Targeting Alternative Splicing as Adjunctive Treatment in EML4-ALK v3a/b+ NSCLC: Knowing Our Socratic Paradox and Learning From Spinal Muscular Atrophy

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After their seminal discovery of EML4-ALK variant 1 (v1) (E13:A20) and v2 (E20:A20) as a transforming driver mutation in NSCLC in 2007, 1 Choi et al. 2 went on to identify EML4-ALK v3 (E6:A20) in 2008 using reverse transcriptase-polymerase chain reaction. Two EML4-ALK v3 isoforms, v3a and v3b, which differs by an inclusion of a cryptic (exon EML4 6b) exon of 33 DNA base pairs into v3b, were identified together in two patients’ samples. Interestingly, the transforming potency on soft agar seemed to be similar between EML4-ALK v3a and v3b, and the ratio of v3a/v3b in the two tumor samples seemed to be identical. 2 Hence, the nomenclature of EML4-ALK v3, while sometimes including the notation of (v3a/v3b), it currently does not differentiate between v3a and v3b nor does the vast majority of the literature distinguish any differences between clinicopathologic characteristic or outcomes between EML4-ALK v3a or v3b variants.

In this issue of the Journal of Thoracic Oncology, Song et al. 3 confirmed that both isoforms of EML4-ALK v3 (v3a and v3b) were detected in most EML4-ALK v3+ NSCLC samples (16 of 19, 84.2%). Among the 16 EML4-ALK v3 samples that contained multi-isoforms, 13 (81%) had v3b as the dominant isoform with the proportion ranging from 53.1% to 91.5%. Among the three single EML4-ALK v3 isoform samples, two were v3b. On the basis of the observation of Song et al., 3 inclusion of the cryptic exon (EML4 6a) seems to be the main splicing process in EML4-ALK v3+ NSCLC. In addition, multiple isoforms were detected in 22% (5 of 23) of the samples among EML4-ALK v1 and 66% (two of three) of the rare EML4-ALK v5’ (E18, A20). 3 The percentage of the dominant isoform in the five v1 samples ranges from 84.7% to 97.7%, at 97.1% and 98.6% among the two v5 samples and 99.8% in the one v2 sample, indicating unlike EML4-ALK v3, in EML4-ALK non-v3 variants, the “namesake” variant is the dominant isoform. The mechanisms that resulted in these multiple isoforms are different with alternative splicing responsible for generating the EML4-ALK v3a and v3b isoforms, 2 whereas among the non-v3 variants, the minor isoforms are likely generated from distinct genomic rearrangements. 4

It is generally recognized that patients with ALK+ NSCLC with EML4-ALK v3 when compared with those with v1 have shorter median progression-free survival when treated with ALK tyrosine kinase inhibitors (TKIs), from review of retrospective case series and post-hoc analyses of randomized phase 3 trials. 5-7 This was also observed in this report by Song et al., 3 where all patients with ALK+ NSCLC received crizotinib as their first ALK TKI. Furthermore, Song et al. 3 reported that patients with tumors harboring multiple EML4-ALK isoforms had a statistically shorter median progression-free survival and overall survival than patients with tumors harboring EML4-ALK single-isoform when treated with crizotinib independent of the specific EML4-ALK variant type. Nevertheless, with the limited number of patients in the study of Song et al., 3 and the fact that multi-isoforms are much more common among v3, in addition to the very low

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proportion of minor isoforms compared to the dominant isoform among non-v3 variants, it calls into question whether the multiple isoform phenotype that had a poorer outcome with crizotinib treatment could be generalized beyond EML4-ALK v3.

Importantly, the most provocative finding by Song et al.\textsuperscript{3} was that inhibition assays with crizotinib, ceritinib, or alectinib in H2228 cell line demonstrated that the ratio of EML4-ALK v3a to v3b increased with time on inhibition. The difference in sensitivity to ALK TKIs between v3a and v3b has been scantily investigated. Heuckmann et al.\textsuperscript{8} reported that EML4-ALK v3a is approximately twofold to fivefold more resistant to crizotinib and TAE684 inhibition than EML4-ALK v3b in transfected cell line growth inhibition experiments. In fact, the growth inhibition (GI\textsubscript{50}) of EML4-ALK v3b by crizotinib and TAE684 is similar to the growth inhibition of EML4-ALK v1 by crizotinib and TAE684. How the differences in 11 amino acids can lead to such a difference in sensitivity to ALK TKIs between EML4-ALK v3a and EML4-ALK v3b remained to be determined but is unlikely underpinned by protein stability.\textsuperscript{8} The single-cell assessment of alternative splicing revealed that each cell contains only either EML4-ALK v3a or v3b did not explain whether there was a clonal selection of the more resistant v3a clones or dynamic changes in alternative splicing mechanism(s) (mutations in the DNA or RNA elements of the splice enhancers/silencers or changes in the cellular dynamics or composition of the spliceosomes) within each cell preferentially favoring the skipping of the cryptic exon generating the more resistant EML4-ALK v3a. Regardless, the observation of Song et al.\textsuperscript{3} indicated that targeting alternative splicing could be a potential adjunctive treatment with ALK TKI against the recalcitrant EML4-ALK v3 exploiting the differential sensitivity between v3b and v3a to ALK TKIs.

Targeting alternative splicing has been successful in treating spinal muscular atrophy (SMA), a group of congenital neurologic disorders.\textsuperscript{9} SMA is caused by alterations of the Survival of Motor Neuron 1 (SMN1) gene resulting in low level of or missing SMN protein. A near identical SMN2 gene could not compensate for the low level of SMN1 protein owing to baseline biological skipping of exon 7 (45 base pairs) of SMN2 leading to a truncated SMN2 protein. Risdiplam, an orally available small-molecule RNA splicing modifier, interacts with the 5’ splice site of exon 7 of SMN2 leading to recruiting of prosplicing factors and inclusion of exon 7 in the mature SMN2 transcript, and the functional SMN2 protein results in the correction of the SMA phenotype. Risdiplam was approved as a treatment for SMA in August 2020.

Similarly, the goal of potential adjunctive treatment targeting alternative splicing in EML4-ALK v3 will be to promote (or to maintain) the inclusion of the cryptic EML4 v6a exon to generate more of the ALK TKI-sensitive EML4-ALK v3b proteins. The trans-elements of splicing can also be manipulated pharmacologically given that the assembly of pro-splicing factors can be controlled by kinases, such as serine arginine protein kinase and CDC2-like kinase (CLK).\textsuperscript{10} Inhibitors to CLK kinases can lead to global suppression of splicing with preferential deleterious effect to the rapid dividing tumor cells.\textsuperscript{10} Nevertheless, global suppression of splicing may lead to unintended consequences in EML4-ALK treatment. In preclinical studies, the use of a CLK inhibitor, TG693, led to skipping of exon 31 of the dystrophin gene in Duchenne muscular dystrophy, in which a mutated premature stop codon is located. By skipping exon 31, it leads to the production of a shortened yet functional dystrophin protein due to bypassing of the premature stop codon located on exon 31 and potentially lead to correction of the Duchenne muscular dystrophy phenotype.\textsuperscript{11} Thus, the use of a CLK inhibitor in EML4-ALK v3+ NSCLC may paradoxically lead to skipping of the cryptic exon and generate the highly resistant EML4-ALK v3a isoform. Therefore, a comprehensive understanding of the basic biology of splicing of EML4-ALK v3a/b is urgently needed to provide the next breakthrough adjunctive treatment to ALK TKI in poor-risk subgroup of EML4-ALK v3, which accounts for 35% to 40% of all EML4-ALK+ NSCLC\textsuperscript{5} at the time of initial treatment rather than at the time of progression to forestall resistances, as most of the recalcitrant acquired ALK mutations, such as G1202R, occur in the background of EML4-ALK v3.\textsuperscript{12}

Targeting alternative splicing in a subset of MET exon14 skipping mutations as adjunctive treatment to the currently approved MET TKIs of capmatinib and tepotinib is a logical consideration as well. Given the diverse MET exon14 alterations in the cis-elements of exon14 from single nucleotide mutations to the frank deletion of exon14,\textsuperscript{13} this will warrant a separate discussion. Another rare yet important actionable target potentially suitable for utilizing alternate splicing is NRG1 fusion. NRG1 requires an intact EGF-like domain to bind to its primary receptor HER3. There are two isoforms of the EGF-like domain (α- and β-) within NRG1 protein generated by differential splicing of one of two exons that encodes part of the EGF-like domain.\textsuperscript{14} Importantly, there are significant differences in binding affinity between the two isoforms with β-isoform in general with approximately 10× higher binding affinity than α-isoform.\textsuperscript{15} The ratio of NRG-1 β/α has only been reported in three cases (0.36, 0.76, 1.15), and the dominant differential splicing processes in selecting
either the α-isofrom or β-isofrom specific exon to generate the full length generating EGF-like domain in NRG1 are unknown. It would not take a huge intellectual leap to speculate that during targeted treatment of NRG1 fusion, the ratio of NRG1 β/α will increase thus favoring the more potent receptor binding isofrom similar to the ratio of EML4-ALK v3a/b reported by Song et al. Deciphering the processes of alternative splicing in EML4-ALK v3 and NRG1α/β fusions may one day enable us to apply therapy targeting alternative splicing to EML4-ALK v3 and beyond.

Currently, no combination therapy has been approved for the treatment of advanced ALK+ NSCLC despite our tremendous understanding of both on-target, and off-target resistances. The clinical approval of "double mutant active" ALK TKIs is at best years away with no guarantee that they will be developed to address the current unmet need of acquired double ALK mutations given that double mutations are only part of the spectrum of acquired resistances to ALK TKIs. The successful clinical development of six ALK TKIs globally leading to long-term survival has raised the expectations among investigators to relentlessly advance the therapeutic success against ALK+ NSCLC. By applying drug-induced splicing using a splicing response cassette to regulate gene expression in the setting of gene therapy, hardcore basic science research understanding splicing mechanism may hold the key to the next quantum leap in the broader field of oncology beyond the treatment of ALK+ NSCLC.

CRediT Authorship Contribution Statement

Misako Nagasaka: Conceptualization, Methodology, Investigation, Writing—original draft, Writing—review and editing, Validation.

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References


