Why technical aspects rather than biology explain cellular heterogeneity in ALK-positive non-small cell lung cancer

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The discovery of anaplastic lymphoma kinase (*ALK*) gene rearrangements in a small subset of lung adenoracimonas in 2007 led to the definition of a new molecular subgroup of non small cell lung cancers (NSCLC) (1). These *ALK*-rearranged tumors are most commonly adenocarcinomas, often with signet ring mucinous cells, without mutation of *EGFR* or *KRAS*, and preferentially arise in non- or light smokers, but some *ALK*-rearranged NSCLC cases do not fit this description. A number of fusion variants have been identified; the most common being *EML4-ALK* fusions which are formed from inversions within the small arm of chromosome 2. The encoded proteins comprise the N-terminal portion of EML4 and the intracellular catalytic domain of ALK. A dimerization or oligomerization of these chimeric proteins leads to a constitutive activation of the ALK kinase domain (2). Other described fusion partners of *ALK* in lung tumors comprise *KIF5B, TFG* and *KLC1* (3-5). Whether the nature of the fusion partner has a biological significance in terms of ALK subcellular localization and activation, and a clinical significance in terms of response to treatment, is not known at the present time.

Dramatic and prolonged responses have been obtained in *ALK*-rearranged patients treated with the ALK and MET inhibitor, crizotinib (PF-02341066), leading to the approval of the drug by the FDA as the first licensed ALK inhibitor for ALK-positive (ALK+) NSCLC.

Even if different methods can be used to detect ALK rearrangements in lung tumors, the presence of more than 15% of tumor cells with an *ALK* fluorescent in situ hybridization (FISH)-positive pattern using break-apart probes remains the reference used to prove the presence of an ALK rearrangement in the patients enrolled in crizotinib studies.

In their study published in Cancer in January 2012 (6), Camidge and colleagues explore the correlations between the percentage of *ALK*+ cells and signal copy number as assessed by FISH, and their association with response to ALK inhibition by crizotinib.

In our routine *ALK* FISH practice, most of us (if not all) have observed variability in the percentage of positive cells in *ALK*+ tumors, as well as the presence of a small percentage of positive cells in non-rearranged tumors. For the authors, this cellular heterogeneity has not a biological but a technical explanation, and they give an astute demonstration of their hypothesis.

To start with, the authors point out that it is easier to detect single red (3') signals than a split pattern within a nucleus, and that a rearrangement is more readily detected when the number of signal copies is increased. Therefore, if cellular heterogeneity is due to technical aspects only, the number of red signals (which contribute to the single red and the split patterns of positivity) should be positively correlated to the percentage of *ALK*+ cells in *ALK*+ tumors, fused signal copy number should not be associated with the percentage of *ALK*+ cells, and there should be a positive correlation between the number of green (5') signals (which contribute to both the split pattern of positivity and the negative single green pattern) with *ALK*+ only in cases with a split pattern of positivity.

Finally, to further rule out any biological explanation, there should be no correlation between the percentage of *ALK*+ cells present in *ALK*+ tumors and tumor shrinkage (as assessed by Response Evaluation Criteria In Solid Tumors, RECIST) after crizotinib therapy.

The authors therefore evaluated the percentage of *ALK*+ cells, patterns of *ALK* positivity (split, single red or both), and signal copy number (fused, isolated red and isolated green signals) in relation with response to ALK inhibition in ninety *ALK*+ NSCLC patients. Out of these 90 patients, 30 received crizotinib (phase 1 study) and had available response outcomes on therapy.

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The main results show that the percentage of *ALK*+ cells was comprised in an 18-100% range (mean =56%) in the 90 *ALK*+ patients, 24-94% (mean =59%) in the 30 crizotinib-treated patients. Confirming the authors' hypothesis, tumors with a single red pattern of positivity, easier to detect, had a significantly higher mean percentage of *ALK*+ cells than those with a split pattern of positivity, even if the range of positive cells was similarly wide. Increased copy number of isolated red signals also strongly correlated with a higher percentage of *ALK*+ cells, and isolated red signal copy number gain was stronger for the single red than for the split pattern of positivity. The percentage of positive cells was strongly correlated to isolated green signal copy number only in tumors with a split pattern of positivity, and fused signal copy number was negatively associated with percentage of cells positive for a rearrangement.

On the biological side, mean maximal tumor shrinkage as assessed per RECIST after Crizotinib treatment was 58%, ranging from 0 to 100%. As expected by the authors, there was no correlation between the percentage of *ALK*+ cells in *ALK*+ tumors and the maximal tumor shrinkage after treatment. However, the number of patients was not sufficient to find any significant difference between the extent of tumor shrinkage and any pattern of positivity or copy number.

This article, together with the paper published by the same team in Clinical Cancer Research in 2010 (7), are two precious papers for all who work in the *ALK* FISH field, giving a certain number of very valuable technical guidelines and explanations.

First of all, contrary to the theory of Martelli *et al*. for who *ALK* rearrangements may represent a late oncogenic event, resulting in the coproliferation of different *ALK*+ and *ALK*-negative clones within established tumors, for Camidge *et al*., the negative cells within a tumor are rather false negatives, and in the same way, the ALK+ cells in ALK negative tissues (tumor or normal tissue) are false-positives. A certain number of technical caveats can explain this phenomenon, but for the authors the main explanation for false-negative signals is interobserver error, especially in the cases of *EML4-ALK* rearrangements, which lead to a separation of the two probes which can be missed. However, the authors consider a separation of the two probes to be positive if the splitting occurs by more than two signal diameters. A splitting by more than one signal diameter seems more appropriate in the case of *ALK* rearrangements, especially for *EML4-ALK*, together with a comparison with another technique; immunohistochemistry and/or RT-PCR, which are very helpful in doubtful cases.

On the other hand, the authors rightly point out that false positive patterns can be due to stretching of the DNA, leading to an artificial separation of the two probes.

Taken together the data in this article show there is a need to assess the response to treatment of the "ALK-positive tumors" in order to define - or not - a positivity threshold above which tumors respond to treatment. However, for the moment little is known about the response to treatment of the various fusions proteins; is the response to treatment the same for EML4-ALK rearranged tumors than for tumors containing another fusion? Do all the EML4-ALK variants respond in the same way to therapy? And what about the response to various inhibitors? A certain number of questions remain. The use of other techniques, especially of RT-PCR or sequencing could allow a better understanding of the biology of these various ALK fusions, leading to another step in personalized medicine, and the advent of high throughput sequencing methodologies will no doubt be very helpful.

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