Review

The *EML4-ALK* oncogene: targeting an essential growth driver in human cancer

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Abstract: Targeting of essential growth drivers represents an ideal approach to cancer treatment. To identify such molecules in clinical specimens, we developed a highly sensitive functional screening system based on the preparation of retroviral cDNA expression libraries. By screening such a library of lung adenocarcinoma with a focus formation assay, we discovered the *EML4-ALK* fusion-type oncogene. A small chromosomal inversion thus leads to fusion of the amino-terminal portion of the microtubule-associated protein EML4 to the intracellular kinase domain of ALK, a receptor-type protein tyrosine kinase. Constitutive dimerization of EML4-ALK mediated by a dimerization motif of EML4 results in kinase activation. Specific inhibitors of the kinase activity of ALK have been developed as therapeutic drugs for *EML4-ALK*-positive lung cancer, three of which (crizotinib, ceritinib, and alectinib) have already been approved for clinical use. An overall clinical response rate of 93.5% for alectinib has shown that agents that target essential growth drivers can become magic bullets for cancer treatment.

Keywords: molecularly targeted therapy, oncogene, protein tyrosine kinase, fusion gene, EML4-ALK

Struggles in the development of targeted therapies for cancer

Conventional chemotherapy with cytotoxic drugs has been widely adopted in the treatment of cancer. Whereas such treatment modalities show marked efficacy for certain hematologic malignancies (for instance, childhood acute lymphoblastic leukemia), their therapeutic value is often limited for other types of cancer, especially for epithelial tumors.¹⁾ This limitation has led to the notion that reagents that target specific molecules related to cell growth might prove to be more effective for cancer treatment. Such targeted therapies would also be expected

to be less toxic than conventional chemotherapy, given that specific modulation of the activity of an individual target molecule is likely to be free of the adverse effects typical of many cytotoxic drugs such as nausea or vomiting, pancytopenia, liver toxicity, and alopecia.

Unfortunately, however, the promise of targeted therapy was not achieved until the remarkable success of imatinib for the treatment of chronic myeloid leukemia (CML). For instance, clinical trials with a farnesyl transferase inhibitor to block the membrane-anchoring of RAS proteins (and thereby to prevent GTP-loading of RAS) failed to yield a progression-free survival (PFS) superior to that for best supportive care in patients with colorectal carcinoma.²⁾ Likewise, early clinical trials with phosphoinositide 3-kinase inhibitors showed little clinical benefit.³⁾ Whereas a monoclonal antibody to vascular endothelial growth factor (VEGF) represents an early success of molecularly targeted therapy, its chief therapeutic benefit is an extension of PFS by only 2 to 3 months when used in combination with cytotoxic drugs.⁴⁾ It thus remained unclear for a while whether molecularly targeted

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Abbreviations: CML: chronic myeloid leukemia; PFS: progression-free survival; NSCLC: non-small cell lung carcinoma; FDA: Food and Drug Administration; FISH: fluorescence *in situ* hybridization; RT-PCR: reverse transcription-polymerase chain reaction.

Fig. 1. Two different approaches to molecularly targeted therapy for cancer. One class of drugs (upper) targets molecules that function in intracellular signaling pathways underlying regulation of cell proliferation. Although such agents are potentially applicable to a wide range of cancers, their efficacy may be modest. The other class of agents (lower) targets essential growth drivers. Such drugs are applicable only to cancers harboring the activated driver, but they may be of high efficacy.

therapies would constitute *bona fide* magic bullets for cancer treatment.

The uncertainty regarding the potential of such therapies was eventually removed by the introduction of imatinib. The tyrosine kinase ABL1 becomes fused to the BCR protein as a result of a balanced chromosome translocation, t(9;22), in individuals with CML. This fusion results in a constitutive elevation of the kinase activity of ABL1. Treatment of CML patients with the ABL1-specific inhibitor imatinib was found to induce a cytogenetic response (disappearance of t(9;22)-positive cells) rate of 76.2%, which is far superior to that of conventional treatments.⁵⁾ Whereas CML was once thought to be an intractable fatal disorder, with the introduction of imatinib it became a manageable chronic disease.

Discovery of the *EML4-ALK* oncogene

What makes imatinib different from earlier molecularly targeted therapies? The marked efficacy of imatinib is likely attributable to the fact that its target, BCR-ABL1, is the essential growth driver in CML. The growth of CML cells is thus dependent on the activity of BCR-ABL1, with the cells not being able to survive without it (Fig. 1). All prior

molecularly targeted reagents failed to show similar efficacy because they were directed at nonessential growth drivers. In such cases, subclones of cancer cells that are able to bypass the targeted pathway and thus override the selective treatment are likely generated as a result of genomic instability.

On the basis of this reasoning, development of the “next imatinibs” will require the identification of essential growth drivers in each cancer type. We therefore developed a highly sensitive functional screening system based on retroviral cDNA expression libraries. In this system, mRNAs are isolated from clinical cancer specimens and converted to cDNAs, which are then incorporated into retroviral expression plasmids. Recipient cells, such as mouse 3T3 fibroblasts, are then infected with recombinant retroviruses generated from the plasmids and are assayed for malignant transformation (Fig. 2).⁶⁾

By coupling the assay of focus formation by 3T3 cells with a retroviral library prepared from a lung adenocarcinoma specimen, we discovered the *EML4-ALK* fusion-type oncogene for non-small cell lung carcinoma (NSCLC).⁷⁾ *EML4* encodes a microtubule-associated protein with a coiled-coil domain, and *ALK* encodes a receptor-type protein tyrosine

Fig. 2. Functional screening for oncogenes with retroviral cDNA expression libraries. The cDNAs are prepared from cancer specimens and cloned into retroviral plasmids. Recombinant retroviruses generated from the plasmids are used to infect assay cells. The selection of fibroblast cell lines as recipient cells, for instance, allows the identification of transforming cDNAs on the basis of the formation of abnormal foci (lower left). The use of hematopoietic cell lines, on the other hand, allows the isolation of cDNAs whose protein products block terminal differentiation of the myeloid lineage (lower right).

Fig. 3. Protein structure of the EML4-ALK fusion kinase. The domain organization of each fusion partner is shown schematically. A chromosomal inversion, *inv*(2)(p21p23), leads to fusion of the amino-terminal portion of EML4 to the intracellular region of ALK. CC, coiled-coil domain; WD, tryptophan and aspartic acid repeats; TM, transmembrane domain.

kinase. Both genes are located on the same short arm of human chromosome 2 but in opposite orientations, and a small inversion involving the two loci, *inv*(2)(p21p23), results in the gene fusion (Fig. 3). EML4-ALK thus comprises the amino-terminal portion of EML4 fused directly to the intracellular kinase domain of ALK, and it undergoes constitutive

dimerization mediated by the coiled-coil domain of EML4. This dimerization results in activation of the tyrosine kinase function of ALK and thereby confers marked oncogenic activity. *EML4-ALK* is found in 4–5% of NSCLC, and enriched in lung adenocarcinoma, tumors of young onset, and never- or light-smokers.^{8,9)} Pathologically, *EML4-ALK*–

positive tumors often exhibit a signet-ring cell pattern or a mucinous cribriform, but other types are also seen.^{10)–12)}

The identification of *EML4-ALK* contradicted the widely believed notion that oncogenesis mediated by chromosome translocations is specific to hematologic malignancies and sarcomas, and does not occur in epithelial tumors.¹³⁾ Together with the *TMPRSS2-ERG* fusion-type oncogene in prostate cancer,¹⁴⁾ our discovery constituted the first evidence against this notion for major epithelial tumors. Furthermore, *EML4-ALK* was also the first example of recurrent tyrosine kinase fusions in such disorders.

Approval of the first ALK inhibitor

To demonstrate that *EML4-ALK* is an essential growth driver for lung cancer, we generated transgenic mice in which *EML4-ALK* is expressed specifically in lung type-II alveolar cells.¹⁵⁾ Unexpectedly, these mice developed hundreds of lung cancer nodules in both lungs soon after birth. In contrast, mice transgenic for other oncogenes usually develop corresponding tumors at 3 to 6 months after birth. These observations thus suggested that oncogenes are not equally competent with regard to transforming ability, and that that of *EML4-ALK* is exceptionally high. Indeed, the lung cancer nodules of *EML4-ALK* transgenic mice disappeared rapidly as a result of treatment with an ALK-specific inhibitor, indicating that *EML4-ALK* is an essential growth driver for lung cancer positive for this fusion gene. Recently, CRISPR/Cas9-mediated *in vivo* generation of *EML4-ALK* was achieved in mice that subsequently bore lung cancer, again confirming the seminal role of *EML4-ALK*.¹⁶⁾

In response to our studies, many pharmaceutical companies started to develop ALK-specific inhibitors. The first such drug to enter clinical trials was crizotinib (formerly known as PF-02341066).¹⁷⁾ The results of a phase I/II trial of crizotinib in patients with NSCLC were first reported in 2010 and showed an overall response rate of ~60% with a median PFS of ~10 months.^{18),19)} Initially, the trial with crizotinib was not conducted in Japan probably owing to the high cost for clinical trials. To expedite the trial in Japan, we constructed a nation-wide clinical network to diagnose *EML4-ALK*-positive lung cancer early in 2009,⁸⁾ and the trial indeed started in Japan within the same year. On the basis of efficacy data in the phase I/II trial, the U.S. Food and Drug Administration (FDA) approved crizotinib as a therapeutic drug on 26 August 2011. It thus took

only 4 years from target discovery to final drug approval, which represents a rapidity not previously achieved in the history of cancer drug development. Following the swift FDA action, crizotinib was also approved for clinical use in Japan in March of 2012.

Two important factors made this rapid development possible. First, the clinical trial accepted only patients with tumors positive for *ALK* rearrangement. Such genotype-based enrollment allowed the phase I/II trial to demonstrate crizotinib efficacy with a relatively small cohort ($n = 82$).¹⁸⁾ A companion diagnostic test based on fluorescence *in situ* hybridization (FISH) with break-apart probes for *ALK* was developed simultaneously with the trial to allow the identification of tumors positive for *ALK* rearrangement. Other diagnostics, such as those based on reverse transcription and polymerase chain reaction (RT-PCR) analysis or on immunohistochemical staining (IHC), were subsequently developed.^{8),20),21)} These three methods (FISH, RT-PCR and IHC) have each specific advantages/disadvantages. FISH/IHC are, for instance, applicable to formalin-fixed paraffin-embedded tissues, but RT-PCR is not. Conversely, liquid specimens (such as pleural effusion and sputum) can be examined by RT-PCR, but not by FISH/IHC. Sensitivity of RT-PCR to detect *EML4-ALK* is far superior to that of the other methods. Therefore, physicians/pathologists should carefully select the methods to clinically diagnose the presence of *EML4-ALK*.

The second important factor was the decision to base the approval of crizotinib on only the phase I/II trial data. Drug approval normally requires additional large-scale phase III studies to compare the efficacy of the new drug with that of standard therapies, with such trials often being time-consuming and expensive. The FDA instead chose to deliver crizotinib swiftly to lung cancer patients for whom standard chemotherapy has a response rate of only 20% to 30%. This approach to clinical trials and drug approval may become a new paradigm for cancer drug development in the era of molecularly targeted therapy.

The second generation of ALK inhibitors

The fact that the median PFS of patients treated with crizotinib is ~10 months suggests that most tumors acquire resistance to the drug within a year. Clarification of the molecular mechanisms underlying such drug resistance might be expected to lead to an improvement in the prognosis of patients with *EML4-ALK*-positive tumors. The first such

mechanism to be revealed was the emergence of a secondary mutation within the kinase domain of *EML4-ALK*.²²⁾ We compared the nucleotide sequences of *EML4-ALK* cDNAs obtained from the lung adenocarcinoma of the same patient during periods of crizotinib sensitivity and crizotinib resistance. Two nonsynonymous mutations were thus identified only in the drug-resistance phase, one resulting in the replacement of Cys¹¹⁵⁶ with Tyr and the other in that of Leu¹¹⁹⁶ with Met within the kinase domain. Interestingly, these mutations arose independently in different subclones of the same tumor, and both were found to confer resistance to crizotinib. Moreover, the position of Leu¹¹⁹⁶ within the three-dimensional structure of the kinase domain corresponds to that of Thr⁷⁹⁰ in the epidermal growth factor receptor (EGFR) and that of Thr³¹⁵ in BCR-ABL1, both of which “gatekeeper” residues are the most frequent sites of mutation underlying resistance to corresponding tyrosine kinase inhibitors.

Several other amino acid changes in *EML4-ALK* have since been identified in crizotinib-resistant tumors, including a Thr insertion at position 1151, Leu¹¹⁵² to Arg, Phe¹¹⁷⁴ to Cys, Gly¹²⁰² to Arg, Ser¹²⁰⁶ to Tyr, and Gly¹²⁶⁹ to Ala.^{23)–26)} Amplification of *EML4-ALK* was also shown to be responsible for crizotinib tolerance in a small proportion of patients. Whereas other genetic alterations (for instance, *KIT* amplification) have been found infrequently in crizotinib-resistant tumors, the mechanisms of crizotinib resistance in about two-thirds of such tumors remain unknown.

Several novel ALK inhibitors have been developed to conquer crizotinib resistance (mostly that mediated by the gatekeeper mutation).²⁷⁾ Such second-generation ALK inhibitors that have already undergone clinical trials include ceritinib, alectinib, AP26113, PF-06463922, TSR-011, RXDX-101, X-396, and CEP-37440, with *EML4-ALK*(L1196M) being sensitive to all of these compounds.

Ceritinib is a highly potent inhibitor of ALK, with a median inhibitory concentration of 0.15 nM, and it is effective against all the mutant forms of *EML4-ALK* mentioned above with the exception of F1174C and G1202R.²⁸⁾ The overall response rate and median PFS of patients with *ALK*-rearranged NSCLC in clinical trials of ceritinib were 58% and 7 months, respectively. The response rate of crizotinib-resistant tumors was surprisingly high (56%), providing an additional therapeutic opportunity for such patients.²⁹⁾ On the basis of these data, the FDA approved ceritinib in April of 2014.

Alectinib is a highly selective and potent inhibitor of ALK, with a median inhibitory concentration of 1.9 nM.³⁰⁾ A phase I/II clinical trial of alectinib enrolled patients with *ALK* rearrangement-positive tumors as judged by either RT-PCR analysis or FISH/immunohistochemistry.³¹⁾ Alectinib showed outstanding efficacy, with a response rate of 93.5%. Treatment of central nervous system involvement with alectinib also showed a high response rate of 52%.³²⁾ Alectinib was approved in Japan in July 2014.

Future perspectives

Discovery of the *EML4-ALK* oncogene revealed that chromosome translocation is an important mechanism of oncogenesis in all types of malignancies, and it triggered attempts to identify other tyrosine kinase fusions in human cancer. Such efforts have already resulted in the identification of fusions for *FGFR1*, *FGFR2*, and *FGFR3*^{33),34)} as well as for *ROS1*,^{35),36)} *RET*,³⁶⁾ and *NTRK1*,³⁷⁾ with clinical trials also having been initiated with corresponding inhibitors.

In the case of *EML4-ALK*, its oncogenic potential is so great that cancer cells are almost completely dependent on its activity for their growth. This addiction explains why alectinib has the potential to become one of the most effective anticancer drugs for an epithelial tumor. Two-year progression-free survival rate of alectinib, for instance, reaches remarkable 76%.³⁸⁾ Compared with *EML4-ALK*-negative lung cancer, *EML4-ALK*-positive cancer cells harbor fewer additional activating mutations of other oncogenes and fewer instances of tumor suppressor gene loss.³⁹⁾

However, ALK inhibitor-monotherapies may not be able to totally eradicate tumor burden. Almost every tumor likely contains a small fraction of cells that do not proliferate and stay at G₀ phase of the cell cycle. It is possible that such fractions (maybe related to cancer stem cells) are not dependent on elevated ALK activity for viability and not sensitive to ALK inhibitors. I assume that cure of cancer patients be possible when we treat cancer with combination therapies of reagents targeting essential growth drivers (ALK inhibitors against *EML4-ALK*-positive tumors, for instance) plus reagents targeting dormant cells such as immunotherapies.

In addition to *EML4*, *ALK* has been found to undergo fusion to *NPM1* in anaplastic large cell lymphoma, to *TPM3* or *TPM4* in inflammatory myofibroblastic tumor, to *VCL* in renal medullary

carcinoma, and to various other partners in other cancers. It is likely that all *ALK*-rearranged tumors are similarly addicted to the transforming activity of *ALK* fusions and will therefore be sensitive to treatment with *ALK* inhibitors. Such efficacy has already been demonstrated in patients with anaplastic large cell lymphoma or inflammatory myofibroblastic tumor, despite small cohort sizes in the clinical trials.^{40),41)} Given that all *ALK*-rearranged tumors share an essential growth driver (highly elevated *ALK* activity), I have proposed that all such cancers be collectively referred to as “*ALKoma*”.⁴²⁾ *ALK* is thus a good (but not the only) example for a system of cancer nomenclature based on essential growth drivers rather than on the affected organ.

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Profile

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He has been awarded The Medical Award from The Japan Medical Association (2008), The Princess Takamatsu Cancer Research Fund Prize (2009), The Academic Award from the Mochida Memorial Foundation (2010), The Takeda Prize for Medical Science from The Takeda Science Foundation (2010), The Uehara Prize from The Uehara Memorial Foundation (2010), The Prize for Science and Technology from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (2011), The Asclepios Award from the Bonnie J. Addario Lung Cancer Foundation (2011), The Takamine Memorial Daiichi Sankyo Prize from The Daiichi-Sankyo Foundation of Life Science (2011), The Keio Medical Science Prize from The Keio University Medical Science Fund (2012), the Medal with Purple Ribbon from The Japanese Emperor (2012), The Shiono Prize from The Cell Science Research Foundation (2013), and Innovator of The Year (2014).