Review Article

Routine clinical use of circulating tumor cells for diagnosis of mutations and chromosomal rearrangements in non-small cell lung cancer—ready for prime-time?

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Abstract: In non-small cell lung cancer (NSCLC), diagnosis of predictive biomarkers for targeted therapies is currently done in small tumor biopsies. However, tumor biopsies can be invasive, in some cases associated with risk, and tissue adequacy, both in terms of quantity and quality is often insufficient. The development of efficient and non-invasive methods to identify genetic alterations is a key challenge which circulating tumor cells (CTCs) have the potential to be exploited for. CTCs are extremely rare and phenotypically diverse, two characteristics that impose technical challenges and impact the success of robust molecular analysis. Here we introduce the clinical needs in this disease that mainly consist of the diagnosis of *epidermal growth factor receptor* (*EGFR*) activating alterations and *anaplastic lymphoma kinase* (*ALK*) rearrangement. We present the proof-of-concept studies that explore the detection of these genetic alterations in CTCs from NSCLC patients. Finally, we discuss steps that are still required before CTCs are routinely used for diagnosis of *EGFR*-mutations and *ALK*-rearrangements in this disease.

Keywords: Circulating tumor cells (CTC); non-small cell lung cancer (NSCLC); *epidermal growth factor receptor* (*EGFR*) mutations; *anaplastic lymphoma kinase* (*ALK*) rearrangement

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Preface

On the basis that ISET filtration offers higher circulating tumor cells (CTCs) recovery and a broader coverage of the phenotypic heterogeneity of CTCs when compared to CellSearch, we utilized ISET for examining *anaplastic lymphoma kinase* (*ALK*)-rearrangement in CTCs. Our group published in 2013 a novel approach in detecting *ALK*-rearrangement in CTCs with high sensitivity and specificity in patients with non-small cell lung cancer (NSCLC) combining ISET filtration (isolation by size of epithelial tumor cells) and a fluorescent in situ hybridization (FISH) assay optimized for CTC characterization on filters called FA-FISH.

Clinical needs

In the last decade, the success of tyrosine kinase inhibitors (TKIs) in selected NSCLC patients has considerably transformed the management of this disease (1,2). NSCLC and in particular adenocarcinoma has been segmented into molecular subsets based on oncogenic 'driver' alterations

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(3-7). The two best characterized oncogene driver paradigms are the *epidermal growth factor receptor* (*EGFR*) activating alterations and *ALK*-rearrangement against which an increasing repertoire of TKI therapies is being developed (8). *EGFR*-activating alterations (the L858R point mutation in exon 21 and exon 19 deletions) present in about 15% of NSCLC patients confer sensitivity to first-line EGFR TKI therapy such as gefitinib, erlotinib or afatinib (9-15). Acquired resistance to first line EGFR TKIs usually developed within 12 months of treatment (15,16), the principal cause being the T790M mutation which is detectable in approximately 60% of patients (17-20). The *ALK*-gene rearrangement involves most often the *echinoderm microtubule-associated protein-like 4* (*EML4*) loci and defines a unique molecular subset in 4% of NSCLC patients (21-24). In 2012, crizotinib was approved for the treatment of patients with previously advanced treated *ALK*-rearranged NSCLC and is now the standard of care in the first line setting (25-28). Acquired resistance develop after a median progression-free survival (PFS) of 8 to 9 months, the most frequently identified secondary mutations being the L1196M, which is analogous to T790M in *EGFR*, and G1296A (29,30). Additional *ALK* secondary mutations distributed throughout the kinase domain (1151Tins, L1152R, C1156Y, G1202R, S1206Y, I1171T, F1174C) have also been detected in crizotinib-resistant *ALK*-positive tumors (31-39). A third molecular entity also targetable by TKIs is a *c-ros oncogene 1* (*ROS1*) fusion gene which has been more recently identified in approximately 1% of NSCLC and can benefit from crizotinib therapy (40,41).

In metastatic NSCLC, surgery is rarely a component of treatment. The availability of tumor tissue is a major hurdle to the identification of genetic alterations and screening of patients eligible for TKI therapies. Genetic profiling is most commonly performed on tumor biopsy which can be invasive, impractical and in some cases associated with risk. Furthermore tissue adequacy, both in terms of quantity and quality, is often insufficient. Importantly, a single biopsy sample may also not reflect the genetic diversity of a patient's tumor (42,43). With the increasing number of EGFR and ALK inhibitors in current clinical development, the identification of alternative noninvasive options to tumor biopsy to diagnose predictive biomarkers of sensitivity to TKIs is becoming increasingly important. Moreover, at the time of disease progression, serial biopsies are needed to identify secondary resistance mutations and re-assess the changing molecular profile of tumors. Subjecting patients to serial biopsies to identify acquired resistance mutations is even more complicated and invasive. Therefore, monitoring the emergence of acquired resistance mutations and tumor evolution is also an important issue for the development of precision medicine in NSCLC (44).

Biological characteristics of CTCs in NSCLC

The development of efficient, non-invasive methods to identify molecular alterations is a key challenge which CTCs have the potential to meet (45). However CTCs are very rare (rate of one cell per 10^6 or 10^7 leucocytes) and their molecular characterization must rely on the combination of successive steps which include an initial enrichment process, the identification of CTCs, and the detection of the genetic alteration itself (46-48). The CellSearch platform is based on the detection of epithelial cells expressing EpCAM, and approved by the FDA as an aid to prognosis in patients with metastatic breast, prostate and colorectal cancers (49-51). CTC levels measured by CellSearch have been reported to be prognostic in NSCLC but the levels are very low in this cancer type and patients are frequently negative even in advanced states (52). We and others groups have reported that CTCs are identified in higher numbers using an enrichment technique based on blood filtration (ISET, isolation by size of epithelial tumor cells) compared to the CellSearch method in NSCLC most likely due to the fact that CTCs expressing markers of epithelial-mesenchymal transition (EMT) which have lost epithelial features can be missed by CellSearch (53-56). Using ISET, the prognostic value of CTCs was also reported in patients with resected NSCLC with CTC thresholds tenfold higher (50 CTC/10 mL) than by the CellSearch (55). Recently our group reported that differential total CTC counts and EMT characteristics can be observed according to different genetic subtypes of NSCLC (57). CTCs from *EGFR*-mutant and *ALK*-rearranged NSCLC patients express epithelial-mesenchymal transition characteristics, not seen in CTCs from patients with *KRAS*-mutant adenocarcinoma.

The biological characteristics of CTCs including rarity and phenotypical heterogeneity impose a number of limitations and technological challenges which currently impact on the success of robust molecular analysis. In NSCLC the studies cited above support the existence of a contingent of CTCs expressing mesenchymal characteristics and a low level of truly epithelial (both EpCAM and pan-keratin positive) CTCs. The absence of a universal detection

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assay capable to embrace the CTC phenotypical diversity imposes technological choices such that the results must take into account the technique used. Here we present studies reporting the detection of *EGFR*-mutations, *ALK*- and *ROS1*-rearrangements in CTCs from NSCLC, including the description of techniques used and results. Their level of validation in the context of routine clinical use is discussed.

Detection of gene-rearrangements

Detection of ALK-rearrangement

Crizotinib was approved with a companion diagnostic test, the Vysis *ALK* Break Apart FISH Probe Kit (Abott Molecular) (58). Therefore the Vysis FISH test performed on tumor biopsies has been the reference assay for years but it tends to be supplanted by immunohistochemistry (IHC) because of the greater technical facility of IHC. The detection of *ALK*-positive CTCs has been reported by four groups including our own (59-62). All groups used FISH testing, Paul Hofman's group using both FISH and IHC (59). In 2012, Ilie *et al.* reported that *ALK* status could be determined in CTCs isolated from patients with NSCLC by IHC and FISH analysis. CTCs from 87 patients with NSCLC were isolated using ISET filtration and screened for their *ALK* status both in tumor biopsy samples and in CTCs. *ALK*-FISH was carried out using the Vysis Probe Kit on two spots of one ISET filter (each spot corresponding to the filtration of one milliliter of blood). IHC analyses were performed using the anti-ALK antibody (5A4 clone) on two other ISET spots. Five patients had *ALK*-rearrangement and strong ALK-protein expression in CTCs and in the corresponding tumor samples. FISH confirmed *ALK*-rearrangement with the break apart of the 5' and 3' probes and multiple signals per cell. Both *ALK*-FISH and ALK-immunoreactivity analyses showed negative results in CTCs and corresponding tumor samples for 82 patients negative in the tumor tissue. For the five patients with *ALK*-rearranged tumors, *ALK*-rearranged CTCs were detected by IHC and FISH in three different CTC samples collected per each patient. On the basis that ISET filtration offers higher CTC recovery and a broader coverage of the phenotypic heterogeneity of CTCs when compared to CellSearch, we utilized ISET for examining *ALK*-rearrangement in CTCs (60). Our group published in 2013 a novel approach in detecting *ALK*-rearrangement in CTCs with high sensitivity and specificity in patients with NSCLC combining ISET filtration and a FISH assay optimized for CTC characterization on filters called FA-FISH. Levels of four or more *ALK*-rearranged CTCs per 1 mL of blood were detected in all 18 *ALK*-positive patients tested, but no or only one *ALK*-rearranged CTC was detected in 14 *ALK*-negative patients.

Using the threshold of four *ALK*-rearranged CTC per 1mL blood, a sensitivity and specificity of 100% was met for predicting the *ALK*-rearrangement status present within the tumors. Diagnosis of *ALK*-rearrangement by FISH on paraffin-embedded tumor samples uses a threshold of 15% of *ALK*-rearranged cells, which represents two standard deviations above the mean cell count in negative tumor samples. Due to the rarity of CTCs in blood, we proposed using the number of *ALK*-rearranged CTCs per volume of blood rather than the overall percentage as a cutoff value for establishing the diagnosis of *ALK*-rearrangement. By combining four-color immunofluorescent-staining (IF) and FISH, *ALK*-rearranged CTCs were found to display a remarkably homogeneous mesenchymal phenotype. A unique *ALK*-FISH pattern (the break apart signal) was consistently identified in CTCs despite the inter-tumoral heterogeneity of *ALK*-rearrangements and the frequency of tumor cells harboring this rearrangement within tumors. In addition this unique *ALK*-rearrangement pattern was detected in CTCs of patients for whom it was not identified within the tumor biopsy. Although a single tumor biopsy sample might not be representative of the entire tumor, CTCs may originate from various metastatic sites. These findings suggested that CTCs that harbor this unique *ALK*-rearrangement and express a mesenchymal phenotype may result from the clonal selection of tumor cells that display migratory properties and higher invasive potential and may possibly contain highly metastatic cells, such as cancer stem cells. In 2016, using a sophisticated NanoVelcro Chip technology based on the selection of EpCAM positive CTCs, He *et al.* reported the detection of *ALK*-rearrangement in 21 *ALK*-positive patients in the tumor biopsy (61). All *ALK*-positive patients had at least three *ALK*-rearranged CTCs per one mL blood while 20 *ALK*-negative patients had no or two *ALK*-rearranged CTCs. The *ALK*-rearrangement status was consistent with that of the tumor. In addition the *ALK*-rearranged CTC ratio was found to correlate to the pTNM stage in *ALK*-positive patients. Although these results may appear similar to ours, it is important to note that *ALK*-rearranged CTCs were epithelial in this study. In our hands, *ALK*-rearranged CTCs collected on ISET filters expressed vimentin and N-cadherin at a level that was generally lower than that

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of leucocytes but were negative for both cytokeratins and E-cadherin. This result was consistent with the absence or very low level of CTCs by the CellSearch in our *ALK*-positive cohort. Another study published in 2016 by Tan *et al.* also reported the detection of *ALK*-rearranged CTCs and concordance between CTCs and tumor biopsies (62). After red blood cell lysis, the ClearCell FX system from Clearbridge Biomedics was used here for CTC enrichment. A fraction of the CTC-enriched fraction deposited on glass slides by cytospin was tested using the Vysis Probe kit after. Using this approach, the authors reported 3 to 15 *ALK*-rearranged CTCs per 1.88 mL in 14 *ALK*-positive patients and 0 to 2 *ALK*-rearranged CTCs per 1.88 mL blood in 12 *ALK*-negative patients and five healthy donors. There was no available information on the phenotype of the CTCs.

In *ALK*-rearranged NSCLC patients, treatment with crizotinib is marked by heterogeneity in the magnitude and duration of clinical response. Response durations vary from a few months to several years, and the long-term effectiveness of crizotinib is invariably limited by the development of acquired resistance (30). In our initial study several CTC subsets harboring distinct *ALK*-FISH patterns—including *ALK*-rearranged CTCs and CTCs with a gain of *ALK*-copy number (*ALK*-CNG)—were identified and were correlated with variable evolution on crizotinib treatment in the five examined patients (60) (*Figure 1A*). Based on this observation, we hypothesized that CTC subsets differing in *ALK*-FISH patterns might be associated with different clinical outcomes in *ALK*-rearranged patients treated by crizotinib. Therefore we recently evaluated whether these CTC subsets monitored on crizotinib in an extended cohort of 39 *ALK*-rearranged patients could inform on treatment benefit (64). CTCs were classified into distinct subsets according to the presence of *ALK*-rearrangement and/or *ALK*-CNG signals. As previously reported in tumors biopsies, no significant association between baseline numbers of *ALK*-rearranged or *ALK*-CNG CTCs, and PFS was observed. However, we observed a significant association between the decrease in the numbers of CTCs with *ALK*-CNG on crizotinib and a longer PFS. In multivariate analysis the dynamic change of CTCs with *ALK*-CNG was the strongest factor associated with PFS. Although not dominant, *ALK*-CNG has been reported to be one of the mechanisms of acquired resistance to crizotinib in tumor biopsies. This study shows that serial FISH analysis of CTCs could identify a predictive biomarker of therapeutic efficacy in *ALK*-rearranged NSCLC and could help to stratify patients at risk of early resistance.

Detection of ROS1-rearrangement

Our group published the detection of *ROS1*-rearrangements in the CTCs of 4 patients with *ROS1*-rearrangement previously detected by FISH on tumor biopsy (65). Detection was performed using ISET filtration and FA-FISH (*Figure 1B*). In *ROS1*-rearranged patients, the median number of *ROS1*-rearranged CTCs at baseline was 34.5 (range, 24–55) per 3 mL of blood while in *ROS1*-negative NSCLC patients, median background hybridization of *ROS1*-rearranged CTCs was 7.5 (range, 7–11) per 3 mL blood. This study provides the proof-of-concept that CTCs can be used for non-invasive, sensitive and specific detection of *ROS1*-rearrangement in NSCLC patients. In this study we also evaluated the effect of crizotinib on *ROS1*-gene copy number; in the two patients who had tumor progression the number of *ROS1*-gene copies present in *ROS1*-rearranged cells increased significantly during treatment. *ROS1*-rearranged CTCs showed considerable heterogeneity of *ROS1*-gene abnormalities and elevated numerical chromosomal instability, which was hypothesized to promote the emergence of drug resistant CTC sub-clones with increased metastatic capacity, offering potential mechanisms of *ROS1*-inhibitor-therapy resistance in *ROS1*-rearranged NSCLC tumors.

Detection of mutations

In 2008, Maheswaran *et al.* reported the detection of *EGFR*-mutations in CTCs isolated from 27 NSCLC patients using the CTC-chip, a microfluidic device containing microposts coated with anti-EpCAM antibodies (66). *EGFR* mutational analysis was performed using allele-specific PCR amplification and was compared to the results from the isolated free plasma DNA and original tumor biopsy. They identified the expected *EGFR*-activating mutation in CTCs from 11 of 12 patients (92%) and in matched free plasma DNA from 4 of 12 patients (P=0.009). They also identified the T790M resistance mutation in CTCs from patients who had already received TKI therapy. Serial analysis also demonstrated that reduction in CTC number under treatment was associated with an improved radiological response and *vice versa*. Interestingly, in patients who developed progression different *EGFR*-activating mutations were identified in CTCs suggesting the emergence of different tumor subclones. Punnoose *et al.* reported on the clinical correlation between change in CTC number detected during therapy and treatment response (67). The study cohort consisted of 41 patients with relapsed

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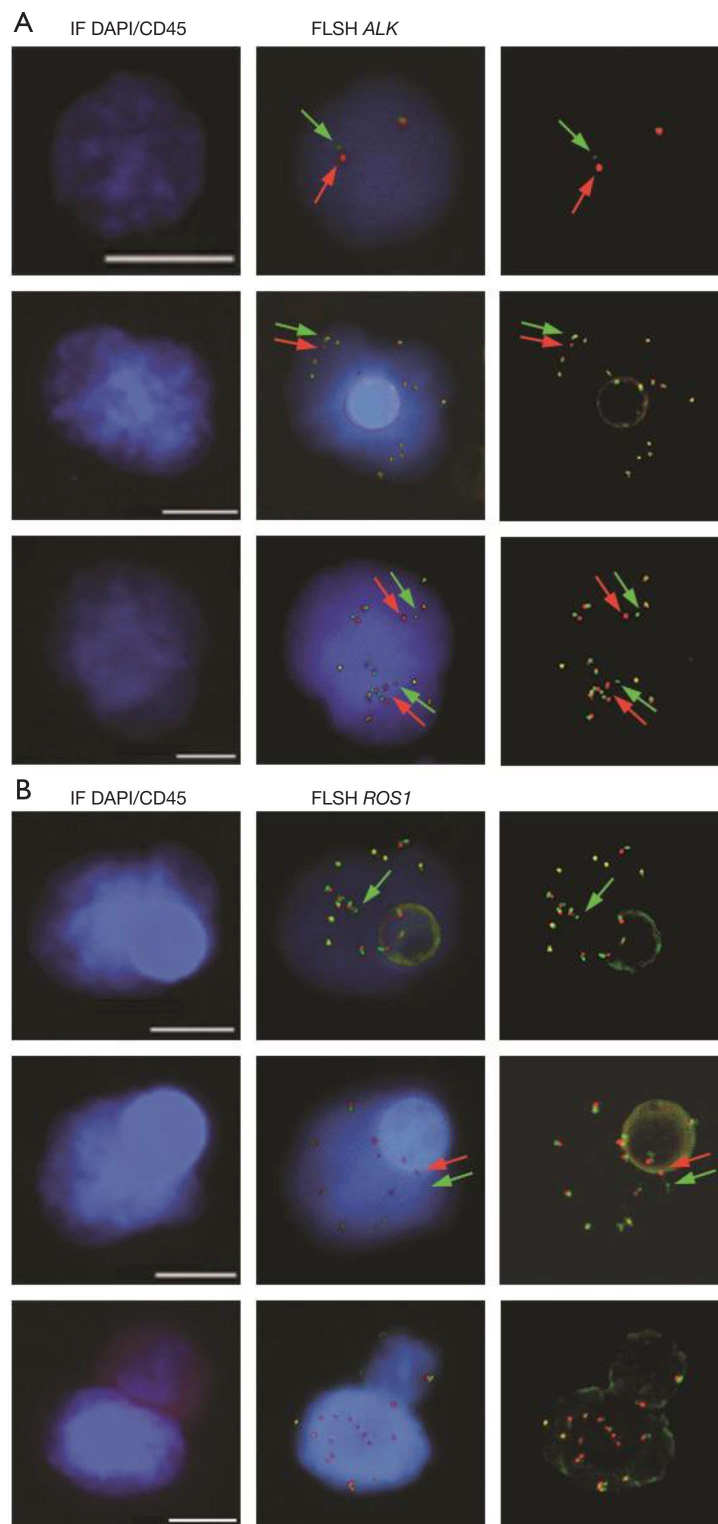

Figure 1 Examples of gene-rearrangement detection in filtration enriched-CTCs from NSCLC patients by combined fluorescent staining and FISH. (A) Example of *ALK*-rearrangement detection in NSCLC patients with an *ALK*-rearranged tumor; (B) example of *ROS1*-rearrangement detection in NSCLC patients with a *ROS1*-rearranged tumor. Adapted with permission from (63). Used under CC BY 4.0.

or refractory NSCLC who were enrolled in a single arm phase II clinical trial of erlotinib and pertuzumab, but only eight patients had confirmed *EGFR*-activating mutations in the tumor tissue. Peripheral blood was analyzed for CTC enumeration using the CellSearch platform at baseline and changes in CTC levels were assessed for correlation with PDG-PET and CT imaging and survival endpoints. The analysis reported a statistically significant correlation between high baseline CTC counts and patient response to treatment according to RECIST (P=0.009). *EGFR* mutational analysis was performed on CTCs captured by the CellSearch profile kit. After DNA extraction and a pre-amplification step, mutations were detected by Taqman PCR using multiplex gene-specific primers. Only one *EGFR* genetic alteration (exon 19 deletion) was detected in CTCs while eight patients had confirmed mutations in archival tumor tissue. In 2014, Marchetti *et al.* reported the results of a multi-center trial of erlotinib treatment in 37 advanced NSCLC patients with activating *EGFR*-mutations in tumor tissue. CTCs were obtained from the CellSearch and subjected to ultra-deep next generation sequencing (NGS) (68). *EGFR*-mutations were in 31 (84%) of the CTC samples examined, 25 (81%) had in frame exon 19 deletions and 6 (19%) had point mutations at exon 21. In 29 (91%) of the 31 cases the mutation type detected by NGS in CTCs corresponded to that found in matching tumor tissue by Sanger sequencing. Interestingly in 4 cases, double or multiple mutations were observed by NGS in CTCs suggesting CTC heterogeneity. No mutations were detected in control samples (CTC from 10 breast cancer patients, 12 healthy subjects). Gorges *et al.* reported the detection of mutations in the *EGFR* and *KRAS* genes in CTCs isolated by the Gilupi CellCollector *in vivo* system (69). In this study the authors provided a proof-of-principle that CTCs captured by this device were suitable for molecular analysis. Captured CTCs from two patients with known mutations in the primary tumor were analyzed using Digital PCR after whole genome amplification. The same *KRAS*- and *EGFR*-mutations found in the primary tumors were detected when one to five CTCs were detected by the system. Recently, Zhang *et al.* reported the L1196M resistance mutation in short-term cultured CTCs from *ALK*-rearranged NSCLC (70).

Clinical use of CTCs: ready for prime-time?

Thus there is still very little data on the detection of these gene aberrations in CTCs from NSCLC patients. All the exploratory studies presented above have only involved a small number of patients. In most cases, different CTC enrichment techniques were used which implies that CTCs identified positive for a genetic biomarker possibly differed from one study to another and the results cannot be compared. Beyond this proof-of-concept studies, there are still many steps that should be taken before CTC assays can be used in routine clinical diagnosis. First of all, because the studies presented above used different CTC enrichment techniques, it is important to identify for each genetic biomarker the most appropriate CTC enrichment technique prior to CTC identification and downstream molecular assays. Such a comparison of test performance, validation of selected assays in cohorts of positive and negative patients and their further clinical qualification will require important collaborative efforts. Such projects can only be carried out within the framework of collaborative groups, for example, the Cancer-ID European consortium whose purpose is to validate and standardize liquid biopsy assays.

Regarding FISH assays, the automation of microscopy analysis is a necessary step to improve reproducibility of analyses, reduce risks of errors, inter-operator differences, and progress towards a standardization and validation of biomarker detection. Our group recently reported a semi-automated method established to analyze filtration-enriched CTCs according to combined fluorescent staining and FISH (63). This method relies on the detection of molecular biomarkers by establishing FISH scanning parameters (z-stacking, step, i.e., distance between two z-stacks, exposure time) for optimal FISH signal identification in filtration-enriched CTCs. For using CTC FISH assays in routine clinical diagnosis, another important point that increases the cumbersome nature of the analytical process is the necessity of the validation of FISH signals by an experienced cytogeneticist.

Mutational analyses have been mainly performed on DNA extracted from enriched CTC populations and in most studies the sensitivity of detection was limited by the presence of leukocyte DNA. The analysis of CTCs at the single cell level will overcome this limitation but imposes important technical challenges for individual CTC isolation, whole genome amplification (WGA), PCR for targeted mutations and NGS. WGA is a process which is prone to amplification bias, polymerase errors and results in nucleotide variations errors and allelic distortion. The distinction of true somatic variants present in CTCs from WGA errors is essential and requires a very rigorous and

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careful assessment.

Assays using circulating tumor DNA (ctDNA) assays for detecting gene aberrations (i.e., mutations, gene rearrangements and gain of copy numbers) are being rapidly implemented in the clinic and several studies have shown the specificity and sensitivity of *EGFR*-mutations detection in ctDNA from NSCLC patients (71-73). Although ctDNA assays indeed offer advantages in terms of simplicity, the molecular analysis of CTCs can provide unique additional information such as the morphology and phenotype. The presence of multiple and different genetic alterations can be identified within the same CTC offering the possibility to infer tumor heterogeneity and evolution. Although the utilization of CTCs is less eminent, CTCs and ctDNA are anticipated to be complementary in their clinical utility. Moreover while tumor tissue biopsies presently remain the gold standard for CTCs and ctDNA analyzes, it is important to question their use as a reference (74). CTCs and ctDNA can be derived from lesions that were not biopsied and may contain a different genetic composition than the tumor biopsies used as a reference.

Although many steps have still to be evaluated and validated, it is anticipated that the progression from "bench-to-bedside" of the molecular analysis of CTCs would be critical to aid the personalized treatment of patients with NSCLC. When considering the path that research in this field will follow over the next 5 years there are some key elements which come to mind; firstly, the validation of CTC detection and characterization techniques on large patient cohorts is necessary and secondly the development of automated platforms to simplify and standardize techniques to aid their passage to widespread clinical utility. Currently several research teams are focusing on the use of highly sophisticated automated platforms and we would hope that over the next 5 years clinical validation of their diagnostic role in NSCLC will be achieved. Thirdly the development of single cell sequencing technologies offers the opportunity to distinguish complex tumor genomes from single CTCs and along intra-tumor heterogeneity and tumor evolution. Ultimately the overall objective is to optimize the tailoring of personalized therapies to NSCLC patients with CTCs potentially utilized in a diagnostic role and predictive assessments.

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Footnote

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

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