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 has been reported in 2–4% of lung adenocarcinoma and recent studies suggest 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
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 $10^5$–$10^7$ 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 immunomorphic 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 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 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
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 reappearance 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 °C. 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...
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 hybridation (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 (METex14) were first reported in primary lung cancer specimens and in a lung cancer cell line (48,49). These METex14 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.

References


analysis in small cell lung cancer: novel juxtamembrane
domain mutations regulating cytoskeletal functions.
mutations lead to an oncogenic deletion of met in lung
of MET via diverse exon 14 splicing alterations occurs
in multiple tumor types and confers clinical sensitivity to
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