression in *ROS1*-rearranged non-small cell lung cancer: A study using simultaneous genotypic screening of *EGFR*, *ALK*, and *ROS1*

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Abstract

Background: The aim of the current study was to investigate the prevalence and clinicopathologic characteristics of *ROS1*-rearranged non-small cell lung cancer (NSCLC) in routine genotypic screening in conjunction with the study of PD-L1 expression, a biomarker for first-line treatment decisions.

Methods: Reflex simultaneous genotypic screening for *EGFR* by peptide nucleic acid clamping, and *ALK* and *ROS1* by fluorescence in situ hybridization (FISH) was performed on consecutive NSCLC cases at the time of initial pathologic diagnosis. We evaluated genetic aberrations, clinicopathologic characteristics, and PD-L1 tumor proportion score (TPS) using a PD-L1 22C3 assay kit.

Results: In 407 consecutive NSCLC patients, simultaneous genotyping identified 14 (3.4%) *ROS1* and 19 (4.7%) *ALK* rearrangements, as well as 106 (26%) *EGFR* mutations. These mutations were mutually exclusive and were found in patients with similar clinical features, including younger age, a prevalence in women, adenocarcinoma, and advanced stage. The PD-L1 assay was performed on 130 consecutive NSCLC samples. High PD-L1 expression (TPS ≥ 50%) was observed in 29 (22.3%) tumors. PD-L1 expression (TPS ≥ 1%) was significantly associated with wild type *EGFR*, while *ROS1* rearrangement was associated with high PD-L1 expression. Of the 14 cases with *ROS1* rearrangement, 12 (85.7%) showed PD-L1 expression and 5 (35.7%) showed high PD-L1 expression.

Conclusion: In the largest consecutive routine Asian NSCLC cohort analyzed to date, we found that high PD-L1 expression frequently overlapped with *ROS1* rearrangement, while it negatively correlated with *EGFR* mutations.

Introduction

Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer death worldwide.[1] Recent advances in the molecular characterization of NSCLC, together with personalized therapies against NSCLC molecular targets, have improved patient prognosis. Testing for activating mutations and rearrangements in the kinase domain of *EGFR*, *ALK*, and *ROS1* is now recommended as part of the routine evaluation of patients with advanced lung adenocarcinoma.[2,3] Recently, immune checkpoint inhibitors against PD-1 have shown remarkable therapeutic activity in NSCLC:[4–6] for example, pembrolizumab has been approved by the United States Food and Drug Administration (USFDA) for first-line systemic therapy in advanced NSCLC with a PD-L1 tumor proportion score (TPS) of > 50% by PD-L1 22C3 assay.[7] However, testing of both genotype changes and PD-L1 biomarker levels in

routine practice has raised questions about first-line treatment options when it comes to a PD-L1 TPS > 50% combined with targetable oncogenic driver mutations.[3] Notably, accumulating evidence has revealed a relationship between PD-L1 expression and the presence of driver mutations in *EGFR*, *ALK*, and *KRAS*.[8,9]

ROS1 is a transmembrane tyrosine kinase receptor that shares a significant amino acid homology to the kinase domain of ALK.[10] Previous studies have reported that chromosomal rearrangements involving *ROS1* occur in 1–2% of NSCLC and are clinically associated with a history of never-smoking, younger age, and adenocarcinoma histologic type.[11,12] There has been debate over the prevalence of ROS1 abnormality and its mutual exclusiveness to other mutations. Previous studies using fluorescence in situ hybridization (FISH), next-generation sequencing, or quantitative PCR (qPCR) have reported that the rearrangements in *ROS1* are generally distinct from and mutually exclusive to *EGFR* and *ALK* mutational status.[13–15] In contrast, other studies using immunohistochemistry (IHC) screening alone, or with FISH, have reported that ROS1-expressing NSCLCs frequently harbor concomitant oncogenic driver mutations.[16,17] The low prevalence of ROS1 aberrations and the diversity in the methods used to detect them are the main reasons for the difficulties in defining the clinicopathologic characteristics of ROS1-aberrant NSCLCs. For example, recent studies assessing PD-L1 expression in ROS1-aberrant cases included no more than three cases and showed no statistical association.[3,18] PD-L1 expression shows limited immunoreactivity in older tissue samples[19] and changes after treatment.[20,21] The assessment of PD-L1 expression in routine clinical practice at the time of the initial pathologic diagnosis, before any antitumor treatment, is the best way to evaluate PD-L1 expression in ROS1-rearranged NSCLCs, especially during first line treatment.

Therefore, we aimed to examine PD-L1 expression using a USFDA-approved companion diagnostic, the 22C3 pharmDx kit (Agilent/Dako, Santa Clara, CA, USA), in a *ROS1*-rearranged population, by simultaneously genotyping *EGFR*, *ALK*, and *ROS1* in unselected consecutive patients with NSCLC.

Methods

Patients

From 2011, the certified Molecular Pathology Center of the Catholic University of Korea Yeouido St. Mary Hospital, South Korea, has been conducting comprehensive genotyping screening for targeted therapy of patients with NSCLC from 36 hospitals in South Korea. From January 2014, *ROS1* FISH has been integrated into the screening platform: a total of 1110 *ROS1* FISH tests have been performed on samples from multiple institutions and 35 (3.2%) cases with *ROS1* rearrangement were found up to December 2017. A total of 407 patients with NSCLC at the Yeouido St. Mary Hospital who consecutively underwent non-sequential, simultaneous genotyping for *EGFR*, *ALK*, and *ROS1* using tissue samples from the initial pathologic diagnosis or from the immediate subsequent surgery (no previous antitumor treatment) were selected for this study. The patients' clinical data were retrieved from the electronic medical record system of the Yeouido St. Mary Hospital. NSCLC tumor pathology was classified according to World Health Organization criteria.[22] A never-smoker was defined as a patient who had never smoked or who had smoked < 100 cigarettes in their lifetime; pack-years of smoking were calculated as packs-day multiplied by the years of smoking. Processing and data analysis were performed on pseudonymized data sets. This study was conducted in accordance with the relevant legislation and approved by the institutional review board of Yeouido St. Mary's Hospital (SC18RNSI0037) and complied with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all participants included in the study.

Simultaneous diagnostic platform for genotyping and immunohistochemistry

We adhered to guidelines previously described for simultaneous genotypic testing and IHC.[23] The sample with the largest tumor proportion and least stroma in each patient was selected for analysis.[24] Additionally, the sample needed to have >100 tumor cells for the PD-L1 test[25] and > 50 tumors cells for ALK and ROS1 FISH tests.[11,26] In a tissue-sparing manner, we also investigated an IHC panel including thyroid transcription factor 1 (TTF-1), p63, p40, and cytokeratin 7; mucin staining using Periodic acid-Schiff stain and Alcian blue for histological confirmation; ALK IHC to confirm the FISH results; and PD-L1 IHC (using the PD-L1 IHC 22C3 pharmDx, Agilent/Dako) for therapeutic purposes.[23,27] PD-L1 IHC was performed on 4 μm thick formalin-fixed paraffin-embedded (FFPE) tissue sections using the PD-L1 clone 22C3 pharmDx kit and Dako Automated Link 48 platform (Agilent/Dako). Immunohistochemical staining for TTF-1 (clone SP141, Ventana Medical Systems, Tucson, AZ, USA), p63 (clone 4A4), p40 (polyclonal anti p40; Diagnostic BioSystems, Pleasanton, CA, USA), cytokeratin 7 (clone SP52, Ventana), and ALK (clone 5A4, dilution 1:50, Leica Biosystems, Newcastle, UK) was performed using the Benchmark Ultra Autostainer (Roche Tissue Diagnostics, Tucson, AZ, USA) with an OptiView Universal DAB detection kit for ALK IHC and UltraView universal DAB detection kit for the other proteins (Ventana Medical Systems).

Detection of *EGFR* mutations

DNA was isolated from three 5 μm thick FFPE tissue sections using the High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions.[23] The extracted DNA was stored at −20 °C until analysis. The PNAClamp EGFR Mutation Detection Kit (PANAGENE Inc., Daejeon, Korea) was used to detect mutations by qPCR, as previously described.[28] Briefly, qPCR was performed in total volume of 20 μL that included template DNA, a primer and peptide nucleic acid (PNA) probe set, and a SYBR Green PCR master mix. The control qPCR lacked the PNA probe and contained the wild-type (WT) template. The qPCR was performed using a CFX96 PCR detection system (Bio-Rad, Philadelphia, PA, USA). PCR cycling conditions were as follows: five minutes at 94°C and 40 cycles of 94°C for 30 seconds, 70°C for 20 seconds, 63°C for 30 seconds, and 72°C for 30 seconds. We used the ΔCt method to quantify the amplification: for this purpose, we used threshold cycle (Ct) values, which were automatically calculated from the PCR amplification plots of fluorescence against the number of cycles. ΔCt values were calculated as the Ct value of the standard minus the Ct value of the sample. Higher ΔCt values indicate more efficient amplification of the mutant. A cutoff value of 2.0 was used to indicate the presence of mutant DNA.

ALK and *ROS1* rearrangements

ALK and *ROS1* rearrangements were detected using FISH without prior screening by IHC. FISH is the preferred method used to investigate *ALK* and *ROS1* mutations and is a prerequisite for crizotinib treatment.[29,30] Additionally, FISH allows the co-detection of *ALK* and *ROS1* rearrangements as opposed to sequential staining in IHC. Briefly, FISH was performed on unstained 4 mm thick FFPE tumor samples using the Vysis ALK FISH Break Apart kit and Vysis ROS1 FISH Break Apart kit (Abbott Molecular, Abbot Park, IL, USA), following the manufacturer's instructions.[31] FISH images were captured using the automated BioView Duet scanning system (BioView, Rehovot, Israel) and scored by either one or two experienced pathologists. The procedure consisted of the following steps: (i) proper tumor tissue sections were selected for automated imaging and analysis using a 10× objective to locate the nuclei; (ii) the system automatically captured and analyzed the nuclei found in those regions using a 60× oil immersion objective and a single band 4′,6-diamidino-2-phenylindole (DAPI)/Spectrum Green/Spectrum Orange filter; and (iii) the target nuclei were selected and scored by a pathologist using a specific algorithm for positive or negative signal patterns based upon the classifications described in the Vysis ALK FISH Break Apart kit instructions.

A minimum of 50 tumor cell nuclei was counted according to the manufacturer's instructions. *ALK* and *ROS1* FISH-positive cases were defined as those with > 25 nuclei (50%) with break-apart (BA) signals or isolated red signals (IRS) for *ALK* and isolated green signals (IGS) for *ROS1*. *ALK* and *ROS1* FISH-negative samples were defined as those with < 5 nuclei (< 10%) with BA signals or IRS (for *ALK*) and IGS (for *ROS1*). *ALK* and *ROS1* FISH cases were considered borderline if 5–25 cells (10–50%) were positive. In the case of borderline results, a second reader evaluated the slide: 50 additional tumor cell nuclei were counted, and a percentage was calculated from a total of 100 cells. If < 15% of the cells were positive, the sample was considered negative. If ≥ 15% of the cells were positive, the sample was considered positive.[26]

PD-L1 tumor proportion score analysis

An experienced pathologist interpreted the expression of PD-L1. PD-L1 protein expression was determined by using the TPS, which is the percentage of viable tumor cells showing partial or complete membranous staining at any intensity. The specimen was considered to be negative or positive for PD-L1 expression at TPS < 1% and TPS > 1%, respectively. PD-L1 expression was subclassified as low PD-L1 expression with TPS ≥ 1% and TPS < 50% and high PD-L1 expression with TPS ≥ 50%.

Statistical analysis

Statistically significant differences between groups were analyzed using the Fisher's exact test. Data are expressed as means ± standard deviations. A two-tailed P value < 0.05 was considered statistically significant. All statistical analyses were performed using R software, version 3.4.1 (R Foundation, Vienna, Austria).

Results

Baseline clinical and pathologic characteristics of patients

Among the 407 patients with NSCLC, 238 (58.6%) were male, and the mean patient age was 66.9 years (Table 1). The predominant histologic type was adenocarcinoma (306 cases, 75.4%), and 183 (46.4%) patients were never-smokers. A high percentage of the study population (56.6%) had advanced stage cancer (stages IIIB and IV). Stage information was not available for 12 patients. Most of the study specimens (96.1%) were obtained by non-surgical biopsy (bronchoscopic biopsy, 10.3%;

Table 1 Demographics of *EGFR*, *ALK*, and *ROS1* status in 407 NSCLC consecutive patients

		EGFR (*n* = 106)		*ALK* (*n* = 19)		*ROS1* (*n* = 14)	
Variables	Total	N (%)	*P*†	N (%)	*P*†	N (%)	*P*†
Age, mean ± SD	66.9 ± 12.07	64.8 ± 12.0	**0.033**‡	55.7 ± 15.7	**< 0.001**†	61.2 ± 15.5	**< 0.001**†
Gender, N (%)							
Male	238 (58.6)	29 (27.4)	**< 0.001**	7 (36.8)	0.058	4 (28.6)	**0.026**
Female	168 (41.4)	77 (72.6)		12 (63.2)		10 (71.4)	
Smoking history							
Never smoker	183 (46.4)	76 (73.8)	**< 0.001**	14 (73.7)	**0.018**	10 (71.4)	0.098
Ever smoker	211 (53.6)	27 (26.2)		5 (26.3)		4 (28.6)	
Packyears	19.5 ± 23.6	5.5 ± 11.6	**< 0.001**‡	6.9 ± 14.8	**0.001**‡	12.1 ± 24.3	0.237‡
Histology							
ADC	306 (75.4)	101 (95.3)	**< 0.001**§	17 (89.5)	0.179 §	14 (100)	**0.026**§
SCC	74 (18.2)	4 (3.8)		1 (5.3)		0 (0.0)	
ADSQ	9 (2.2)	1 (0.9)		0 (0.0)		0 (0.0)	
Other NSCLCs	17 (4.2)	0 (0.0)		1 (5.3)		0 (0.0)	
Stage							
IA	60 (15.2)	20 (19.6)	**< 0.001**¶	2 (10.5)	**0.006**¶	1 (7.1)	**0.001**¶
IB	37 (9.4)	10 (9.8)		0 (0.0)		1 (7.1)	
IIA	18 (4.6)	7 (6.9)		1 (5.3)		0 (0.0)	
IIB	18 (4.6)	2 (2.0)		1 (5.3)		1 (7.1)	
IIIA	38 (9.6)	7 (6.9)		2 (10.5)		0 (0.0)	
IIIB	32 (8.1)	6 (5.9)		0 (0.)		0 (0.0)	
IV	191 (48.5)	50 (49.0)		13 (68.4)		11 (78.6)	
Type of sample							
Biopsy			0.258††		1.000††		1.000††
Bronchoscopy	42 (10.3)	6 (5.7)		3 (15.8)		1 (7.1)	
PCNB	298 (73.4)	87 (82.1)		13 (68.4)		9 (64.3)	
Metastatic site	35 (8.7)	7 (6.6)		3 (15.8)		3 (21.4)	
Cell block	5 (1.2)	3 (2.8)		0 (0.0)		1 (7.1)	
EBUS-TBNA	10 (2.5)	1 (0.9)		0 (0.0)		0 (0.0)	
Surgical resection							
Lobectomy	13 (3.2)	2 (1.9)		0 (0.0)		0 (0.0)	
Wedge resection	3 (0.7)	0 (0.0)		0 (0.0)		0 (0.0)	

†*P* value was evaluated compared to wild type for the gene. ‡Evaluated by *t*-test. §Adenocarcinoma versus all others. ¶Stage I–IIIa versus IIIb–IV. ††Biopsy versus surgical resection. Values in bold are statistically significant. ADC, adenocarcinoma; SCC, squamous cell carcinoma; ADSQ, adeno-squamous carcinoma; NSCLC, non-small cell lung cancer; PCNB, percutaneous needle aspiration biopsy; EBUS-TBNA, endobronchial ultrasound-transbronchial needle aspiration biopsy.

percutaneous needle aspiration biopsy, 73.4%; metastatic site biopsy, 8.7%; cell block from pleural effusion, 1.2%; and endobronchial ultrasound-guided transbronchial needle aspiration, 2.5%); the remainder was obtained by surgical resection (lobectomy, 3.2%; wedge resection, 0.7%).

Genetic alterations of *EGFR*, *ALK*, and *ROS1*

As shown in Table 1, among the 407 patients, we identified 106 (26.0%) *EGFR* mutations, and 19 (4.7%) *ALK* and 14 (3.4%) *ROS1* rearrangements. Among the squamous cell carcinoma cases, none had a *ROS1* rearrangement, one had an *ALK* mutation, and four had an *EGFR* mutation. In this cohort, coexisting genetic alterations were detected in two patients (0.7%): one had an *EGFR* mutation with an *ALK* rearrangement, the other had an *EGFR* mutation with a *ROS1* rearrangement.

When comparing patients with WT or mutated oncogenes, we found that younger age and advanced stage (IIIB and IV) were significantly associated with the three oncogene mutations. *EGFR* mutations were significantly more frequent in patients with the following characteristics: younger age ($P = 0.033$), female gender ($P < 0.001$), never-smoker ($P < 0.001$), shorter smoking timeframe (pack-years, $P < 0.001$), adenocarcinoma ($P < 0.001$), and advanced stage (IIIB and IV; $P < 0.001$). A similar pattern was observed in patients with *ALK* and *ROS1* rearrangements. Specifically, younger age ($P < 0.001$), never-smoker ($P = 0.018$), shorter smoking timeframe ($P < 0.001$), and advanced tumor stage ($P = 0.006$) were also significantly associated with *ALK* rearrangements, while *ROS1* rearrangements were significantly associated with younger age ($P < 0.001$), female gender ($P = 0.026$), adenocarcinoma ($P = 0.026$), and advanced tumor stage ($P < 0.001$).

Association of *EGFR*, *ALK*, and *ROS1* gene mutation with PD-L1 expression

Table 2 shows the relationship between *EGFR*, *ALK*, and *ROS1* gene mutations and PD-L1 expression. From September 2016 onwards, the PD-L1 22C3 test was performed on 130 consecutive patients with NSCLC; we identified 29 cases (22.3%) with high PD-L1 expression, 59 cases (45.4%) with low PD-L1 expression, and 42 cases (32.3%) with no PD-L1 expression. *EGFR* WT status was significantly associated with PD-L1 expression (TPS > 1%, $P = 0.031$). *ALK* rearrangement showed no association with PD-L1 expression, while *ROS1* rearrangement was significantly associated with high PD-L1 expression ($P = 0.031$).

Clinicopathologic profiles of patients with *ROS1* gene mutations

The characteristics of the patients with *ROS1* rearrangements are shown in Table 3 and Figure 1. Most of the patients were female (71.4%), never-smokers (71.4%), at advanced tumor stage (IV, 78.6%). All patients had adenocarcinoma. Two coexisting mutations (*EGFR* and *ROS1* mutations) were detected in one patient. Because of the unexpectedly strong association between high PD-L1 expression and *ROS1* mutation in four *ROS1*-rearranged cases, we additionally performed PD-L1 assay on the 10 earlier cases with *ROS1* rearrangement that had not been tested for PD-L1 expression. Out of the 14 *ROS1* mutated patients, five (35.7%) had high PD-L1 expression, 7 (50.0%) had low PD-L1 expression, and 2 (14.2%) were negative for PD-L1 expression.

Table 2 Oncogenic aberration and PD-L1 expression in 130 consecutive NSCLC patients

	PD-L1 expression			
Type of mutation	PD-L1 < 1% ($n = 42$)	1% ≤ PD-L1 < 50% ($n = 59$)	PD-L1 ≥ 50% ($n = 29$)	P†
EGFR, N (%)				
Negative	26 (61.9)	45 (76.3)	26 (89.7)	0.051‡
Positive	16 (38.1)	14 (23.7)	3 (10.3)	**0.031**§
Exon 19 deletion	5 (11.9)	6 (9.0)	2 (10.5)	
Exon 21 L858R	7 (16.7)	4 (2.6)	0 (0.0)	
Others	4 (9.5)	4 (0.7)	1 (5.3)	
ALK, N (%)				
Negative	41 (97.6)	55 (93.2)	27 (93.1)	0.652‡
Positive	1 (2.4)	4 (6.8)	2 (6.9)	0.427§
ROS1, N (%)				
Negative	42 (100.0)	58 (98.3)	26 (89.7)	**0.031**‡
Positive	0 (0.0)	1 (1.7)	3 (10.3)	0.304§

†P was evaluated by comparison with the wild type gene. ‡PD-L1 < 50% versus PD-L1 ≥ 50%. §PD-L1 < 1% versus PD-L1 ≥ 1%. Values in bold are statistically significant.

Discussion

To the best of our knowledge, our study is the first to simultaneously diagnose genetic alterations in *EGFR*, *ALK*, and *ROS1* in an unselected Asian population. Previous studies have reported that the frequency of *ROS1* rearrangement in *EGFR*/*KRAS*/*ALK* WT and never-smoking patients with lung adenocarcinoma is 5.7–8.3%.[32,33] In an unselected NSCLC population, the rate of *ROS1* mutation is 0.6–4.5%.[11,14,34–36] Consistently, in this study, the rate of *ROS1* rearrangement was 3.4% (14/407).

There were no cases of co-occurring *ROS1* and *ALK* mutation, but one case of co-occurring *ROS1* and *EGFR* mutation was found. These findings are similar to those of previous studies suggesting that *ROS1* rearrangements are mutually exclusive to other oncogenic changes.[11,14,36,37] However, others have reported that *ROS1* rearrangements occur in conjunction with other driver mutations.[16,17] This inconsistency may be a result of differences in *ROS1* testing techniques. Updated molecular testing guidelines recommend that, while ROS1 IHC may be used as a screening test, any finding should be confirmed by molecular (qPCR) or cytogenetic (FISH) testing.[2] Wiesweg *et al.* reported that 36% of *ROS1* IHC-positive cases have a concomitant *EGFR* mutation; however, only about half of the cases showed *ROS1* rearrangement by FISH.[17] This result suggests that IHC results include a high number of false-positives. The significance of ROS1 IHC differs from that of ALK IHC: ALK expression is highly specific to tumors with *ALK* rearrangement,[31] whereas ROS1 is expressed at typically low and occasionally high levels in tumors lacking *ROS1* rearrangement.[15,38,39] Therefore, we chose *ROS1* FISH testing as the initial screening test.

In this study, *ROS1*-rearranged NSCLCs were exclusively adenocarcinomas and were predominantly observed in women and younger patients, in line with previous studies.[11,14,36,40]

As a result of the use of pembrolizumab in first-line therapy, its companion diagnostic PD-L1 22C3 assay has become a major tool in the routine clinical testing of NSCLC.[3] Increasing costs and medical complexity are significant challenges in organizing the diagnostic platform of biomarkers in NSCLCs, especially in relation to the prevalence and mutual exclusiveness of these biomarkers.[41–43] Accumulating evidence has revealed a relationship between PD-L1 expression and driver mutations. Previous studies have shown that activating *EGFR* mutations induce PD-L1 expression in cell lines and animal models.[8,44] Moreover, some studies have shown more frequent PD-L1 expression in NSCLC with *EGFR* mutations.[8,45] These observations

Table 3 Clinicopathologic profiles of patients with *ROS1* rearrangement

Patient	Age	Gender	Smoking	Pack-years	Cell type	Sample	Stage	*EGFR*	*ALK*	PD-L1 (%)	*ROS1* (%)
1	66	Male	Y	50	Adenocarcinoma	Bronchoscopy	IV	*WT*	Negative	10	28
37	79	Female	N	0	Adenocarcinoma	Cell block	IV	*WT*	Negative	80	54
46	59	Female	N	0	Adenocarcinoma	PCNB	IV	*WT*	Negative	5	29
70	76	Male	Y	75	Adenocarcinoma	Metastatic site	IV	*WT*	Negative	0	59
275	72	Male	Y	40	Adenocarcinoma	PCNB	IB	*WT*	Negative	30	58
276	79	Female	N	0	Adenocarcinoma	PCNB	IV	*WT*	Negative	95	27
282	43	Female	N	0	Adenocarcinoma	PCNB	IA	*WT*	Negative	25	88
300	80	Female	N	0	Adenocarcinoma	PCNB	IV	Exon 21 L858R	Negative	25	49
360	69	Female	N	0	Adenocarcinoma	PCNB	IV	*WT*	Negative	2	31
375	57	Female	N	0	Adenocarcinoma	PCNB	IV	*WT*	Negative	0	86
396	47	Female	N	0	Adenocarcinoma	PCNB	IIB	*WT*	Negative	1	62
404	44	Female	N	0	Adenocarcinoma	Metastatic site	IV	*WT*	Negative	66	88
406	33	Male	Y	5	Adenocarcinoma	Metastatic site	IV	*WT*	Negative	90	62
407	53	Female	N	0	Adenocarcinoma	PCNB	IV	*WT*	Negative	88	86

WT, wild type; PCNB, percutaneous needle aspiration biopsy.

support the theory of an immunologic evasion mechanism by *EGFR*-mutated lung cancer and suggest that therapeutic blockade of the PD-1/PD-L1 pathway could improve the treatment of patients with NSCLC carrying an *EGFR* mutation. On the contrary, a previously published study revealed the lack of high PD-L1 expression in *EGFR*, *ALK*,

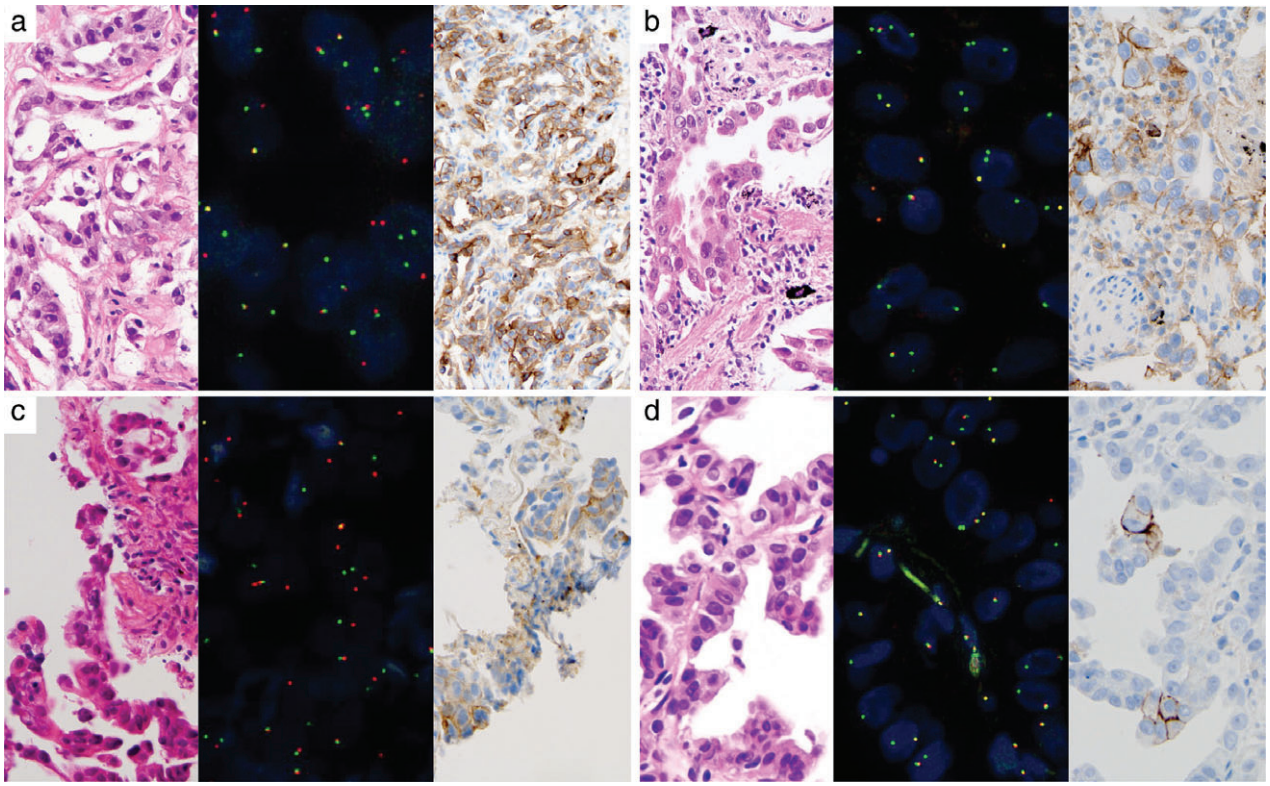

Figure 1 PD-L1 tumor proportion score (TPS) in several cases of adenocarcinoma with positive fluorescence in situ hybridization (FISH) ROS1 split signal. (**a**) Case no. 407 showed adenocarcinoma histology (left panel), positive *ROS1* FISH, mainly break-apart signal (middle panel), and high PD-L1 expression (right panel). (**b**) Case no. 404 showed adenocarcinoma histology (left panel), positive *ROS1* FISH, mainly isolated green signal (middle panel) and high PD-L1 expression (right panel). (**c**) Case no. 282 showed adenocarcinoma histology (left panel), positive *ROS1* FISH, mainly break-apart signal (middle panel), and low PD-L1 expression (right panel). (**d**) Case no. 396 showed adenocarcinoma histology (left panel), positive *ROS1* FISH, mainly isolated green signal (middle panel), and low PD-L1 expression (right panel).

or *ROS1*-mutated lung adenocarcinoma and proposed therapeutic subgrouping as *EGFR/ALK/ROS1* affected, high PD-L1 expressing, and biomarker negative groups.[3] Another large meta-analysis reported that PD-L1 expression is associated with gender, smoking status, histology, and tumor size; *EGFR* WT status was associated with PD-L1 expression, while *ALK* or *KRAS* mutations were not.[8] These data are consistent with the correlation between PD-L1 expression and *EGFR* WT status we found. PD-L1 expression in *ROS1*-rearranged NSCLC has not been assessed prior to our study. Interestingly, 5 (35.7%) of the 14 patients with *ROS1* rearrangement had high PD-L1expression, and high PD-L1 expression was also significantly associated with *ROS1* rearrangement in our consecutive cohort, which is the largest PD-L1 study cohort so far. Based on our results, *ROS1* rearrangement and high PD-L1 expression (≥ 50%) are not mutually exclusive but correlate. Further studies are necessary to establish the exact relationship between PD-L1 expression and *ROS1* rearrangement.

There are some limitations to this study. First, we have no data on the immune checkpoint inhibitor treatment response in patients with oncogenic driver mutations because our national insurance only covers pembrolizumab as a second-line NSCLC treatment, while it covers other tyrosine kinase inhibitors as first-line treatment. Second, PD-L1 expression was only tested in 130 consecutive patients and the PD-L1 test in *ROS1* rearrangement cases was partly performed on a simultaneous platform because PD-L1 has been only recently integrated into the routine diagnostic platform. Third, the number of *ROS1* rearrangements was small even though this was the largest cohort analyzed so far. Thus, further research is needed to generalize our findings. Despite these limitations, this study is a large-scale screen using simultaneous panel genotyping of *EGFR, ALK*, and *ROS1* in unselected patients, which show mutual exclusiveness with other targetable mutations and demonstrates a strong association between *ROS1* rearrangement and high PD-L1 expression.

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Disclosure

No authors report any conflict of interest.

References

1 Cersosimo RJ. Lung cancer: A review. *Am J Health Syst Pharm* 2002; **59**: 611–42.
2 Lindeman NI, Cagle PT, Aisner DL *et al.* Updated molecular testing guideline for the selection of lung cancer patients for treatment with targeted tyrosine kinase inhibitors: Guideline from the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology. *Arch Pathol Lab Med* 2018; **142**: 321–46.
3 Rangachari D, VanderLaan PA, Shea M *et al.* Correlation between classic driver oncogene mutations in EGFR, ALK, or ROS1 and 22C3-PD-L1 50% expression in lung adenocarcinoma. *J Thorac Oncol* 2017; **12**: 878–83.
4 Borghaei H, Paz-Ares L, Horn L *et al.* Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. *N Engl J Med* 2015; **373**: 1627–39.
5 Garon EB, Rizvi NA, Hui R *et al.* Pembrolizumab for the treatment of non-small-cell lung cancer. *N Engl J Med* 2015; **372**: 2018–28.
6 Herbst RS, Baas P, Kim DW *et al.* Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer KEYNOTE-010: A randomised controlled trial. *Lancet* 2016; **387**: 1540–50.
7 Reck M, Rodriguez-Abreu D, Robinson AG *et al.* Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. *N Engl J Med* 2016; **375**: 1823–33.
8 Azuma K, Ota K, Kawahara A *et al.* Association of PD-L1 overexpression with activating EGFR mutations in surgically resected nonsmall-cell lung cancer. *Ann Oncol* 2014; **25**: 1935–40.
9 Zhang M, Li G, Wang Y *et al.* PD-L1 expression in lung cancer and its correlation with driver mutations: A meta-analysis. *Sci Rep* 2017; **7**: 10255.
10 Chin LP, Soo RA, Soong R, Ou SHI. Targeting ROS1 with anaplastic lymphoma kinase inhibitors: A promising therapeutic strategy for a newly defined molecular subset of non-small-cell lung cancer. *J Thorac Oncol* 2012; **7**: 1625–30.
11 Bergethon K, Shaw AT, Ou SH *et al.* ROS1 rearrangements define a unique molecular class of lung cancers. *J Clin Oncol* 2012; **30**: 863–70.
12 Hirsch FR, Suda K, Wiens J, Bunn PA Jr. New and emerging targeted treatments in advanced non-small-cell lung cancer. *Lancet* 2016; **388**: 1012–24.
13 Lin JJ, Ritterhouse LL, Ali SM *et al.* ROS1 fusions rarely overlap with other oncogenic drivers in non-small cell lung cancer. *J Thorac Oncol* 2017; **12**: 872–7.
14 Takeuchi K, Soda M, Togashi Y *et al.* RET, ROS1 and ALK fusions in lung cancer. *Nat Med* 2012; **18**: 378–81.
15 Yoshida A, Tsuta K, Wakai S *et al.* Immunohistochemical detection of ROS1 is useful for identifying ROS1 rearrangements in lung cancers. *Mod Pathol* 2014; **27**: 711–20.
16 Warth A, Muley T, Dienemann H *et al.* ROS1 expression and translocations in non-small-cell lung cancer:

Clinicopathological analysis of 1478 cases. *Histopathology* 2014; **65**: 187–94.
17 Wiesweg M, Eberhardt WEE, Reis H *et al.* High prevalence of concomitant oncogene mutations in prospectively identified patients with ROS1-positive metastatic lung cancer. *J Thorac Oncol* 2017; **12**: 54–64.
18 Jiang L, Su X, Zhang T *et al.* PD-L1 expression and its relationship with oncogenic drivers in non-small cell lung cancer (NSCLC). *Oncotarget* 2017; **8**: 26845–57.
19 Giunchi F, Degiovanni A, Daddi N *et al.* Fading with time of PD-L1 Immunoreactivity in non-small cells lung cancer tissues: A methodological study. *Appl Immunohistochem Mol Morphol* 2018; **26**: 489–94.
20 Han JJ, Kim DW, Koh J *et al.* Change in PD-L1 expression after acquiring resistance to Gefitinib in EGFR-mutant non-small-cell lung cancer. *Clin Lung Cancer* 2016; **17**: 263–70.
21 Kim TJ, Hong SA, Kim O *et al.* Changes in PD-L1 expression according to tumor infiltrating lymphocytes of acquired EGFR-TKI resistant EGFR-mutant non-small-cell lung cancer. *Oncotarget* 2017; **8**: 107630–9.
22 Travis WD, Brambilla E, Nicholson AG *et al.* The 2015 World Health Organization classification of lung tumors: Impact of genetic, clinical and radiologic advances since the 2004 classification. *J Thorac Oncol* 2015; **10**: 1243–60.
23 Kim TJ, Park CK, Yeo CD *et al.* Simultaneous diagnostic platform of genotyping EGFR, KRAS, and ALK in 510 Korean patients with non-small-cell lung cancer highlights significantly higher ALK rearrangement rate in advanced stage. *J Surg Oncol* 2014; **110**: 245–51.
24 Ku BM, Heo MH, Kim JH *et al.* Molecular screening of small biopsy samples using next-generation sequencing in Korean patients with advanced non-small cell lung cancer: Korean lung cancer consortium KLCC-13-01. *J Pathol Transl Med* 2018; **52**: 148–56.
25 Heymann JJ, Bulman WA, Swinarski D *et al.* PD-L1 expression in non-small cell lung carcinoma: Comparison among cytology, small biopsy, and surgical resection specimens. *Cancer Cytopathol* 2017; **125**: 896–907.
26 Gainor JF, Varghese AM, Ou SH *et al.* ALK rearrangements are mutually exclusive with mutations in EGFR or KRAS: An analysis of 1,683 patients with non-small cell lung cancer. *Clin Cancer Res* 2013; **19**: 4273–81.
27 Sterlacci W, Savic S, Schmid T *et al.* Tissue-sparing application of the newly proposed IASLC/ATS/ERS classification of adenocarcinoma of the lung shows practical diagnostic and prognostic impact. *Am J Clin Pathol* 2012; **137**: 946–56.
28 Yeo CD, Park KH, Park CK *et al.* Expression of insulin-like growth factor 1 receptor IGF-1R predicts poor responses to epidermal growth factor receptor EGFR tyrosine kinase inhibitors in non-small cell lung cancer patients harboring activating EGFR mutations. *Lung Cancer* 2015; **87**: 311–7.
29 Ou SH, Bazhenova L, Camidge DR *et al.* Rapid and dramatic radiographic and clinical response to an ALK inhibitor crizotinib, PF02341066 in an ALK translocation-positive patient with non-small cell lung cancer. *J Thorac Oncol* 2010; **5**: 2044–6.
30 Shaw AT, Solomon BJ. Crizotinib in ROS1-rearranged non-small-cell lung cancer. *N Engl J Med* 2015; **372**: 683–4.
31 Thunnissen E, Bubendorf L, Dietel M *et al.* EML4-ALK testing in non-small cell carcinomas of the lung: A review with recommendations. *Virchows Arch* 2012; **46**: 245–57.
32 Kim HR, Lim SM, Kim HJ *et al.* The frequency and impact of ROS1 rearrangement on clinical outcomes in never smokers with lung adenocarcinoma. *Ann Oncol* 2013; **24**: 2364–70.
33 Kim MH, Shim HS, Kang DR *et al.* Clinical and prognostic implications of ALK and ROS1 rearrangements in never-smokers with surgically resected lung adenocarcinoma. *Lung Cancer* 2014; **83**: 389–95.
34 Davies KD, Le AT, Theodoro MF *et al.* Identifying and targeting ROS1 gene fusions in non-small cell lung cancer. *Clin Cancer Res* 2012; **18**: 4570–9.
35 Rimkunas VM, Crosby KE, Li D *et al.* Analysis of receptor tyrosine kinase ROS1-positive tumors in non-small cell lung cancer: Identification of a FIG-ROS1 fusion. *Clin Cancer Res* 2012; **18**: 4449–57.
36 Yoshida A, Kohno T, Tsuta K *et al.* ROS1-rearranged lung cancer: A clinicopathologic and molecular study of 15 surgical cases. *Am J Surg Pathol* 2013; **37**: 554–62.
37 Shaw AT, Ou SHI, Bang YJ *et al.* Crizotinib in ROS1-rearranged non-small-cell lung cancer. *N Engl J Med* 2014; **371**: 1963–71.
38 Mescam-Mancini L, Lantuéjoul S, Moro-Sibilot D *et al.* On the relevance of a testing algorithm for the detection of ROS1-rearranged lung adenocarcinomas. *Lung Cancer* 2014; **83**: 168–73.
39 Li C, Fang R, Sun Y *et al.* Spectrum of oncogenic driver mutations in lung adenocarcinomas from east Asian never smokers. *PLoS ONE* 2011; **6**: e28204.
40 Go H, Kim DW, Kim D *et al.* Clinicopathologic analysis of ROS1-rearranged non-small-cell lung cancer and proposal of a diagnostic algorithm. *J Thorac Oncol* 2013; **8**: 445–50.
41 Jackman DM, Zhang Y, Dalby C *et al.* Cost and survival analysis before and after implementation of Dana-Farber clinical pathways for patients with stage IV non-small-cell lung cancer. *J Oncol Pract* 2017; **13**: e346–52.
42 Shim HS, Choi YL, Kim L *et al.* Molecular testing of lung cancers. *J Pathol Transl Med* 2017; **51**: 242–54.
43 Wong DWS, Leung ELH, So KKT *et al.* The EML4-ALK fusion gene is involved in various histologic types of lung cancers from nonsmokers with wild-type EGFR and KRAS. *Cancer* 2009; **115**: 1723–33.
44 Akbay EA, Koyama S, Carretero J *et al.* Activation of the PD-1 pathway contributes to immune escape in EGFR-driven lung tumors. *Cancer Discov* 2013; **3**: 1355–63.
45 D'Incecco A, Andreozzi M, Ludovini V *et al.* PD-1 and PD-L1 expression in molecularly selected non-small-cell lung cancer patients. *Br J Cancer* 2015; **112**: 95–102.

