On: 20 May 2015, At: 01:54
Publisher: Taylor & Francis
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK

Expert Review of Molecular Diagnostics
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/iero20

The rationale for liquid biopsy in colorectal cancer: a focus on circulating tumor cells

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Published online: 15 May 2015.

To cite this article: Paola Gazzaniga, Cristina Raimondi, Chiara Nicolazzo, Raffaella Carletti, Cira di Gioia, Angela Gradilone & Enrico Cortesi (2015): The rationale for liquid biopsy in colorectal cancer: a focus on circulating tumor cells, Expert Review of Molecular Diagnostics

To link to this article: http://dx.doi.org/10.1586/14737159.2015.1045491

EXPERT REVIEWS

The rationale for liquid biopsy in colorectal cancer: a focus on circulating tumor cells

Expert Rev. Mol. Diagn. Early online, 1–8 (2015)

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Capturing circulating tumor cells (CTCs) and/or circulating tumor DNA from blood, which represents a precious source of biological material derived from both primary and metastatic tumors, has been named a 'liquid biopsy'. While the circulating tumor DNA might be more representative of the bulk of the metastatic tumor, CTCs are thought to reflect more of the metastases-initiating cells. Consequently, a liquid biopsy made of tumor cells and tumor DNA that is able to track cancer evolution, as a fingerprint of the patient's individual tumor, and is easy to perform at every stage of the disease course, sounds attractive. This article mainly focuses on the applications of CTCs to track tumor dynamics in real time using colorectal cancer as a model system. The analysis of viable CTCs at DNA, RNA and protein levels, as well as their expansion *in vitro,* may allow deep investigation of the features of metastases-initiating cells.

Keywords: cancer stem cells • circulating tumor cells • colorectal cancer • epithelial–mesenchymal transition • liquid biopsy • targeted therapy

The innovative and exciting idea that most solid tumors spread tumor cells into the bloodstream, which can be quantified through enumeration and provide information about prognosis, recently celebrated its tenth birthday. Capturing circulating tumor cells (CTCs) and/or circulating tumor DNA (ctDNA) from blood, which represents a precious source of biological material derived from both primary and metastatic tumor, has been named 'liquid biopsy'. While the ctDNA might be more representative of the bulk of the metastatic tumor [1], CTCs are thought to reflect more of the metastases-initiating cells. Compared to the traditional tissue biopsy, which represents a static snapshot of a tumor, a liquid biopsy has a unique, great advantage: to provide a 'real time' information on disease burden, shedding light on tumor evolution over time and on its heterogeneity. Due to the difficulty of repeated sampling of tumors and corresponding metastatic lesions, liquid biopsy is expected to improve understanding and monitoring of changes in tumor cell populations during disease progression and, more crucial, in response to therapies. In fact, although it is largely accepted that intra-tumor heterogeneity might influence the effectiveness of molecular targeted therapy, the major barrier to investigate the acquired drug resistance is the frequent unavailability of post-treatment tumor samples. Consequently, a liquid biopsy made of tumor cells and tumor DNA able to track cancer evolution, as a fingerprint of patient's individual tumor, easy to perform in every stage of the disease course, sounds attractive. This perspective mainly focuses on the applications of CTCs to track tumor dynamics in real time using colorectal cancer (CRC) as a model system. The analysis of viable CTCs at DNA, RNA and protein levels, as well as their expansion *in vitro*, may allow us to deeply investigate the features of metastases-initiating cells.

Liquid biopsy & new therapeutic targets: lessons from CRC stem cells

Dreaming about the optimal anti-cancer treatment, the ultimate goal of therapies should be the radical elimination of all tumor cells. In the face of the rapidly emerging and complex

 10.1586/14737159.2015.1045491 ISSN 1473-7159

cancer heterogeneity now coming into focus, it is not surprising that this goal is hard to be reached [2]. In the last few decades, the old model of cancer clonal evolution, stating that all cancer cells have the same ability to acquire genetic and/or epigenetic changes, conferring growth advantage, has been challenged by the cancer stem cells (CSCs) hypothesis [3]. The new model suggests that tumors are highly hierarchical with a unique self-renewing population of putative CSCs at the top of the hierarchy, which are thought to drive tumor propagation and metastatic relapse, mainly due to their ability to self-renew and escape traditional therapies. Accumulating evidence has suggested that both models might be easily combined towards a dynamic model where clonal evolution and CSC models are both involved in cancer progression [3]. In CRC, it has been suggested that these cells may sustain colon cancer progression [4]. The self-renewal capacities of colon CSCs have been ascribed to different pathways, including that of Wnt, Notch and HGF. Poorly differentiated CRCs have an increased number of CSCs, and the epithelial–mesenchymal transition (EMT) induced by the tumor microenvironment seems to favor CSCs expansion [5]. To date, the search for CSC markers is based on the presence of antigens on subpopulation of cells with stem-like properties. Despite the powerful search for surface markers specific for CRC CSC, the results are currently inconsistent. This failure may be ascribed to the plasticity of CRC CSCs phenotype, which strongly depends upon several variables, including the selective pressure of therapies, the type of therapy and unpredictable microenvironmental factors. In this interpretation, the originating CSC that sustained the first oncogenic mutation gives rise to subclones with self-renewal capabilities that accumulate epigenetic and genetic changes over time. Each different CSC subclone gives rise to intermediate transit-amplifying progenitors that lack self-renewal capabilities. This instability allows CSC to unceasingly move from a functional state to another, even floating between a stem and non-stem state [6]. This suggests that, being both CSCs and non-CSCs highly adaptable populations capable of transient evolution and plasticity, targeting both is imperative. CD133, CD44, CD24, CDCP1, CXCR4, CD44v6, CD44v9 and CD26 have been identified as colon CSC surface antigens, but it is not well defined which are the best markers to identify a tumor stem cell [7–11]. From a mere experimental point of view, the CSC model has been always tested using mouse transplantation assays, which undoubtedly underestimate the frequency of cells with metastatic potential due to the persistence of xenogeneic immune response even in immunocompromised animals, and to the different microenvironment, being human cancer cells forced to grow in a mouse environment (e.g., inability of murine growth factor to bind human receptor). In CRC, the use of xenotransplantation in immunocompromised mice as functional assays for studying CSCs recently leads to the demonstration that tumorigenic activity is confined in the CD44v6 population, which also shares EMT features [9]. Following this observation, our group is currently investigating whether CD44v6 might be retained in CTCs isolated through a filtration method (ScreenCell) from patients with metastatic CRC (mCRC). Our preliminary data suggest that a subpopulation of CTCs expressing CD44v6 are detectable through a liquid biopsy, thus supporting the hypothesis that the CTC population contains putative CSCs, and lightening the possibility of an easily reproducible liquid biopsy to develop new CSC-targeted drugs in CRC (data submitted for publication). Obviously, in a liquid biopsy what we expect to find is a mixed population of innocent, bystander CTCs and more aggressive circulating CSCs with EMT traits. Evidence exists that in CRC dedifferentiated cancer cells combine EMT properties with a stem cell-like phenotype, leading to the hypothesis that these cells combine traits that are necessary for acquiring motility and a stem-like phenotype [12]. Therefore, the identification of 'circulating CSCs' in the entire pool of tumor cells disseminated in the bloodstream would, at least theoretically, provide a unifying hypothesis on CTCs and CSCs. In this context, recent data indicate that CSCs and CTCs may represent different functional statuses of the same pathogenically relevant subpopulation of cancer cells [13] and proteomic research, as well as single cell sequencing may contribute to differentiate bystander CTCs from cancer circulating stem cells with rapid clinical applications in diagnosis, prognosis and therapies. It is conceivable that the elimination of bystander CTCs by immune system or chemotherapy may impair the balance between CTCs and CSCs thus selecting aggressive subclones, similar to what observed in primary and metastatic tumors. The more lines of therapy one patient receives, the more likely the circulating CSCs may be selected. Thus, trying to imagine an ideal liquid biopsy-directed therapy in CRC, the combination of existing chemotherapeutic drugs with a CSC targeting agent might be conceived to eliminate both bystander CTCs and circulating CSCs, including those that may have become dormant. Unfortunately, the identification of circulating CSCs relies on the same putative stem cell markers previously identified in CSC. These markers are highly imperfect so that even isolating a marker-positive subpopulation that is CSC rich, we cannot exclude the presence of CSC in the marker-negative subpopulation, which will re-establish a marker-positive subpopulation in the tumor. Furthermore, the use of a broad spectrum of different antigen-dependent and antigen-independent methods, which may capture different populations of CSCs in the pool of CRC CTCs, may add complexity in the identification of specific stem-like markers [14–16].

Liquid biopsy & molecular subtypes of CRC: questioning about EMT

The recent molecular subtype classification of CRC highlighted three distinctive hallmarks: EMT, higher mutation frequency and cellular proliferation [17]. The mismatch repair deficient subtype (A type) displays strong microsatellite instability phenotype and epithelial features; it accounts for 20–30% of all CRC and benefits from 5-fluorouracil-based chemotherapy. More than half cancers with epithelial phenotype are characterized by high proliferation rate and low overall mutation

frequency, consistent with the absence of DNA mismatch repair deficiency. Patients with this subtype (B type) show a relatively poor baseline prognosis but benefit most significantly from adjuvant chemotherapy, due to the high proliferation rate of tumor cells. The third class (C-type), which has a mesenchymal gene expression phenotype, is enriched with genes indicative of a stem-like state and characterized by low proliferative index, thus resistant to conventional treatments. Further studies have now well established that tumors defined by these three groupings can be additionally stratified into further molecularly defined subtypes, with unifying feature being the identification of a CRC subtype significantly enriched for genes associated with a poorly differentiated, mesenchymal/invasive phenotype, often co-enriched with genes indicative of a stem-like state [18]. Thus, regardless of the molecular classification used, it is accepted that a mesenchymal signature is a defining feature of poor prognosis in CRC. EMT is involved in all steps of metastatic cascade, from the escape from primary site, where microenvironmental and genetic factors endorse the activation of EMT program, to the intravasation where epithelial–mesenchymal-transitioned cells are transported in the circulation to the latter formation of micrometastases. The consequent colonization in distant organs requires the reversion of EMT and/or activation of the MET program to establish secondary tumors [19]. The frequent association between EMT and peritoneal carcinomatosis in CRC is a further proof that EMT-positive tumors have high metastatic potential, which is particularly important even at early stages of tumor growth [20]. The mechanisms for maintenance of mesenchymal state in CTCs are not fully understood, although it has been described that mesenchymal CTCs may be clustered with platelets, which represent the major source of circulating TGF-β production, a primary inducer of EMT [21]. It is conceivable that CTCs need to maintain the EMT program to prevent anoikis while in circulation, and use their mesenchymal features to protect themselves from many chemotherapy as well as molecularly targeted drugs. EMT is associated with resistance to gemcitabine, 5-fluorouracil, cisplatin, tyrosine kinase inhibitors and hormonal therapy [22]. The presence of mesenchymal cells has been consistently found associated with resistance to monoclonal anti-EGFR antibodies (Cetuximab), as mesenchymal cells regulate AKT activation through EGFR-independent pathways like integrin-linked kinase. Consistent with these findings, several studies have highlighted the prognostic significance of CRC CTCs with EMT features, suggesting that the genetic makeup of these cells would be useful for the discovery of new 'druggable' targets [23,24]. Actually, since it is emerging that both epithelial and mesenchymal tumor cells need to cooperate to successfully form metastatic tumors (only the epithelial-like are able to generate macrometastases, which are consequently largely epithelial), targeting both cell types, combining standard chemotherapies against cycling cells with EMT inhibitors would improve therapeutic success. Among the EMT-inhibiting approaches, targeting circulating mesenchymal cells could be considered. For example, Twist silencing, which has been demonstrated to reduce the number of CTCs with EMT phenotype in prostate cancer, might be exploited therapeutically, even if it might elicit an acceleration of epithelial cell deposits with increased metastasis formation [25]. Recent studies performed in breast cancer have provided evidence of a striking association between expression of mesenchymal markers and clusters of CTC rather than single migratory cells [26]. These clustered CTCs, which have the cell junction component plakoglobin highly differentially expressed compared to single CTCs, are oligoclonal precursors of breast cancer metastasis. Whether the presence of CTC clusters may have a role in CRC has been poorly investigated, although this item should deserve more attention [27]. Through the filtration-based method ScreenCell we were able to detect CTC clusters from patients with mCRC (Figure 1). These clusters were found enriched with mesenchymal-like cells (Figure 2). CTC clusters are described to travel into the bloodstream 'protected' by platelets, which are believed to promote their survival within the circulation allowing them to escape the immune surveillance. Furthermore, recent studies have provided evidence that platelets-derived TGF-β and PDGF are powerful activators of EMT in CTCs [21]. In this scenario, and thinking about the prolonged survival of CRC patients treated with aspirin, a new appealing role of aspirin as a modulator of platelet-induced EMT in CTC clusters could be speculated, which may contribute to the intriguing antimetastatic potential of this drug. The use of new filtration methods capable of viable enrichment of single and clustered CTCs from clinically

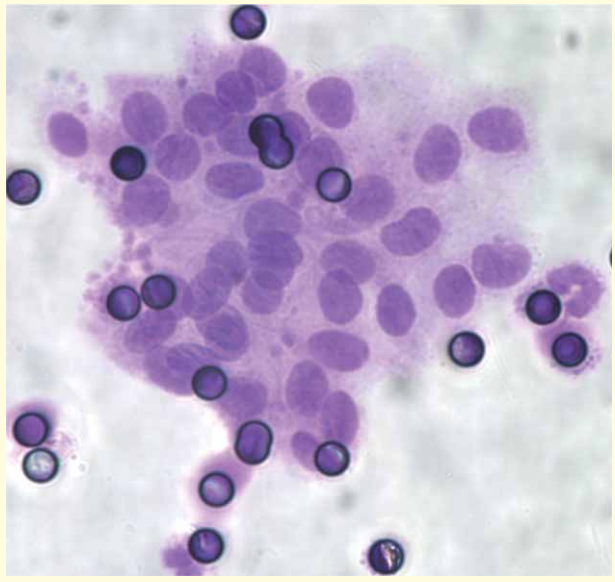

Figure 1. Cluster isolated from a liquid biopsy of a metastatic colorectal cancer patient. CTCs were isolated through a filtration-based method (ScreenCell). After blood filtration, the filter was stained with Giemsa, and analyzed by a pathologist. Giemsa staining enables morphological analysis of potential CTCs.
CTCs: Circulating tumor cells.

doi: 10.1586/14737159.2015.1045491

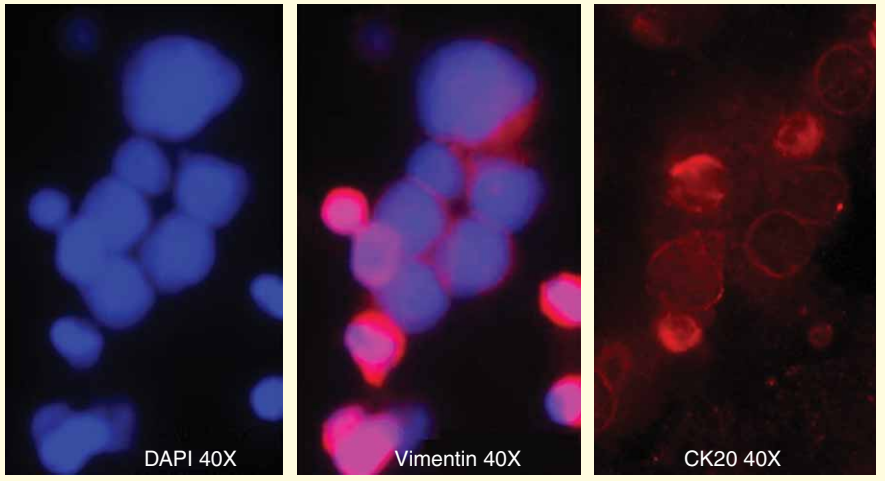

Figure 2. Immunofluorescence of liquid biopsy from one mCRC patient showing vimentin expression as a hypothetical marker of EMT, and CK-20 as a marker of colorectal cancer. The blue counterstaining (DAPI) shows the location of nuclei (Magnification ×40).
mCRC: Metastatic colorectal cancer; EMT: Epithelial–mesenchymal transition.

relevant volumes of whole blood could address this crucial question [28].

Liquid biopsy & targeted therapy: catching a 'moving target' in CRC

The improved understanding of CRC molecular biology and the recent advances in diagnostic techniques and tailored therapies have approximately tripled median survival of patients with mCRC in the last two decades. Cetuximab and panitumumab, both targeting the EGFR, have largely contributed to this success. Both drugs are indicated for wild-type (WT) KRAS mCRC, due to the recognition that activating mutations in KRAS is a strong predictor of resistance to EGFR-targeted mAbs. Thus, routine testing of all patients with CRC for KRAS mutations is now recommended; only those harboring WT KRAS should be candidates for such therapies, thus improving outcomes, and minimizing unnecessary toxicity and cost. Although KRAS testing has facilitated the selection of patients who are most likely to have a response to anti-EGFR therapy, a substantial fraction of patients with WT-RAS tumors do not benefit from treatment [29]. Resistance to anti-EGFR therapies, which typically occur within 3–18 months after treatment initiation, is partly ascribed to oncogenic activation of other intracellular signaling pathways (RAS/RAF/MAPK; PI3K/PTEN/AKT), acting downstream and/or independently of EGFR [30]. Thus, although EGFR-targeting therapies are key approaches in the treatment of mCRC patients, the adaptation of tumor cells to these molecularly targeted agents still represents a significant barrier towards an effective treatment, as demonstrated by the short duration of clinical benefit in the majority of patients with advanced disease. The success of individualized cancer treatments is furthermore limited by the significant variability of biomarkers expression within primary and metastatic tumors. Although at present KRAS mutational status is the only predictive marker for targeted therapy in mCRC, the concordance in KRAS mutation status between primary colorectal tumors and metastases still remains a controversial issue. Notably, although most studies agree on high rate of concordance, which is congruent with the notion that KRAS mutations are considered early driving events in CRC progression, a recent meta-analysis showed that around 11% of patients with mutant KRAS in the primary tumor, thus not candidate for targeted therapy, have WT-KRAS in the metastases, and that 9% of patients with WT-KRAS in the primary tumor have mutant KRAS in the metastatic site [31]. The clinical relevance of these percentages seems considerable, in light of the 40% of patients harboring KRAS mutations within the entire population of patients with CRC. The explanations for such variability in biomarker expression between primary tumor and metastasis might be numerous. Anti-EGFR therapy has been shown to promote the selection of RAS mutant subclones in primary tumors that are otherwise KRAS, NRAS and BRAF WT. The complex cancer heterogeneity translates in a substantial instability of cancer genomes, enabling cancer cells to adapt to any hostile environment under selective pressures, including the application of targeted therapies, and driving the evolution of resistant subclones. Often clinical practice is guided by the molecular analysis of primary tumors assuming that all primary tumor characteristics are carried over to metastases later in the disease course. Nevertheless, according to the two models of metastatic dissemination, a late dissemination following a subclonal selection at the primary site should be taken into account to explain the deviation of genetic makeup from a primary to a metastatic cancer. Single biopsy specimens of primary tumors performed for diagnostic purposes may not be sufficiently sensitive to detect genetic events in tumor subclones. In the case of mKRAS metastases derived from WT-KRAS primary tumors, the hypothesis that cancer cells may have detached from the primary tumor before the acquisition of KRAS mutation should also be taken into account. Recent studies support that relapse in mCRC patients treated with anti-EGFR therapies may be due to the growth of cells with low, thus undetected, KRAS mutant tumor cell content [29,32]. In this perspective, the authors conclude that few if any colon tumors are WT, with intriguing implications for effective targeted therapy. All these data together confirm that, being cancer genotypes highly plastic and unstable, an adjustment of molecularly targeted therapies to reflect such continuous genetic alterations is strongly needed and that some mCRC patients may require serial monitoring of their tumor genome's makeup

to warrant that the therapy is still 'hitting the target' or to detect whether new predictive biomarkers are emerging. The recent observation that WT-KRAS primary tumors shed into circulation circulating free tumor DNA with mKRAS sequencing few months after starting treatment suggests that a liquid biopsy may address the temporal heterogeneity of activating KRAS mutations, identifying early the appearance of KRAS mutant tumor cells over the course of the disease [33]. The very high concordance found in the first blinded prospective study comparing the determination of KRAS and BRAF mutation status from ctDNA to that determined from tumor-tissue analysis is a demonstration that liquid biopsy through ctDNA analysis could advantageously replace tumor-section analysis. A key question to be solved concerning ctDNA is which populations of cells it actually reflects. Is it released from apoptotic/necrotic tumor cells (if this is the case its burden would reflect the efficacy of therapies) or is it actively secreted (if this is the case its burden would reflect the resistance to therapies)? This last hypothesis to date is still enigmatic. Our group recently investigated KRAS pathway activating mutations in CTCs and determined the degree of heterogeneity for KRAS oncogenic mutations between CTCs and tumor tissues [34]. Whether KRAS gene amplification may represent an alternative pathway responsible for KRAS activation was also explored. *KRAS* gene amplification emerged as a functionally equivalent and mutually exclusive mechanism of KRAS pathway activation in CTCs, possibly related to transcriptional activation. Discordance in KRAS mutational profile has been widely demonstrated between primary CRC and CTCs. In this case, the serial assessment of CTCs mutational profile may represent a clear biomarker for treatment resistance, able to overcome the intrinsic limit of ctDNA discussed above. The fact that CTCs are live cells is the main advantage of their use in liquid biopsy. In fact, literal meaning of the term 'liquid biopsy' should be restricted to a blood test that is associated with cytopathological assessment, by analogy to the definition of a 'tissue' biopsy. By the same analogy, a liquid biopsy should theoretically be able to provide clear images of tumor cells to the pathologist, using conventional staining methods, and to allow pathologist to perform a diagnosis through the identification of morphological features and immunohistochemical markers of prognostic relevance. Of note, we might use liquid biopsy to verify whether the well-defined molecular subtypes of CRC, each with distinct features and prognostic significance, are maintained in CTCs throughout the metastatic process, with the additional possibility for downstream genetic analyses using whole genomic amplification. To this purpose, novel blood filtration techniques suited for cytomorphological classification, immunocytochemical and molecular characterization of CTCs, where staining, cell enumeration, immunocytochemistry and FISH assays can be conducted directly on the filter, represent an easy, economic, rapid tool to deep insight microheterogeneity of CRC [35–39]. Angiogenesis is the other one validated therapeutic target in CRC patients with macroscopic metastases. On the molecular level, it is controlled by a number of pro- and anti-angiogenic factors, among which the VEGF signaling plays a pivotal role [40]. Despite the proven benefit of VEGF-targeting therapy in metastatic setting, the modest effects observed in adjuvant setting, the high variability in individual patient response and the frequent appearance of acquired resistance again reflect the complex CRC heterogeneity in angiogenesis pathways. This heterogeneity, again, might not be captured by the tumor biopsy performed before first treatment, since single portions of a tumor may be differently vascularized. Whether metastatic tumors may be more dependent on angiogenesis than micrometastases is one of the unsolved questions which might partially explain the failure of the anti-VEGF monoclonal antibody Bevacizumab in adjuvant setting [41]. The role of circulating endothelial cells as a potential biomarker of efficacy of antiangiogenic therapies has been widely investigated through CellSearch system, which reached a sufficient level of sensitivity for circulating endothelial cell quantification and monitoring during the course of the disease and in response to treatment [42]. Results from clinical trials investigating the predictive role of circulating endothelial cell in CRC patients treated with antiangiogenic therapies are conflicting and thus deserve further attention.

Expert commentary

Investigating complexities of cancer heterogeneity both at primary and metastatic sites is increasingly coming into focus. Recent evidence has been provided that a high degree of heterogeneity is also observed in cancer cells traveling in the bloodstream. The concept of CTCs heterogeneity hampers the necessity to isolate the different entities found in the blood and to analyze them comparatively to investigate the tumorigenic potential of different subsets, thus moving away from the prognostic significance of their enumeration, well proven in different tumor types, including colon cancer. To this purpose, the identification of a recently proved stem-like marker of colon cancer, CD44v6, in CTCs from mCRC patients who failed second line therapies may suggest that this specific CTCs subpopulation could theoretically drive CRC progression. Nevertheless, to be confirmed, this hypothesis needs to be assessed in CTCs-derived xenograft tumor models, which to date represent the most reproducible approximation of human tumors. In this context, the recent establishment of a stable CTC line from the blood of a metastatic colon cancer patient, which showed a complex genetic makeup made of epithelial, mesenchymal and stem-like features, as well as tumorigenic potential after xenografting into immunodeficient mice, has particular relevance [43]. Established CTC lines from CRC patients will be really helpful to investigate drug resistance as well as in future might facilitate new drug development. Nevertheless, the low success rate of CTC lines generation, the rarity of metastasis initiating cells frequency within CTCs pool, the lack of specific CSCs markers and the divergence between murine and human microenvironments hamper the necessity to optimize the *ex vivo* culture conditions of these cells. Further studies exploring the tumorigenic potential of single CTCs compared to clusters are also

doi: 10.1586/14737159.2015.1045491

warranted. To date, the role of CTC clusters in colon cancer has been poorly investigated. To this purpose, the cross talk between platelets and CTCs, the effects of specific anti-platelets compounds to block this cross talk, as well as the role of platelets-derived endostatin and TGF-β in CTCs cluster maintenance might represent a fertile ground of investigation.

Five-year view

Cancer research is rapidly moving. Patients are increasingly treated with molecularly targeted approaches aimed to block crucial cancer drivers, based on the genomic characteristics of the primary tumor. In light of the increasing knowledge about tumor heterogeneity, it is clear that single biopsy tumor resection does not accurately reflect the overall portrait of a patient's cancer, thus hampering the search for solid biopsy surrogates that may accurately represent the real-time status of the patient's tumor. In CRC, studies performed using ctDNA and CTCs suggest that in a next future we may use a combination of both as liquid biopsies to provide new insight into targeted-therapy efficacy, metastatic competence and cancer heterogeneity. The use of liquid rather than conventional biopsy might circumvent the spatial bias of sample collection, and the limited access to matched pretreatment and post-treatment tissues. Nevertheless, whether this approach may lead to a positive difference for patient's care is still to be determined with large clinical trials. The continuous plasticity of CTCs, continuously floating between EMT/non-EMT as well as stem-like/non-stem-like state, represents a major obstacle to their identification and targeting for clinical purpose. CTCs heterogeneity and plasticity hamper the use of combination of agents, including blockage of various self-renewal pathways (Wnt, Notch, PTEN and Hedgehog) or new approaches which may force cells to escape EMT induction, thus contributing to the depletion of cells with stem-like/EMT features, such as CD44v6 positive clones. The high degree of heterogeneity described in CTCs also implies that new multimarker assays are necessary to reveal the real diagnostic potential of blood-based tests. Furthermore, innovative imaging technologies should be developed to determine the effects of hypothetical CSC-targeting drugs, since tumor shrinkage cannot be considered a measure for their efficacy. Finally, the development of nanoparticles and nanoformulations to be used for CTCs neutralization is an innovative, appealing tool [44].

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Key issues

- Liquid biopsy might provide 'real time' information on disease burden, shedding light on tumor evolution over time and on its heterogeneity.
- In colorectal cancer (CRC), the enumeration of circulating tumor cells (CTCs) for prognostic purpose needs to be explored in non-metastatic setting, specifically high risk stage II CRC patients, where the role of adjuvant therapy is debated.
- In metastatic setting a comparison between the 'all-RAS' mutational status of primary tumor and that of liquid biopsy is potentially useful to follow cancer evolution as a 'moving target'.
- The finding of CD44v6 positive CTCs in bloodstream of metastatic CRC patients supports the hypothesis that the CTC population contains CSCs, and lightens the possibility of an easily reproducible liquid biopsy to develop new CCSC-targeted drugs in CRC.
- CTC clusters are described to travel into the bloodstream 'protected' by platelets, which are believed to be powerful activators of epithelial–mesenchymal transition in CTCs through TGF-β and PDGF pathways.
- The analysis of circulating clusters in metastatic CRC through liquid biopsies might allow us to explore a new role of aspirin and anti-platelet drugs as modulators of epithelial–mesenchymal transition features in CTCs.

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