Fusion gene and splice variant analyses in liquid biopsies of lung cancer patients

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Abstract: Obtaining a biopsy of solid tumors requires invasive procedures that strongly limit patient compliance. In contrast, a blood extraction is safe, can be performed at many time points during the course disease and encourages appropriate therapy modifications, potentially improving the patient's clinical outcome and quality of life. Fusion of the tyrosine kinase genes anaplastic lymphoma kinase (*ALK*), C-ROS oncogen 1 (*ROS 1*), rearranged during transfection (*RET*) and neurotrophic tyrosine kinase 1 (*NTRK1*) occur in 1–5% of lung adenocarcinomas and constitute therapeutic targets for tyrosine kinase inhibitors. In addition, a *MET* splicing variant of exon 14, has been reported in 2–4% of lung adenocarcinoma and recent studies suggests that targeted therapies inhibiting *MET* signaling would be beneficial for patients with this alteration. In this review, we will summarize the new techniques recently developed to detect *ALK*, *RET*, *ROS* and *NTRK1* fusions and *MET* exon 14 splicing variant in liquid biopsy using plasma, serum, circulating tumor cells (CTCs), platelets and exosomes as starting material.

Keywords: Lung cancer; splice variant; liquid biopsy; fusion gene

Introduction

Lung cancer is the leading cause of cancer death worldwide and non-small cell lung cancer (NSCLC) accounts for approximately 80–85% of cases (1). The majority of the patients are diagnosed when the disease is locally advanced or metastatic and the overall 5-year survival rate is less than 20% (2). In the last years, driver alterations have been discovered in several oncogenes (3) such as *KRAS*, *EGFR*, *ALK*, *MET*, *BRAF*, *PIK3CA*, *ROS1*, *HER2*, *RET*, *PTEN*, *NRG1*, *NTRK1*, *DDR2* or *FGFR1*. These alterations render the tumor sensitive to targeted agents that, in most cases, have significantly better clinical outcomes compared to standard chemotherapy (4-6).

Among the above mentioned driver alterations, ALK, *ROS-1* or *RET* fusion transcripts occur in 1–5% of stage IIIB-IV lung adenocarcinomas and constitute therapeutic targets for tyrosine kinase inhibitors such as crizotinib or alectinib. The splicing variant of *MET* exon 14 is present in 2–4% of advanced NSCLC (3,7) and recent studies in small series of

patients have demonstrated the efficacy of MET tyrosine kinase inhibitors in this population (8).

Tissue biopsy is the traditional source of material for tumor genotyping. However, tissue specimens are not always available or tumor material may be insufficient for molecular testing, especially when cancer is diagnosed at advanced stages on small biopsy specimens. On other occasions, due to tumor location or small size, tissue sampling can be challenging and potentially risky, particularly in extensively treated patients (9). But, even when available, tissue biopsies have other limitations. A single biopsy at presentation does not necessarily reflect the molecular characteristics of the tumor at the time of disease progression, highlighting the need of additional, more dynamic and less invasive sources for continuous genotyping of cancer patients (10).

Circulating tumor cells (CTCs), exosomes and nucleic acid fragments such as cell-free and platelet-bound RNAs, and circulating cell-free DNA (cfDNA) (11) have emerged as useful biosources that are complementary or even alternative to tissue biopsy when a tissue biopsy is not available. The non-invasive liquid biopsies allow frequent sampling and treatment monitoring (12).

EML4-ALK fusions

The *EML4-ALK* fusion (13) is the result of a chromosome rearrangement between the N-terminal portion of the *EML4* gene and the tyrosine kinase domain of the *ALK* gene. *ALK* rearrangements are more prevalent in younger NSCLC patients, females, never or former light smokers (14). *EML4-ALK* positive patients derive clinical benefit from ALK tyrosine kinase inhibitors such as crizotinib (6), ceritinib (15) or alectinib (16). However, despite initial good responses, all patients eventually relapse due to the emergence of different mechanisms of resistance (17).

EML4-ALK fusion transcripts in CTCs

CTCs are viable cells released into the circulatory system (18), but they constitute a minor fraction of the cell population in the peripheral blood of cancer patients, both in terms of absolute (<10 cells/mL) and relative numbers compared to other blood cells (one CTC per 10^6–10^7 leukocytes) (19). CTC counting has been reported to have a prognostic value in metastatic breast (20), colorectal (21) and castration resistant prostate cancer (22). They may also have a role in other solid tumors (20), including NSCLC (23) and SCLC (24).

Several techniques have been developed for the isolation and characterization of CTCs that differ in enrichment, staining and detection methods (25) as well as sensitivity, specificity and reproducibility (26). CTCs enrichment can be based on physical or immunomagnetic approaches. Magnetic beads and ferrofluid-based systems are examples of magnetic affinity cell sorting while filters and density gradients are examples of physical approaches (27). Both of them have been commercialized. The CellSearch System (CSS; Veridex, Warren, USA) is a semi-automated CTCs detection method, approved by the FDA, where CTCs are enriched enrichment using magnetic microbeads covered by antibodies against the epithelial marker EpCAM (epithelial cell adhesion molecule). The Isolation by Size of Epithelial Tumor Cells (ISET) is a kit where CTCs are isolated by a combination of enrichment and filtration. Regarding the identification of CTCs, it can be performed either by direct cytometry techniques like immunocytochemistry, immunofluorescence and flow cytometry or by indirect, nucleic acid-based methods (26,27).

Using the ISET technology for CTCs isolation, Ilie *et al*. (11) were able to detect *ALK*-gene rearrangements on CTCs by immunochemistry (IHC) and fluorescence in situ hybridization (FISH). When comparing the results with paired biopsies in 87 lung adenocarcinoma patients, they found a total of five patients with *ALK*-gene fusions by FISH and strong ALK protein expression by IHC on both CTCs and in the corresponding tumor sample. This seminal study demonstrated that CTCs can be a reliable source for the detection of *ALK*-gene rearrangement in lung cancer patients (11). Pailler *et al*. analyzed by filter-adapted FISH the CTCs of 18 *ALK*-positive patients isolated with the ISET technology, finding four or more *ALK*-rearranged CTCs per milliliter of blood in all cases (28). In contrast, only one or zero *ALK*-rearranged CTCs where detected in 14 *ALK* negative NSCLC patients. In the same study, variations in the levels of *ALK*-rearranged CTC were detected when monitoring over time five patients treated with crizotinib (28).

Enrichment and characterization techniques other than ISET have also been successfully used for CTC isolation in order to detect EML4-ALK fusions by FISH (29,30). In a recent study, an antibody-independent system was employed for the isolation of CTCs from 31 patients (14 *ALK* positive, 12 *ALK*-negative lung cancer and five healthy controls) with paired tissue biopsy (31). Using CTCs of blood samples from healthy donors, a 'false positive' cutoff for *ALK* FISH of ≤2 cells per 1.88 mL of blood was established and
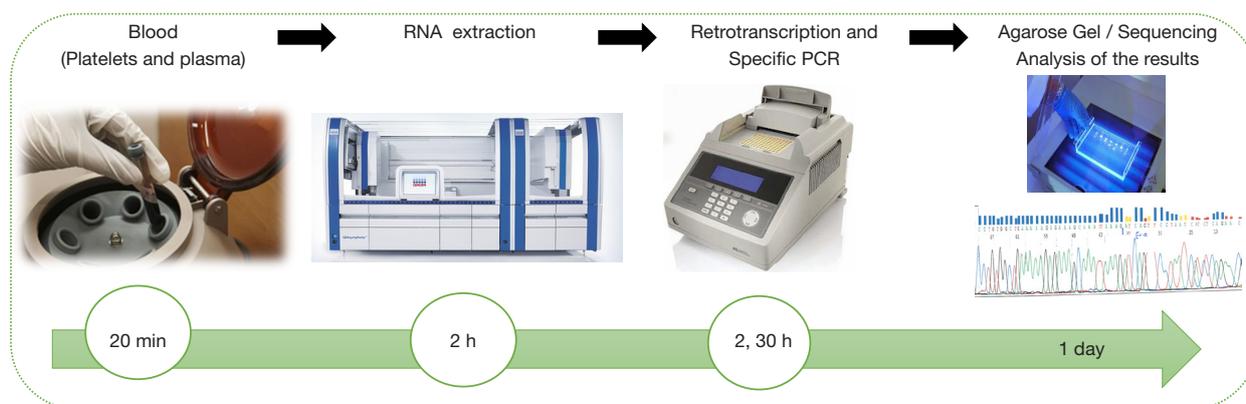
Figure 1 Workflow of the fusion gene analyses in plasma and platelets-derived RNA using automatic extraction followed by RT-PCR. RT-PCR, reverse transcription-polymerase chain reaction.

validated. Using this cut-off, a ≥90% concordance was found between the detection of *ALK* rearrangements in CTCs versus tissue biopsy. The same study reported differences in the *ALK* pattern between CTCs collected at baseline and at the time of disease progression in one patient treated with crizotinib (31). Finally, using the NanoVelcro chip system for CTCs isolation, ≥3 ALK-rearranged CTCs per mL of blood could be detected in *ALK*-positive patients, versus ≤2 in *ALK*-negative cases (32).

EML4-ALK fusion transcripts in platelets

Isolation of other blood components like plasma, serum or platelets is simpler than CTCs and can also provide material for biomarker analysis (33). Recent studies have shown that platelets can sequester tumor related RNA by a microvesicle dependent mechanism, resulting in the generation of tumor-educated platelets (TEPs) (34,35). Our group has demonstrated 65% sensitivity and 100% specificity for the detection of *EML4-ALK* rearrangements in RNA isolated from platelets in patients at presentation (36). During the course of the disease, the presence of *EML4-ALK* fusion transcripts in platelets correlated with clinical outcome to crizotinib treatment response. Monitoring of *EML4-ALK* rearrangements in the platelets of one index patient revealed re-appearance of fusion transcripts associated with crizotinib resistance two months prior to radiographic disease progression. Our data showed that platelets are a valuable source for the non-invasive detection of EML4-ALK fusion transcripts in lung cancer patients, useful for predicting and monitoring outcome to ALK targeted therapies (36).

EML4-ALK fusion transcripts in plasma

cfDNA purified from plasma is commonly used for mutational analysis in advanced NSCLC and other malignancies (37). However, RNA circulating in plasma has not been so widely used for gene fusion detection due to a variety of reasons. Unlike cfDNA, cfRNA degrades very quickly and plasma samples need to be processed rapidly after blood extraction. The usual procedure is adding an RNA preservative such as Trizol and immediately freezing the sample at –80 ℃. However, this procedure is not easily accessible to many clinical sites.

Our research group has 5-year experience in detection of *EML4-ALK* fusion transcripts by reverse transcription-polymerase chain reaction (RT-PCR) in plasma, and we have demonstrated 22% sensitivity and 100% specificity for the detection of this alteration (36). We are currently working to improve the method for plasma cfRNA extraction and *EML4-ALK* fusion transcript analysis. Submitting the plasma samples to automated extraction within 24 h after blood collection, plasma cfRNA can be obtained in the shortest possible time avoiding degradation and without the need of an RNA preservative. Using these new protocol, we have analyzed more than 200 plasma samples and we have significantly improved the sensitivity of RT-PCR for the detection of EML4-ALK fusion transcripts (unpublished data) (*Figure 1*).

KIF5B-RET, ROS-1 and NTRK gene fusions

Additional fusions involving *ROS1*, *RET* and *NTRK* oncogenes have been identified in 1–2% of advanced

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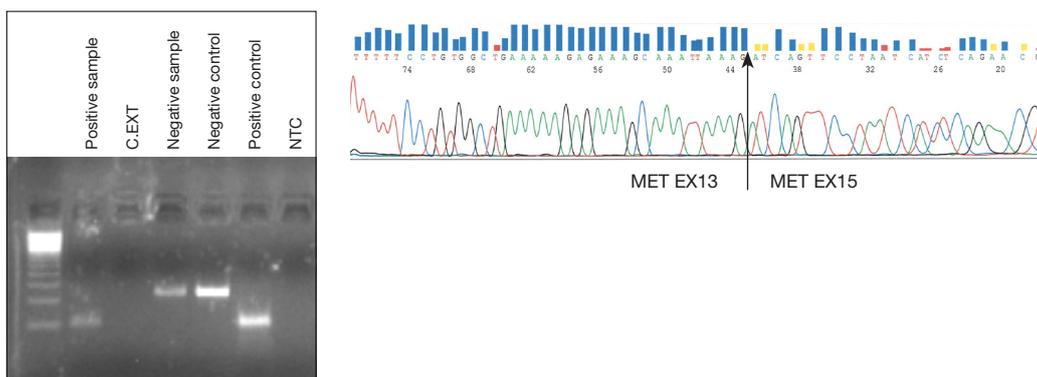
Figure 2 Agarose gel (left) and sequence (right) of the RT-PCR products products of a patient positive for exon 14 MET splicing variant in platelet-derived RNA. RT-PCR, reverse transcription-polymerase chain reaction.

NSCLC patients in the case of RET and ROS1 (3) and 3.3% for NTRK1 (38,39). Despite these low frequencies, these alterations offer the possibility for targeted therapies with better clinical outcomes than chemotherapy.

Similarly to *EML4-ALK*, *ROS1* rearrangements can be detected on the CTCs of NSCLC patients using filter-adapted-fluorescent in situ hybridization (FA-FISH). In a recent study, *ROS1* rearrangements were studied in the CTCs of patients with paired tumor tissue (31). The median number of *ROS1*-rearranged CTCs at baseline was 34.5 per 3 mL blood (range, 24–55) in *ROS1* positive patients versus 7.5 per 3 mL blood (range, 7–11) in *ROS1*-negative patients. New generation platforms, such as Cancer Personalized Profiling by deep Sequencing (CAPP-Seq) (40) or NEOliquid® (41), have also been used to detect gene fusions in the circulating free DNA purified from advanced NSCLC patients. NEOliquid® is a next-generation sequencing assay, based on hybrid-capture, that covers clinically relevant genomic alterations, such as point mutations, small insertions and deletions, selected gene fusions and copy number alterations within a panel of more than 30 genes. Using NEOliquid®, Heukamp *et al*. identified KIF5B-RET gene fusions in two patients previously tested negative for the rest of mutations (41).

Alternative splicing variants

Alternative splicing in cancer is emerging as a growing and promising field in basic and translational oncology and is an object of active investigation (42,43). Although alternative splicing in several genes have been associated with lung cancer (44), MET 14 is so far the only splicing variant with clinical relevance.

MET splicing variant in exon 14

c-MET alterations, including overexpression and amplification, have been described in a number of solid tumors such as papillary renal, gastric and lung cancer, and hepatocellular carcinoma (45-47). Somatic mutations affecting splice sites of exon 14 of the *MET* gene (*MET*ex14) were first reported in primary lung cancer specimens and in a lung cancer cell line (48,49). These *MET*ex14 alterations were shown to promote RNA-splicing-based skipping of *MET* exon 14, which results in activation of MET kinase activity through a unique mechanism (50).

MET ex14 alterations in NSCLC are not necessarily associated with MET amplification occur in approximately 3% of lung adenocarcinoma cases (51) and have also been observed in neuroblastoma (52) and gastric (53) cancer cell lines. Mutational events of MET leading to exon 14 skipping are frequent and potentially targetable events. *In vitro* preclinical studies indicated that MET-targeted agents can counteract oncogenesis resulting from MET exon 14 loss and the efficacy of these agents in NSCLC patients with MET 14 positive tumors has recently been demonstrated (50,54).

There are no reports so far about detection of exon 14 MET splice variant in liquid biopsies, but great progress is expected in the near future due to the relevance of this alteration for the treatment of lung cancer patients. In our laboratory, we have started to analyze the exon 14 MET splice variant by RT-PCR in RNA isolated from plasma and platelets with the same methodology used for *EML4-ALK* rearrangements. Recently, we have detected the alteration in the platelet-derived RNA of a NSCLC patient who harbored the MET exon 14 splicing variant in tumor tissue (*Figure 2*). The patient attained a partial response to

crizotinib (unpublished data).

Conclusions

Gene fusions and splicing variants have been recognized as tumor drivers for more than three decades, providing new targets for personalized therapy. The accurate detection of these alterations in blood will be a significant step for treatment selection at the time of the diagnosis, monitoring treatment outcome and predicting disease progression in NSCLC patients.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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