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Breaking down *RET* breakpoints in lung adenocarcinoma

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Abstract

The work by Mizukami et al., published in this issue, describes precise genomic breakpoints on 18 lung adenocarcinoma samples with oncogenic *RET* rearrangements, which occur in ~2% of lung adenocarcinomas. Sequence analysis indicates that breakpoints occur at non-specific sites, using variable mechanisms for DNA repair. This study provides important information for the development of molecular tests for this genomic rearrangement.

In 1960, the first recurrent chromosomal rearrangement in human malignancies was identified by Nowell and Hungerford in chronic myelogenous leukemia and described as the Philadelphia chromosome, which is currently recognized as the *BCR-ABL* translocation. The activated ABL tyrosine kinase, the fusion protein product from this rearrangement, also became the first defined molecular target against which a tyrosine kinase inhibitor was successfully developed[1]. Since the introduction of chromosomal banding techniques in the 1970s that enabled easier detection of these chromosomal rearrangements, numerous recurrent rearrangements have been reported across many types of hematopoietic malignancies as well as in solid tumors. In fact, recurrent *RET* rearrangements in papillary thyroid carcinomas were the first discovered chromosomal translocations involving a tyrosine kinase in solid tumors[2,3].

In lung cancer, there has been a recent profusion of new activating gene fusion discoveries, beginning with the discoveries of *EML4-ALK* fusion genes and *ROS1* fusions[4,5]. Recently, with the advent of systematic genome-wide sequencing technologies, chromosomal rearrangements of other tyrosine kinases such as *RET*[6–9], *NTRK1*[10], and *FGFR3*[11–13], as well as *NRG1* ligand fusions[14] have been identified. With many ongoing preclinical and clinical studies to test the efficacy of kinase inhibitors for such aberrantly activated molecules, a rapid introduction of accurate and sensitive diagnosis of those chromosomal

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rearrangements has become increasingly important in order to identify patients who may benefit from these therapeutics. Current techniques to detect these chromosomal rearrangements include a break-apart fluorescence *in situ* hybridization (*FISH*) assay, a fusion *FISH* assay on unstained tissue slides, and RT-PCR on RNA samples from fresh frozen tissue specimens. Additionally, targeted sequencing using hybrid capture followed by next-generation sequencing on DNA samples has been used as a detection method (See Box 1 for details).

The diagnosis of *RET* fusions is of increasing potential clinical importance because response to cabozantinib has already been reported in some patients[15] and numerous small molecule Ret inhibitors are currently under investigation. Analyzing fusion breakpoints not only can lead to a better understanding of the molecular mechanisms that generate these rearrangements but also can help in the design of better detection methods. The study by Mizukami et al. in this issue describes the structures of breakpoint junctions involving the *RET* oncogene of 16 lung adenocarcinoma samples. Breakpoints from 14 samples with documented *KIF5B-RET* fusion transcripts by RT-PCR were identified using targeted genomic PCR followed by Sanger sequencing. Breakpoints from two samples with documented *CCDC6-RET* fusions by break-apart and fusion *FISH* assays were identified using targeted next-generation sequencing, with capture probes targeting the exons and introns spanning across exons 7–12 of the *RET* gene. This study also analyzed two previously published DNA sequences from lung adenocarcinomas with rearrangements involving the *RET* gene. The authors' findings are in concordance with previous studies that observed genomic breakpoints in a few confined genomic regions spanning kilobases, but not at specific genomic loci, and without significant enrichment of nucleotide motifs or chromatin features that would make them susceptible to breaks[16–19].

Efforts to analyze these breakpoints at the sequence level have identified relatively consistent reciprocal rearrangements with small insertion or deletion of a few base-pairs in chemotherapy-related leukemias or radiation-induced papillary thyroid carcinomas, whereas broader range stretches of DNA insertion, deletion, or duplication have been observed in spontaneous cancers[20,21]. Mizukami et al. further analyzed the breakpoint sequences to infer which DNA repair mechanisms were involved to illegitimately rejoin the DNA ends of fusion partners, and found that while the majority of the events are reciprocal inversions, there are cases of non-reciprocal rearrangements. Unfortunately, the authors were unable to analyze sequences on the other end of non-reciprocal breakpoint junctions, as captured-based sequencing data for these cases were not available. Nevertheless, these findings have significant implication to the interpretation of results from break-apart *FISH* assays in a clinical setting. Similar to the cases of *EML4-ALK* fusions[22], their results suggest it is of positive diagnostic value to only detect a probe corresponding to the 3' end of the *RET* gene.

This study identified diverse DNA repair mechanisms for breaks. In most cases, a lack of sequence homology at breakpoint junctions implicated non-homologous end joining (NHEJ) as the repair mechanism. In four cases with reciprocal rearrangements, duplication of sequences at both ends of the breakpoint junctions implicated break-induced replication (BIR) repair. This is in contrast to repair mechanisms observed in papillary thyroid carcinomas, where NHEJ has only been observed[20,21]. While the exact factors that cause

DNA breaks or choice of DNA repair pathways is unknown, the difference in repair mechanisms between lung adenocarcinoma and papillary thyroid carcinomas may be explained by a difference in carcinogenic insults in these two cohorts. Evidence of BIR repair in lung adenocarcinoma suggests single-stranded breaks occur in addition to double-stranded breaks, however, there was no difference in features of breaks or DNA repair mechanisms between never-smokers and ever-smokers; therefore, the role of smoking-related carcinogens in the genesis of *RET* fusions is unclear.

The work by Mizukami et al. provides rich information on breakpoints involving the *RET* oncogene. The information provided by Mizukami et al. should aid in the design of more sensitive molecular detection of these rearrangements in lung adenocarcinoma samples. The development of sensitive methods to detect rearrangements for DNA samples is particularly important for cases where RNA is not available or is of low quality. However, while the majority of rearrangements are observed within relatively confined genomic regions, this study and other studies have shown that rearrangements do not occur at recurrent breakpoint positions nor retain specific sequence features characteristics of a single repair mechanism. This highlights the challenge for designing genomic PCR based methods to cover all probable breakpoints, unless highly multiplexed. Therefore, this study implies that unbiased approaches using next-generation sequencing, including whole genome sequencing, sequencing following capture of selected regions of RNA or DNA encompassing the relevant breakpoints in *RET*, or transcriptome sequencing of RNA may be the best methodologies for the detection of *RET* chromosomal rearrangements in lung adenocarcinoma.

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Box 1: Description of methods used to discover chromosomal rearrangements between two hypothetical genes, gene *A* and gene *B*

Method	Target nucleic acid	Approach to detect a rearrangement
Break-apart fluorescence *in situ* hybridization (FISH)	DNA	Hybridization probes corresponding to the 5'-end of gene *A* and the 3' end of gene *A* are detected in separate chromosomal locations.
Fusion FISH	DNA	A red-color probe corresponding to gene *A* and a green-color probe corresponding to gene *B* are detected at the same locus, and visualized as a merged yellow color.
RT-PCR	RNA	A forward primer corresponding to the 5'-end of a transcript for gene *A* and a reverse primer corresponding to the 3' end of a transcript for gene *B* will only generate a PCR product when the fusion exists.
Capture followed by next-generation sequencing	DNA	Customized probes corresponding to targeted exons and introns of gene *A* and/or gene *B* are used to capture DNA only from these segments and then subjected to next-generation sequencing. Rearrangements are detected computationally from sequencing reads.

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