Review Article

Beyond *ALK-RET*, *ROS1* and other oncogene fusions in lung cancer

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Abstract: Fusions of the *RET* and *ROS1* protein tyrosine kinase oncogenes with several partner genes were recently identified as new targetable genetic aberrations in cases of non-small cell lung cancer (NSCLC) lacking activating *EGFR*, *KRAS*, *ALK*, *BRAF*, or *HER2* oncogene aberrations. *RET* and *ROS1* fusion-positive tumors are mainly observed in young, female, and/or never smoking patients. Studies based on *in vitro* and *in vivo* (i.e., mouse) models and studies of several fusion-positive patients indicate that inhibiting the kinase activity of the RET and ROS1 fusion proteins is a promising therapeutic strategy. Accordingly, there are several ongoing clinical trials aimed at examining the efficacy of tyrosine kinase inhibitors (TKIs) against *RET* and *ROS1* proteins in patients with fusion-positive lung cancer. Other gene fusions (*NTRK1*, *NRG1*, and *FGFR1/2/3*) that are targetable by existing TKIs have also been identified in NSCLCs. Options for personalized lung cancer therapy will be increased with the help of multiplex diagnosis systems able to detect multiple druggable gene fusions.

Keywords: RET fusion; ROS1 fusion; oncogene fusion; tyrosine kinase inhibitor (TKI); multiplex diagnosis system

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Introduction

Oncogene activation is a critical step toward the development of non-small cell lung cancer (NSCLC), particularly lung adenocarcinoma (LADC); these activated genes are called driver oncogenes (1-3). Representative driver oncogenes include *EGFR*, *KRAS*, *BRAF*, and *HER2/ERBB2*, which are activated by missense and/or insertion/deletion mutations, and the *ALK* gene, which is activated by fusion to other genes (called partner genes) (*Figure 1*). Aberrations of these genes are mutually exclusively detected in LADC; therefore, they are believed to drive LADC development. Suppressing the activity of aberrant gene products inhibits the growth of LADC cells harboring oncogenic aberrations in the corresponding driver genes. Indeed, tyrosine kinase inhibitors (TKIs) have become the standard drug treatment for advanced cases of LADC harboring *EGFR* mutations and *ALK* fusions (1,4,5).

In 2012, two additional oncogenes, *RET* and *ROS1*, were added to the list of driver oncogenes that are targetable with existing TKIs (*Figure 1A*) (1,6-8) and clinical trials investigating the efficacy of such TKIs have been conducted. Furthermore, analysis of lung cancer genome and/or transcriptome has identified other gene fusions, including the *NTRK1* (9), *NRG1* (10,11), and *FGFR1/2/3* fusions (12-14), as novel targetable driver genes in a minor fraction of NSCLC cases. *In vitro* and *in vivo* experimental data show that existing TKIs are a promising therapy for lung cancer cases that are positive for these novel oncogenic fusions. Here, we review the oncogenic fusions associated with NSCLC and discuss the issues surrounding personalized therapy.

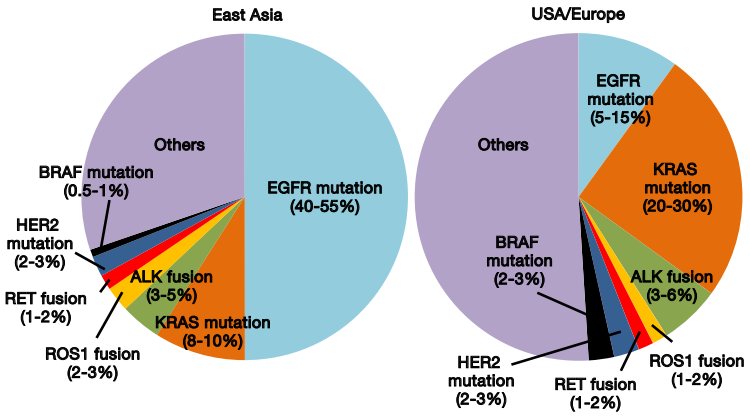

Figure 1 Pie charts showing the proportion of LADC harboring aberrations in driver oncogenes. Data from patients in East Asia (Japan, Korea, and China) and from those of European descent were generated by summarizing the results from previous reports (2-4). LADC, lung adenocarcinoma.

The *RET* fusion in LADC

The link between the oncogenic *RET* fusion and LADC was discovered by several groups (including our own) in 2012. The *RET* gene was fused to the *KIF5B* and *CCDC6* genes in 1-2% of LADC cases (6-8,15,16); none of these positive cases harbored *EGFR*, *KRAS*, *BRAF*, or *HER2/ERBB2* mutations or *ALK* fusions. The *RET* fusion is mainly detected in young, female, and/or never/light-smoker patients (6,7,17-19). Also, it occurs in adenocarcinoma but not in squamous and small cell lung cancers (SQLC and SCLC) (2,7). LADCs harboring the *RET* fusion show well- or moderately-differentiated histological features, similar to those of LADCs harboring *EGFR* mutations; however, a subset of LADCs harboring the *RET* fusion show mucinous cribriform features, similar to those of ALK fusion-positive LADCs (6,17-19).

Oncogenic *RET* variants fused to six partner genes have been identified in lung cancers (10,20,21) (*Figure 2*). In all of these variants, the coiled-coil domains of the partner proteins induce dimerization of the RET fusion proteins, resulting in constitutive activation of the RET kinase (as in the case of oncogenic *ALK* fusions). The tumorigenic activity of the *RET* fusion gene was illustrated by transformation of NIH3T3 cells (6-8) and in a transgenic mouse model in which the *KIF5B-RET* gene was specifically expressed in lung epithelial cells (22); The tumorigenic activity was suppressed by RET TKIs, indicating its dependence on the kinase activity of the RET protein. Consistent with this, a human LADC cell line derived from a Japanese patient, which carries the *CCDC6-RET* fusion gene, is sensitive to RET TKIs (23,24). Therefore, LADC cells harboring the *RET* fusion are in a state of "oncogenic addiction" to constitutive RET kinase activation. This makes the *RET* fusion a promising therapeutic target.

The US Food and Drug Administration (FDA) has approved two multi-kinase inhibitors with RET TKI activity, vandetanib (ZD6474) and cabozantinib (XL184), for the treatment of advanced medullary thyroid cancer in which activating *RET* mutations are observed in >50% of cases (16). Five phase II clinical trials are currently examining the therapeutic effects of RET TKIs against *RET* fusion-positive NSCLCs (*Table 1*). These trials have single-arm open-label designs, with response rate as the primary endpoint. Our own group is conducting one of these phase II clinical trial in Japan (UMIN00001009). This trial, designated "LURET (lung cancer with *RET* rearrangement study)", is designed to investigate the therapeutic efficacy of vandetanib against NSCLC. We are using a RT-PCR-based screening method to select patients with *RET* fusion-positive tumors. This process is being carried out in >170 hospitals via a consortium called "LC-SCRUM (lung cancer genomic screening project for individualized medicine in Japan)", and >1,000 patients with advanced NSCLC without *EGFR* mutations have been screened as of Aug 31, 2014 (2). A trial conducted at Memorial Sloan-Kettering Cancer Center (NCT01639508) reported promising responses in the first three patients treated with cabozantinib (20). In addition, another study reported that one patient with LADC harboring a *KIF5B-RET* fusion showed a positive response to vandetanib (25). Although the number of patients in these studies is small and follow-up is

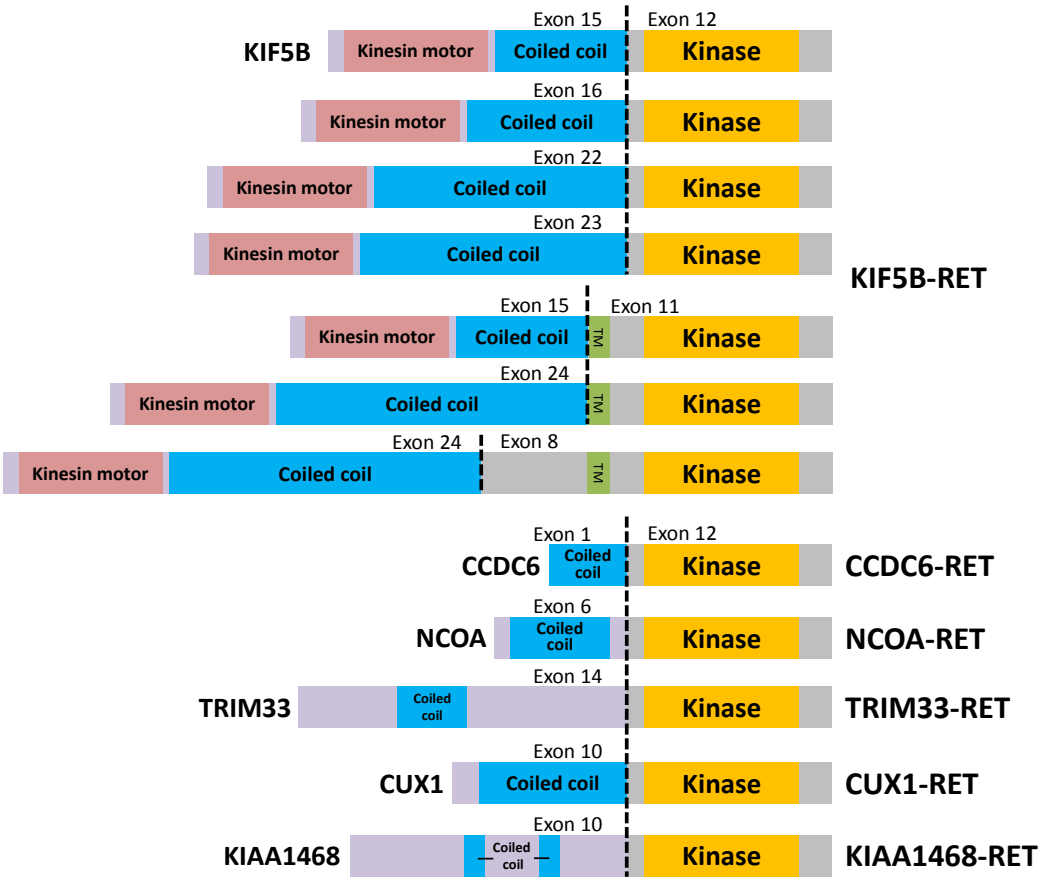

Figure 2 Schematic diagram showing *RET* fusion proteins in LADC. The domains are highlighted in different colors: RET tyrosine kinase domain (orange), RET transmembrane domain (TM; green), and coiled-coil domain (blue) in fusion partners. LADC, lung adenocarcinoma.

Table 1 Clinical trials of TKIs in patients with *RET* and *ROS1* fusion-positive non-small cell lung cancer (NSCLC)

Gene fusion	Trial number*	Drug	Pharmaceutical company	Phase	Location	Primary endpoint	Enrollment	Start date
RET	NCT01639508	Cabozantinib/XL184	Exelixis	II	USA	Response rate	25	July 2012
ROS1, *NTRK1*, and others**	NCT01639508	Cabozantinib/XL184	Exelixis	II	USA	Response rate	25	August 2014
RET	UMIN000010095	Vandetanib/ZD6474	AstraZeneca	II	Japan	Response rate	17	February 2013
RET	NCT01823068	Vandetanib/ZD6474	AstraZeneca	II	Korea	Response rate	17	April 2013
RET	NCT01877083	Lenvatinib/E7080	Eisai	II	Global	Response rate	20 or more	April 2013
RET	NCT01813734	Ponatinib/AP24534	ARIAD	II	USA	Response rate	20	June 2013
ROS1	NCT01945021	Crizotinib	Pfizer	II	Asia	Response rate	110	September 2013
ROS1	NCT01964157	Ceritinib/LDK378	Novartis	II	Korea	Response rate	32	October 2013
ROS1 and *ALK*	NCT01970865	PF-06463922	Pfizer	I/II	Global	Response rate (phase II)	200	October 2013
ROS1	NCT02183870	Crizotinib	Pfizer	II	EU	Response rate	30	June 2014

*, detailed information is available at http://clinicaltrials.gov/ or https://upload.umin.ac.jp; **, including MET (overexpression, amplification, or mutation) and AXL (overexpression, amplification, or mutation). TKI, tyrosine kinase inhibitor.

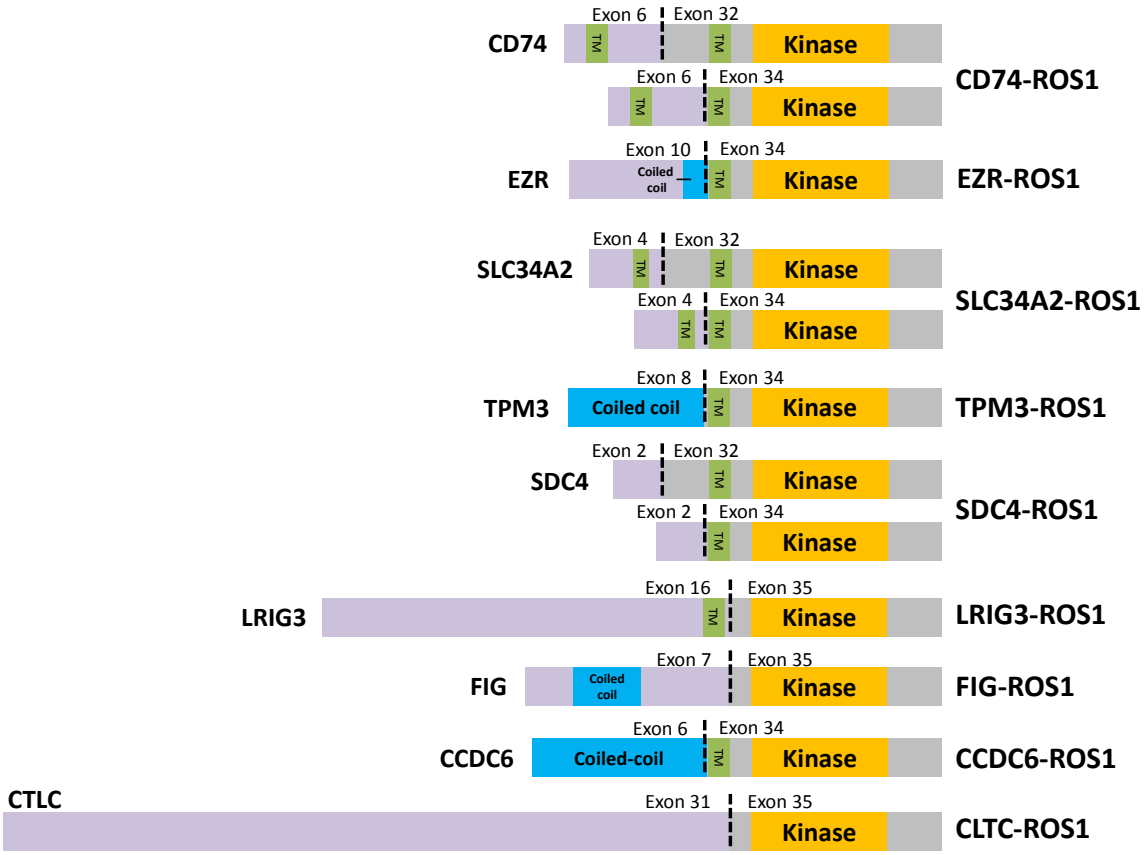

Figure 3 Schematic diagram showing ROS1 fusions in LADC. The domains are highlighted in different colors: ROS1 tyrosine kinase domain (orange), ROS1 transmembrane domain (TM; green), and coiled-coil domain (blue) in fusion partners. LADC, lung adenocarcinoma.

limited, the results provide early proof-of-principle that the *RET* fusion is targetable by existing TKIs.

The *ROS1* fusion in LADC

The oncogenic *ROS1* fusion is present in 1-2% of LADC cases (6), and is likely to be specific for adenocarcinoma (26). The *ROS1* gene fuses to several partner genes, although *CD74* is the most common (*Figure 3*) (27-29). As is the case for the *RET* fusion, the *ROS1* fusion occurs in a manner that is mutually exclusive with other known driver oncogene mutations and fusions. The *ROS1* fusion is preferentially detected in young, female, and/or never/light-smoker patients (6,18,30-32). LADCs harboring the *ROS1* fusion often show mucinous cribriform features (6,18,30,31), similar to those of *ALK* fusion-positive LADCs. The *ROS1* fusion is also likely to be specific for LADC (6,18,30,32).

The transforming activity of the *ROS1* fusion gene has been demonstrated *in vitro* using NIH3T3 cells (6,33) and *in vivo* using a transgenic mouse model in which the *EZR-ROS1* gene is specifically expressed in lung epithelial cells (33). Crizotinib, a TKI approved by the FDA for ALK fusion-positive lung cancer, also inhibits the ROS1 protein due to the structural similarity of the kinase domains of ROS1 and ALK proteins. In fact, the LADC cell line, HCC78, which harbors a *SLC34A2-ROS1* fusion, is sensitive to crizotinib (26,32). Thus, LADC cells harboring the *ROS1* fusion are in a state of "oncogenic addiction" to constitutive ROS1 kinase activation. In contrast to the *RET* and *ALK* fusions, constitutive activation of the ROS1 kinase protein is unlikely due to dimerization of ROS1 fusion proteins since the majority of ROS1 partner proteins lack dimerization domains (27) (*Figure 3*).

A phase I trial (NCT00585195) examining the efficacy of crizotinib against ROS1 fusion-positive NSCLC showed an objective response rate of 60% (27). Other studies (32,34-36)

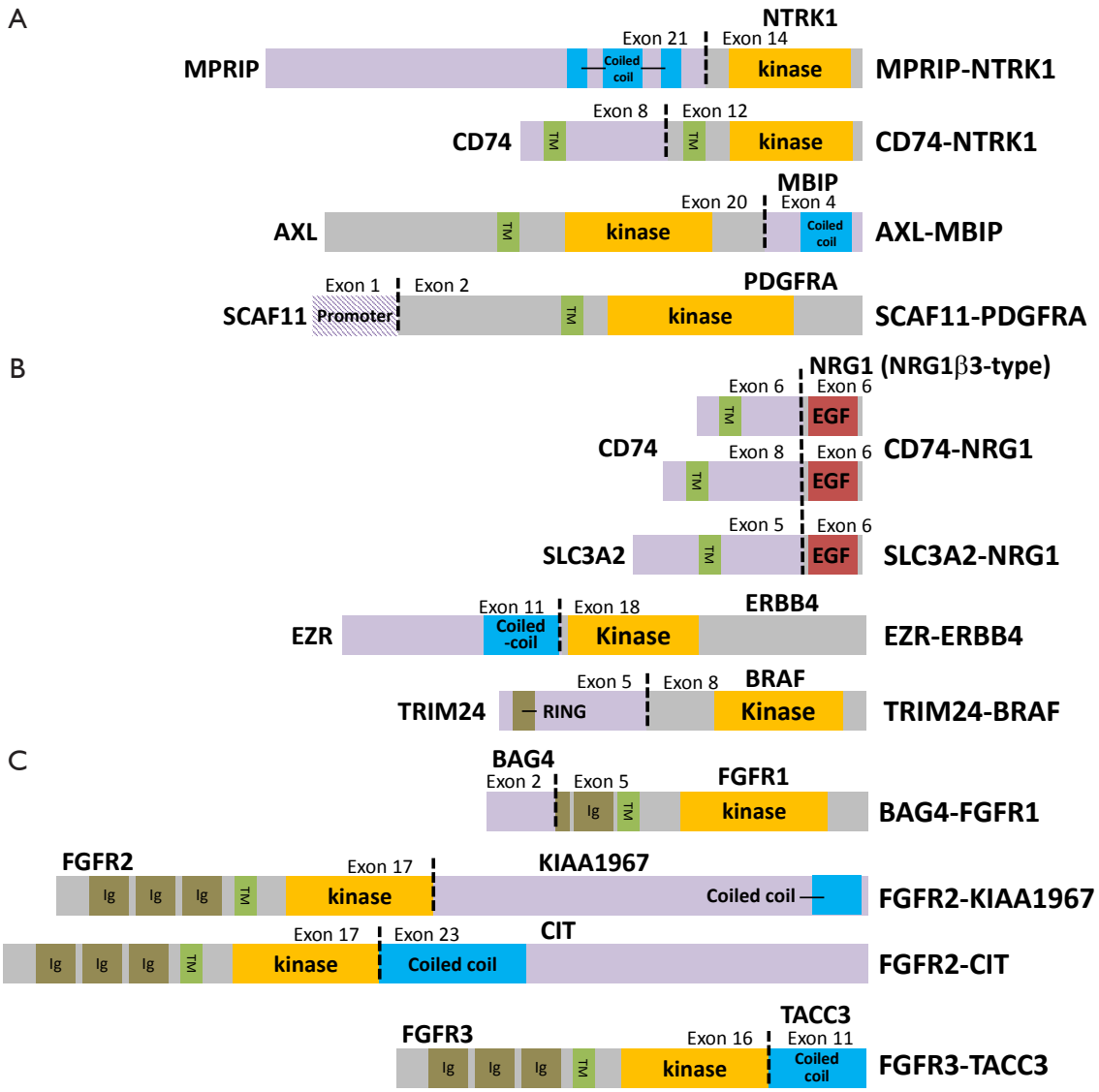

Figure 4 Schematic diagram of other fusion proteins in non-small cell lung cancer. (A) Fusion proteins in LADC. TM, transmembrane domain; (B) fusion proteins in IMAs. EGF, EGF-like domain; (C) FGFR fusion proteins in SQLC. The domains are highlighted in different colors: tyrosine kinase domain (orange), transmembrane domain (TM; green), immunoglobulin-like domain (dark green), coiled-coil domains (blue). LADC, lung adenocarcinoma.

report that patients with LADC harboring a *ROS1* fusion show a near-complete or partial response to crizotinib. Therefore, molecular-targeted therapy using crizotinib (and other ROS1 TKIs) appears promising. Five phase II or I/II clinical trials have been conducted to examine the therapeutic effects of ROS1 TKIs against *ROS1* fusion-positive NSCLCs (*Table 1*). The LC-SCRUM consortium is currently screening *ROS1* fusion-positive tumors in Japan and *ROS1* fusion-positive patients are being enrolled in a crizotinib trial (NCT01945021).

Other protein kinase fusions in LADC

Other oncogenic fusions of protein kinase genes have been detected in LADCs that are negative for known driver oncogene aberrations (*Figure 4A*). Oncogenic fusions of the *NTRK1* gene (which encodes a nerve growth factor receptor, TRKA) with the *CD74* and *MPRIP* genes were

recently identified in 3% of patients within an American cohort (9). However, other LADC cohorts, including a TCGA-USA cohort (n=230), a Korean cohort (n=87), and our own NCC-Japan cohort (n=200, unpublished data), contained no *NTRK1* fusion-positive cases (9). Thus, the prevalence of *NTRK1* fusion remains unclear. A few TKIs (ARRY-470, CEP-701, and crizotinib) that suppress the activity of the TRKA protein kinase also suppress the NIH3T3-transforming activity of the *NTRK* fusion gene (9). Notably, a LADC patient harboring the *MPRIP-NTRK1* fusion showed a minor therapeutic response to crizotinib (9). An ongoing clinical trial (NCT01639508) includes not only patients positive for the *RET* and *ROS1* fusions, but also patients positive for the *NTRK1* fusion (*Table 1*).

The *AXL-MBIP* and *SCAF11-PDGFRA* fusions, two more protein kinase gene fusions (*Figure 4A*), were each detected in a single case of LADC in a Korean cohort of 200 patients (29). Since these fusions were not detected in either the TCGA-USA cohort (n=230) (28) or our own NCC-Japan cohort (n=200, unpublished data), they may only occur in a very small subset of LADC cases.

Multiple oncogenic fusions in invasive mucinous LADC

Invasive mucinous adenocarcinoma (IMA) of the lungs, which accounts for 2-10% of all LADC cases in Japan, the USA, and Europe, is thought to be a distinct histological type of LADC that commonly (>50%) harbors *KRAS* mutations (37,38). We recently identified multiple oncogene fusions involving the *NRG1* (*neuregulin*), *ERBB4*, *BRAF*, *ALK*, and *RET* genes as drivers for the development of IMA in the absence of *KRAS* mutations (10) (*Figure 4B*). Among these, the *CD74-NRG1* fusion was the most common (5-15%). The *CD74-NRG1* fusion has also been detected in another Japanese IMA cohort and in a Taiwanese IMA cohort (11,39). The fusion product acts as a ligand for HER2:HER3 and causes anchorage-independent growth of NIH3T3 cells (9,10,11). Its transforming activity is suppressed by HER2 inhibitors that are approved for clinical use, including lapatinib and afatinib (10), suggesting that IMAs may be amenable to personalized therapy.

FGFR1/2/3 fusions in SQLC

Amplification of the *FGFR1* gene has been identified as a major oncogene aberration in approximately 10% of SQLC cases (40), whereas activating mutations in *FGFR1*, *FGFR2*, and *FGFR3* are detected in a small subset of SQLC cases (41). Recent studies have detected fusions of the *FGFR1*, *FGFR2*, and *FGFR3* genes to several partner genes in SQLC (*Figure 4C*) (13,14,28). In particular, the *FGFR3-TACC3* fusion, which is detected in 3% of glioblastoma multiforme cases (42), was recurrently observed in a 2-3% of LSQC cases. The *FGFR3-TACC3* fusion gene induces cell transformation and accelerated growth. Both cell growth and tumorigenicity are suppressed by FGFR TKIs (13). Importantly, several clinical trials examining the efficacy of FGFR TKIs against SQLC harboring mutation/amplification of the *FGFR* genes are ongoing, although broadening the inclusion criteria for such clinical trials would be beneficial.

Diagnosis of fusion-positive cases

The findings discussed to date provide a strong rationale for developing precision medicine approaches based on targeting oncogene fusions in LADC and LSQC. Since this form of therapy is applicable only to a subset of LADC and LSQC cases, it is important that we develop suitable diagnostic methods that are able to identify fusion-positive cases (43). The diagnosis of ALK fusion-positive lung cancer is based on fluorescence *in situ* hybridization (FISH) either with or without immunohistochemistry (IHC) (44). FISH and IHC are also suitable for the diagnosis of ROS1 fusion (45,46); however, IHC is not suitable for the diagnosis of *RET* fusion (7,8,19).

Because only very small amounts of material can be obtained from biopsies, there is a need to develop diagnostic systems that enable simultaneous examination of multiple gene fusions in routine formalin-fixed and paraffin-embedded (FFPE) clinical specimens. However, because the FFPE technique damages DNA, the robustness against DNA qualities is needed for the diagnostic systems. In addition, most of the samples that are subjected to testing are small biopsies; therefore, the system must also be able to deal with limited amounts of tissue and/or extracted DNA/RNA. Accurate and sensitive profiling must be achieved, even when the proportion of tumor cells within the specimens is low.

Representative systems are currently being developed that will enable multiple, robust, and sensitive diagnoses (*Table 2*). Some employ the method of target re-sequencing of tens to hundreds of genes using DNA or RNA extracted from tumor tissues (47,48), while others employ quantitative RT-PCR or RNA molecule counting (21,49,50). Optimizing these (or other equivalent) systems for use in the clinic will

Table 2 Multiplex diagnostic systems for gene fusions

Method	Material	Detectable fusions
Target capture followed by next-generation sequencing (8,9,47)	Genomic DNA	ALK, RET, ROS1, NTRK1, and others
Target capture followed by transcript counting (21)	RNA	ALK, RET, and ROS1
Multiplex RT-PCR followed by next-generation sequencing (48)	RNA	ALK, ROS1, and others
Multiplex ARMS RT-PCR (49)	RNA	ALK, RET, and ROS1
Anchored multiplex RT-PCR (50)	RNA	ALK, RET, ROS1, NTRK1, and others

greatly facilitate the progress toward precision medicine for lung cancer.

Perspective: issues still to be investigated

In vitro/*in vivo* experiments and the responses of the few patients examined in trials suggest that the therapies described in this review hold promise. However, innate and acquired resistance to TKIs may become a problem, as is the case for TKIs targeting the ALK and EGFR proteins. The mechanisms underlying resistance are beginning to be unraveled and several next-generation TKIs have been developed to treat resistant ALK fusion and *EGFR* mutations (5,51). This is good news because some ROS1 fusion-positive cases also have acquired resistance to crizotinib (52). Further studies should be done on the resistance of other fusions to TKIs so that lung cancers harboring novel fusions can be treated effectively.

Preventing the development of lung cancer via oncogenic fusions is another issue to be tackled by those involved in lung cancer medicine. LADCs harboring oncogene fusions are mainly observed in never/light smokers; therefore, preventive methods other than smoking cessation are necessary. We have been investigating the molecular mechanisms underlying chromosome inversions that generate oncogenic *RET* fusions in LADC by cloning genomic segments that contain breakpoint junctions (53). We found that inversions were most likely caused by the mis-repair of DNA strand breaks, which occurred in a region spanning a few Kb within the *RET* gene (the region in which DNA strand breaks leading to *RET* rearrangements in papillary thyroid tumors also frequently occur) (53). Thus, tobacco-independent DNA strand breaks are likely to trigger development of the *RET* fusion. To the best of our knowledge, no studies have elucidated the structure of the breakpoints in *ALK*, *ROS1*, and other fusions. Further examination of the molecular processes underlying gene fusion, as well as identifying the endogenous/exogenous factors that cause DNA breaks, will provide the key to preventing the development of lung cancers harboring oncogenic gene fusions.

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