Editorial

A story of ALK variants and the efficacy of ALK inhibitors: moving toward precision oncology

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In the era of precision medicine, rearrangement of the anaplastic lymphoma kinase (*ALK*) gene has been proven to be a targetable oncogenic driver in 3–7% of patients with advanced non-small cell lung cancer (NSCLC) (1). Multiple clinical trials have demonstrated the superiority of ALK inhibitors compared with chemotherapy for treating patients with *ALK*-rearranged NSCLC; however, the responses to ALK inhibitors have varied in each study (2-7). Fluorescence in situ hybridization (FISH) or VENTANA anti-ALK (D5F3) immunohistochemistry, which are widely used as standard tests for ALK detection for enrollment in clinical trials, are unable to distinguish between the different variants or fusion partners of the *ALK* gene. The impact of ALK variants on the heterogeneity of the response to ALK inhibitors has not been fully elucidated.

One major mechanism may be that various portions of the echinoderm microtubule-associated protein-like 4 (*EML4*) are fused to ALK in different variants, which may be identified by real time-polymerase chain reaction (RT-PCR) or next-generation sequencing (NGS). More than a dozen different variants of *EML4-ALK* variants and non-*EML4* fusion genes have been detected in NSCLC (8-12). Among the variants known thus far, three of the *EML4-ALK* variants identified in NSCLCs are most commonly reported, including variant 1(V1), variant 2 (V2), and variant 3a/3b (V3a/b) (13-15).

The biological basis for the differential activity of *EML4-ALK* has been typically correlated with the distinct stability of the EML4-ALK protein. The primary sequence of the *EML4* portion comprises different domains, including a hydrophobic EMAP-like protein (HELP) domain that is linked to a variable number of tryptophan-aspartic acid (WD) repeats separated from an N-terminal coiled coil by a basic region consisting of serine, threonine, and basic residues. The tertiary structure of the HELP-WD region creates a tandem atypical propeller EML (TAPE) domain in which the HELP motif is part of the hydrophobic core and is crucial for maintaining the folding of the TAPE region. The TAPE domain influences protein stability. Variants 1 and 2 in which the break point occurs within the N-terminal and the C-terminal β-propeller, respectively, include only a partial TAPE domain. This domain determines the exposure of the hydrophobic core, thus rendering the protein unstable and requiring binding with a chaperone to avoid the protein misfolding. By contrast, variants 3a/b and 5 lack the TAPE domain and are more stable (16). The protein stability of the EML4-ALK variants influence the overall fusion protein stability, inhibitor-induced protein degradation, and drug sensitivity (17).

One highlight of the recent study by Woo *et al.* published in Annals of Oncology was categorization of *EML4-ALK* variants based of differential protein stability rather than clinical frequency (15). A total of 51 patients with advanced NSCLC harboring an *EML4-ALK* fusion were subdivided into two groups: variants 1/2/others (27, 52.9%) and variants 3a/b (24, 47.1%). Among the patients treated

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with crizotinib, the 2-year progression-free survival rate (PFSR) was 76.0% (95% CI: 56.8–100) for the *EML4-ALK* variants 1/2/others group, and this was significantly higher than the 26.4% (95% CI: 10.5–66.6) for the variants 3a/b group (P=0.034). Of note, this report also established specific *EML4-ALK* variant-expressing cell lines for evaluating the response of various ALK inhibitors. In line with the clinical findings, the *in vitro* results have demonstrated that all three ALK inhibitors suppressed the growth of V1- or V2-expressing Ba/F3 cells, but had weak inhibition in V3a- or V5a-expressing cells. Contrary to the abovementioned results, another retrospective study in which patients were categorized based on the frequency of ALK variants, no statistically significant correlation between the ALK variants and median PFS of crizotinib was demonstrated by two types of categorization (*EML4-ALK* V1 *vs.* *EML4-ALK* V3a/b *vs.* other uncommon *ALK* variants or common *EML4-ALK* variants including V1 and V3a/b *vs.* other rare *ALK* variants) (18). Recently, *Yoshida et al.* retrospectively analyzed the efficacy of crizotinib in 35 patients with ALK-positive NSCLC categorized by the presence of *EML4-ALK* V1 versus non-V1 variants. Although there was a statistically significant difference in the disease control rate (95% *vs.* 63%, respectively; P=0.0318), and median PFS (11 *vs.* 4.2 months, respectively; P<0.05) (14), the biological rationale for categorizing patients based on the presence of *EML4-ALK* V1 is somewhat artificial (19). According to an *in vitro* study using the EML4-ALK variant-expressing Ba/F3 cell line, variants 1 and 3b exhibited intermediate sensitivity, V3a was least sensitive, and V2 was most sensitive to ALK inhibitors (17). To take it a step further, Hrustanovic *et al.* also discovered differential sensitivity to *EML4-ALK* V1 and V3b in cell lines. Compared with H3122 (harboring V1), crizotinib failed to suppress RAS-GTP, p-ERK, or cell viability in H2228 cells harboring V3b), and thus the half-maximal growth inhibitory concentration for crizotinib was higher in H2228 than in H3122 cells (20). This difference was caused by the lack of a HELP domain in EML4 variant 3, which enhances activation of the RAS-MAPK signaling pathway. These findings suggested that *EML4-ALK* V1 and *EML4-ALK* variant 3a/b might represent two distinct diseases, and patients with *EML4-ALK* V1 achieved a longer PFS from crizotinib than that found with the *EML4-ALK* variant 3a/b; thus, the type of ALK fusion may partially determine the initial sensitivity to ALK inhibition.

In addition to the abovementioned progress in determining the correlation between *EML4-ALK* variants and response to ALK inhibitors, some limitations of this study need to be addressed. First, the small enrollment size might not reflect the true landscape of *EML4-ALK* variants. With more than ten different *EML4-ALK* variants identified, the genetic landscape of *EML4-ALK* variants could be characterized by distinct mountains and hills. Data from earlier studies have demonstrated that *EML4-ALK* V1 and V3a/3b are the most frequent variants, and they have been detected in 33% and 29% of NSCLCs respectively (13), suggesting that both are mountains in the heterogeneous landscape of *ALK* variants, while other *ALK* variants, such as V2 and V7, account for 9% and 3%, respectively, and might be categorized as hills. Such a complicated landscape for ALK variants has posed a tough challenge for discriminating various variants in retrospective analysis of small sample sizes.

In addition to the analysis by *Woo et al.* (15), there were three other retrospective studies analyzing the correlation between ALK variants and the efficacy of ALK inhibitors (14,18,21). It was intriguing to find that distinct ALK variants demonstrated heterogeneous landscapes across these studies, particularly for the common *EML4-ALK* variants 1 and 3a/b. In addition to the *EML4-ALK* variants, the percentage of non-*EML4* variants also remains controversial, ranging from 3.3% to 36.5% across these four studies. Due to the small sample size of each study, whether patients enrolled with a specific subtype of ALK variants could represent the true genetic landscape of this subpopulation deserves further investigation.

Consequently, results from these retrospective analyses have to be carefully interpreted. With regards to the complexity of ALK variant subtypes and small sample sizes of enrollment, whether such controversial results could be simply attributed to the different categorizations in each study and/or the small sample sizes, which might not represent the true genetic landscape of *ALK* variants, is largely unsettled. A multi-center, prospective study with a larger cohort is warranted to provide answers to this question.

Second, whether *EML4-ALK* V3a/3b is truly important for the resistance to ALK inhibitors deserves further investigation. The study by Woo *et al.* draws the conclusion that *EML4-ALK* V3a/3b might be a major source of resistance to ALK inhibitors, which was supported by clinical efficacy analyses and viability tests using established *in vitro* cell lines (15). It appears that this is the first report on clinical data that recognizes the impact of *ALK* variants in generating resistance to ALK inhibitors. Previous

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retrospective analyses have mostly demonstrated the differential or similar role of *ALK* variants in predicting response to crizotinib or ALK inhibitors (14,18,21). Whether such a conclusion could be directly drawn is still worth discussing.

Multiple acquired resistance mechanisms to ALK inhibitors have been identified, including *ALK* gene alterations, such as *ALK* point mutations and copy number gain (22,23) and the bypass activation of other oncogenic genes (24,25). In this study, we noticed that only a small percentage of patients (7/23) underwent rebiopsies at disease progression, and there were none with ALK mutations. Thus, without comprehensive data on the ALK mutations that have been considered as a major resistance mechanism to ALK inhibitors, it still needs to validate the role of *EML4-ALK* V3a/3b in modulating resistance to ALK inhibitors despite evidence from *in vitro* tests. The emergence of next-generation sequencing techniques will possibly allow for the detection of various *ALK* variants and mutation screening in a single test in the near future. Further studies employing NGS-based tests might help determine a more precise correlation between specific *ALK* variants and the efficacy of ALK inhibitors.

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