Editorial

Association of epithelial-to-mesenchymal transition circulating tumor cells in non-small cell lung cancer (NSCLC) molecular subgroups

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Provenance: This is an invited Editorial commissioned by the Section Editor Ji-Gang Wang (Department of Pathology, The Affiliated Hospital of Qingdao University, Qingdao University, Qingdao, China).

Comment on: Lindsay CR, Faugeroux V, Michiels S, *et al.* A prospective examination of circulating tumor cell profiles in non-small-cell lung cancer molecular subgroups. Ann Oncol 2017;28:1523-31.

Submitted Sep 16, 2017. Accepted for publication Nov 13, 2017.
doi: 10.21037/jtd.2017.11.106
View this article at: http://dx.doi.org/10.21037/jtd.2017.11.106

The early detection of cancer is the hallmark of a successful treatment. However, many tumors remain clinically occult until they are far advanced. The high metastatic potential of the disease is attributed to the dissemination of tumor cells through the hematogenous and/or the lymphatic vasculature (1). To this direction, circulating tumor cells (CTCs) constitute the "seed" for the promotion of the general metastatic potential in distant organs ("soil"), triggering a mechanism that is responsible for the vast majority of cancer-related deaths (2). CTC detection, in the peripheral blood, has been proposed and is being tested as a liquid biopsy material; it is a non-invasive procedure and the presence of CTCs and their molecular characteristics has been associated with disease prognosis, treatment effectiveness and, eventually, with early detection of cancer (3).

Recent technology advances for CTCs enrichment and detection have shown a correlation of CTC numbers and patients' clinical outcome in various cancer types, including non-small cell lung cancer (NSCLC) (4,5). Among these technologies, CellSearch (Veridex, Warren, NJ, USA) is the only assay approved by FDA for the detection of CTCs representing, thus, a "reference standard" among the different CTC-detection platforms (6). Using the CellSearch assay, CTCs were initially isolated through their binding to ferrofluid particles coated with antibodies against the epithelial cell adhesion molecules (anti-EpCAM) and, subsequently, are stained with phycoerythrin-conjugated anti-cytokeratin (recognizing predominantly cytokeratins 8, 18, and 19) and allophycocyanin-conjugated anti-CD45 (recognizing hematopoietic cells) antibodies, followed by 4',6-diamidino-2-phenylindole (DAPI) staining to fluorescently label the cell nuclei. The recent knowledge concerning the significant genetic and molecular heterogeneity that has been demonstrated both between patients (inter-tumor) and within a single tumor (intra-tumor) as well as the tumor evolution (7,8), requires the possibility of re-biopsies of the primary tumor as well as of the metastatic sites in order to have a real-time image of the molecular landscape of the disease; however, such re-biopsies are often difficult or even impossible in most patients. Therefore, the molecular and genetic analysis of CTCs along with study of the plasma cell tumor DNA (ctDNA), which are originated from both the primary tumor as well as the different metastatic sites, have been emerged as important tools to obtain real-time information for the molecular characteristics of the tumor. Despite the fact that CellSearch cannot provide information regarding the molecular background of the CTCs, it can be used to isolate the cells for further molecular profiling which, in the era of personalized medicine, could aid the prediction of therapeutic effectiveness, the detection of drug resistance,

the identification of CTC subpopulations/gene signatures allowing patients' stratification and a thorough evaluation of patients for clinical trial eligibility (9).

In the current issue of *Annals of Oncology*, Lindsay and colleagues (10), report results from a prospective evaluation of the prognostic value of CTCs based on their number, phenotype and molecular profiling, in patients with advanced (stage IIIb–IV) NSCLC. In fact, following an initial evaluation of the prognostic value of the CTC number using an already recognized cutoff of ≥5 CTCs/10 mL of blood, the authors evaluated the prognostic value of the detection of vimentin-positive (vim+) CTCs during treatment while, in addition, they correlated the detection of such cells with the presence of *EGFR*, *ALK* and *KRAS* mutations. Among all enrolled patients, those with ≥5 CTCs/10 mL of blood was emerged as an independent factor associated with a lower overall survival (OS, P=0.022) but not progression-free survival (PFS, P=0.118). This is the largest clinical study of CTCs analyzed by CellSearch in advanced NSCLC patients and the presented results are in accordance with previous studies, even when analyzing samples from different patient cohorts (stage IIIA and/or IV) (5). Indeed, Krebs *et al.* in a study that enrolled 40 chemotherapy-naive patients with advanced NSCLC, demonstrated that the number of CTCs as well as its change during the treatment course had prognostic value in patients with stage IIIA or IV NSCLC. The authors concluded that CTCs are among the most valuable predictor for OS (5). These findings were also confirmed by subsequent studies which demonstrated that CTC number can be emerged as independent prognostic factor for both PFS and OS (11). The use of OS, as a primary endpoint, has some disadvantages such as the delayed announcement of study results, the high cost and longer duration because of the need of larger patients' cohorts and longer follow-up. On the other hand, OS is considered the "gold standard" for the evaluation of new therapies for cancer given that death is easily defined, not arbitrary and is not prone to investigator bias (12). In addition, OS is a better indicator of the overall tumor biological behavior.

One major disadvantage of many studies using the CellSearch platform is its failure to detect low or no EpCAM-expressing tumor cells such as those derived from NSCLC (13). Recent studies have identified a process termed epithelial-to-mesenchymal transition (EMT) as a prerequisite for invasion and metastasis of cancer cells. One of the hallmarks of EMT in tumor cells is the progressively loss of epithelial antigens, including EpCAM and cytokeratins (14), while developing mesenchymal markers, such as vimentin (15). In order to overcome this limitation in the present study, Lindsay *et al.* took advantage of the free channel of the CellSearch platform and added a FITC-labelled anti-vimentin antibody aiming to further characterize patients' CTCs according to their vimentin expression during treatment; a patient was considered as CTC-positive if at least one vim+ cell was detected (10). Twenty-four (19.2%) patients had ≥5 CTCs/10 mL of blood and 5.6% of them were vim+. Using only vimentin as a CTC marker, patients' positivity was raised up to 23.3%. Nevertheless, the authors failed to demonstrate any significant difference between the vimentin expression and the clinical or pathological characteristics of the patients. Moreover, no difference was observed between baseline and paired samples during the treatment of patients with vim+ CTCs (P=0.373); in addition, there was no difference for those patients with dynamic change of vim+ and vim– CTCs expression during treatment (P=0.696) and no difference was observed for the ratio of vim+: total CTCs (P=0.645). Nevertheless, a meta-analysis of observational studies including 4,118 cases, showed that overexpression of vimentin on CTCs may have both predictive and prognostic value in NSCLC (16).

Apart from the enumeration of the CTCs, their molecular characterization may also provide a strategy for monitoring tumor cell genotypes in serial samples during treatment. It is documented that over 64% of all lung cancers have an underlying driver mutation that is responsible for proliferation of the cancer cells while, most of these mutations are mutually exclusive (17). For about 20–30% of these cases a specific targeted therapy has been developed and approved by the authorities. The most common ones are associated with mutations in the *EGFR* gene or *ALK*-rearrangements (18). Treatment with the appropriate tyrosine kinase inhibitors provides rapid induction of responses, prolonged disease control although, inevitably, disease progression due to the emergence of diverse resistance mechanisms will be occurred.

Currently, several other oncogenes, including *RAS*, as well as the potential therapeutic value of their inhibition are under evaluation. So far, there are no specifically approved targeted therapies for the largest genomically defined subset of *KRAS* mutant NSCLC patients. Indeed, the prognostic and predictive value, and thereby the clinical value of *KRAS* mutations in NSCLC are questioned. In a recent article, several data regarding the biology of *KRAS* mutations and their prognostic and predictive value in patients with

NSCLC were reviewed (19). A study conducted in early NSCLC patients demonstrated the prognostic value of *KRAS* mutations (PFS, P=0.038; OS, P=0.002), but these results could not be reproduced in subsequent studies, concluding that such mutations could serve as a prognostic tool in specific patient cohorts, such as stage I NSCLC, women or Asians. However, studies in advanced NSCLC patients presented more promising data, showing a negative impact of *KRAS* mutations on survival (19).

In the Lindsay's *et al.* current study (10), the authors also correlated the total CTC number as well as the vim+ subgroup with the presence of *EGFR*, *ALK* and *KRAS* mutations. Of the patients tested for genetic aberration, 24.4% were *KRAS* mutated, 22.3% were *EGFR* mutated, and 14.4% *ALK* rearranged. According to the analysis, high number of CTCs with EMT characteristics could be identified only in patients with *EGFR* mutant (42.9%) but not from *KRAS* mutant or *ALK* rearranged adenocarcinomas that presented no or low vim+ CTC numbers. In line with previous studies about mutual exclusivity, *KRAS* mutant patients were considered as *EGFR* and *ALK* wild type (wt). The same approach was also used in the cases with *EGFR* mutations or *ALK* rearrangements. However, given the fact that concomitant mutations have been reported in some studies, this approach could be considered as a limitation of the study since it has been reported that a small number of patients may carry *EGFR-KRAS*, *EGFR-ALK* or *KRAS-ALK* concomitant mutations (1.1%, 1.6% and 2.5%, respectively) (20). In the Lindsay's *et al.* study, no statistically significant difference was observed between *KRAS* mutated and *KRAS* wt patients when analyzing either the total CTC positive patients (P=0.315) or only the vim+ CTC patients (P=0.086). A complete absence of vim+ CTCs was revealed in *KRAS* mutant patients (0% of vim+ CTCs in *KRAS* mutants) while in *KRAS* wt 32.2% of patients had vim+ CTCs (P=0.004). These results are in accordance with previous studies, showing a complete absence of vimentin expression in tumors resected from *KRAS*-driven lung cancer mouse models (21). This could be explained by the fact that *KRAS* dependency is linked to the epithelial differentiation status. Upon EMT, *KRAS* dependency is reduced, and conversely, by mesenchymal-to-epithelial transition (MET), *KRAS* dependency is gained (22). Similarly, patients with *ALK*-rearrangements presented lower CTC positivity, although not significant (P=0.122); even so, the patients with *ALK*-rearranged tumors had significantly lower total CTC counts (P=0.029). All patients with *ALK*-rearrangements had higher proportion of vim+ CTCs in relation to total CTCs. However, no significant correlation with *ALK*-rearrangement and vimentin expression was observed which is in discordance with the data from other studies where EMT was associated with the response to treatment with *ALK* inhibitors (23,24). In the *EGFR*-assessed group, *EGFR* mutated patients had both significantly higher total CTC and vim+ CTC positivity than EGFR wt patients (P=0.038 and P=0.013, respectively). The present study, despite the enrolment of treatment-naïve patients, confirmed the already reported data that for some patients, acquired resistance to EGFR inhibitors is associated with the phenomenon of EMT (25).

In conclusion, Lindsay and colleagues prospectively evaluated the prognostic value of CTC detection using the CellSearch platform and the ≥5 CTCs/10 mL of blood as a cutoff, both in terms of PFS and OS. Moreover, it was demonstrated that the number of CTCs as well as the EMT phenotypes may vary among the different molecular subtypes of NSCLC, with EMT characteristics being expressed mainly in *EGFR* mutant NSCLC. Although this is the largest study investigating the role of CTCs in NSCLC patients, more studies, with additional patient cohorts and with sequential samples during treatment are required, in order, more safely, to conclude for the precise value of CTCs in this disease.

Acknowledgements

We would like to sincerely thank the scientific secretary Vasso Athanasaki for her attentive editing of this manuscript.

Footnote

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

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Cite this article as: Messaritakis I, Kotsakis A, Georgoulias V. Association of epithelial-to-mesenchymal transition circulating tumor cells in non-small cell lung cancer (NSCLC) molecular subgroups. J Thorac Dis 2017;9(12):4817-4820. doi: 10.21037/jtd.2017.11.106

