PERSPECTIVAS

CIRCULATING TUMOUR CELLS: A PORTUGUESE CONTRIBUTION TOWARDS PRECISION MEDICINE

CÉLULAS TUMORAIS CIRCULANTES: CONTRIBUIÇÃO PORTUGUESA PARA A MEDICINA DE PRECISÃO

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ABSTRACT

In the context of cancer, liquid biopsy refers to the capture and subsequent analysis of tumour material, such as circulating tumour cells (CTCs), circulating tumour nucleic acids and tumour-derived extracellular vesicles, present in the blood of patients with cancer, or even in other body fluids. CTCs are shed from primary tumours or metastatic sites and have a short half-life in circulation, therefore providing information about the biology of cancer in real time and holding great potential as a biomarker for cancer diagnosis, management, and prognosis. As a result, several technologies have been developed over the years in order to efficiently capture these cells with the ultimate goal of revolutionizing cancer assessment. A great focus is deserved on microfluidic-based approaches for CTC isolation, as they provide unprecedented sensitivity and purity, while keeping low cost. In this article, we discuss the huge impact that CTCs could have in oncology and ultimately in precision medicine regarding its greatest advantages against other circulating biomarkers, but we also consider its main limitations and current challenges to be implemented into the clinic.

Keywords: *Circulating tumour cells, CTCs, Cancer, Metastasis, Liquid Biopsy.*

RESUMO

No contexto do cancro, a biopsia líquida é uma metodologia que se baseia na captura e análise de material de origem tumoral, tal como células tumorais circulantes (CTCs), ácidos nucleicos e vesiculas extracelulares, que se encontram em circulação no sangue de doentes com cancro, ou até mesmo noutros fluídos corporais. As CTCs são libertadas pelo tumor ou por lesões metastáticas, permitindo a obtenção de informação em tempo real sobre a biologia do cancro, conferindo-lhes um grande potencial para se tornarem biomarcadores úteis para o diagnóstico, gestão e prognóstico do cancro. Nos últimos anos, várias metodologias têm sido desenvolvidas com vista à captura eficiente destas células. Em particular as metodologias baseadas em microfluídica têm merecido especial atenção, uma vez que permitem obter elevada sensibilidade e pureza a baixo custo. Neste artigo, discutimos o grande impacto que as CTCs podem ter, não apenas na oncologia clínica, mas em última instância na medicina personalizada salientando as vantagens que as destacam comparativamente a outros biomarcadores circulantes. Temos, ainda, em consideração as suas principais limitações e atuais desafios à sua implementação na clínica.

Palavras-chave: *Células tumorais circulantes, CTCs, Cancro, Metástases Biopsia Líquida.*

Over the past years, liquid biopsy has emerged as a promising diagnostic and prognostic tool in cancer, since it allows a better insight into tumour heterogeneity through a non-invasive type of sampling. This biopsy refers mainly to the extraction of a peripheral blood sample from a cancer patient and the subsequent isolation of a diversity of tumour-associated components, such as circulating tumour cells (CTCs), circulating nucleic acids and tumour-derived extracellular vesicles that are released from the primary or metastatic tumours into the bloodstream[1]. There are several sources of nucleic acids that can be found in cancer patients' blood including circulating cell-free DNA (cfDNA) and cell-free RNA (cfRNA), despite not being necessarily directly released from the tumour, a subset of these represent circulating tumour DNA (ctDNA), which is tumour-derived fragmented DNA.

CTCs, presented in the blood as cells or clusters, are viable non-hematological cells with malignant features and short life-span that are ultimately responsible of the metastatic process. Metastasis is however an extremely inefficient process and most CTCs die once in the bloodstream by suffering apoptosis, due to the action of the innate immune system, shear forces or even oxidative stress. However, those that survive are the most interesting ones due to their ability to escape the immune response, resist the harsh conditions, move to a distant location, undergo mesenchymal-to-epithelial transition (MET) and adapt, colonize and survive in the new tissue-specific microenvironment, where they finally grow to form metastasis[1].

Despite the CTCs and their role has been known since the nineteenth century, to date the capture of CTCs remains technically challenging due to the very low concentration of these tumour cells in the background of millions of blood cells. Therefore, extremely sensitive, and specific analytic methods are required first for the isolation/capture, and then for the enumeration or characterization of these cells[2]. Different approaches for CTC isolation have been reported and they can be mainly classified according to physical or biological properties of the CTCs[3]. CTC isolation methods based on physical properties are label-free and rely either on cell size, density, electric charge, or deformability to allow efficient separation from blood cells. On the other hand, technologies based on biological properties include mainly recognition of proteins expressed in the cell-surface and involve immunologic procedures to enrich the cellular fraction of the sample[4].

Up until now, the CellSearch® system is the most widely used technology for CTC isolation and enumeration and the only method approved by the FDA in the context of metastatic breast, prostate and colorectal cancer. It operates through immunomagnetic antibodies against the protein EpCAM, which is expressed in the membrane of epithelial CTCs, but not in blood cells. Then a cross characterization is performed by staining the captured cells to demonstrate the presence of the nucleus and by immunofluorescence analysis with antibodies against cytokeratin (CK) and CD45. Hence, in order to be considered a CTC, a cell must have a nucleus, possess an intact membrane, be positive for CK – proving its epithelial origin-,and negative for CD45 to be excluded as a blood cell[5]. Nonetheless, there are some limitations regarding the CellSearch® system that are related to the EpCAM dependent-enrichment, since this protein is downregulated in CTCs during epithelial to mesenchymal transition, the process that enables cells with increased plasticity and capacity for migration and invasion[5]. In addition, not all cancers have an epithelial origin and, in those, EpCAM is not expressed, therefore other cell phenotypes will not be detected, including mesenchymal and EMT CTCs. This limitation in combination with necessary sample pre-processing steps, accounts for significant cell loses, which results in low CTC capture efficiency using this system[6].

In order to improve CTC capture and identification, several techniques have been developed in the past years. Still, microfluidic-based approaches lead the technological advancements in the field, allowing the separation of CTCs from other blood components mainly based on their physicochemical

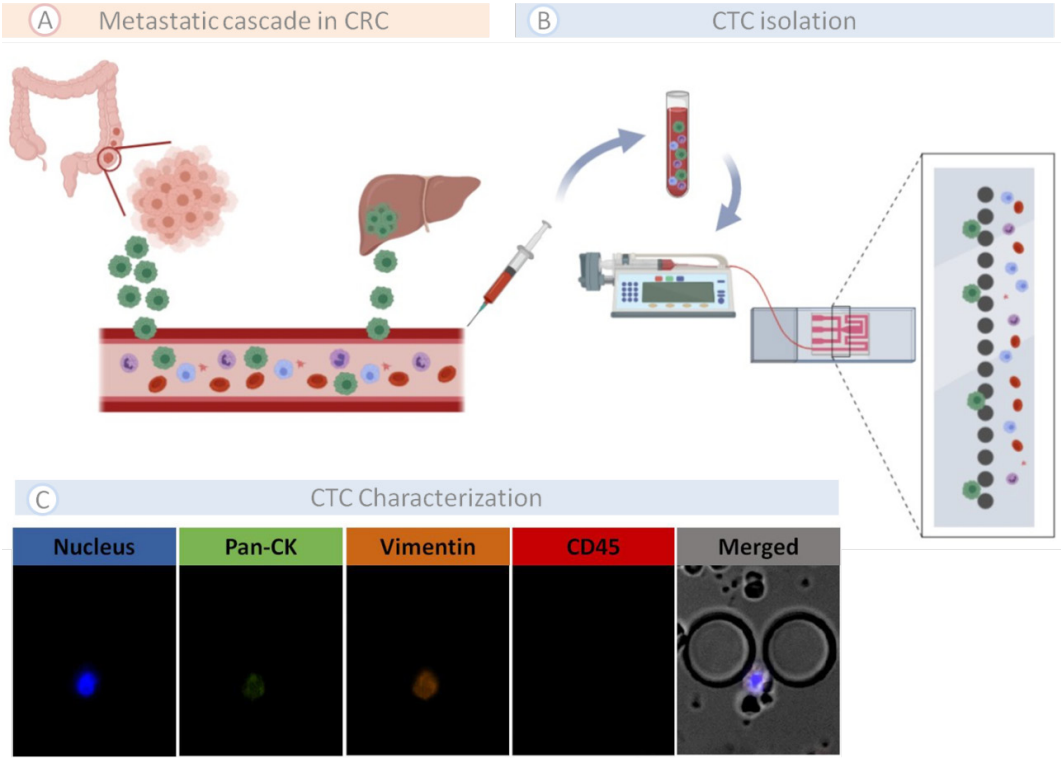

Figure 1 – ***A) Metastatic cascade in colorectal cancer. Cancer cells from the primary tumour travel though the bloodstream to distant organs. Circulating tumour cells (CTCs) must be able to evade the innate immune response, adapt to the microenvironment and initiate proliferation at the secondary site in order to develop a metastatic lesion. Metastatic tumours can also shed CTCs into circulation. B) Peripheral blood is an ideal source for the detection of CTCs. Size-based microfluidic CTC isolation has recently demonstrating its advantages compared to standard methodologies and has the potential to lead new developments to demonstrate the clinical utility of CTCs. C) Immunocytochemistry protocols can be optimised to discriminate the cells trapped inside the microfluidic device. CTCs are classified as intact nucleated cells (DAPI-blue) that express CK (green) and do not express CD45 (red).*** Figures were created with BioRender.com

characteristics, often without the need of prior sample processing, and enabling either *in situ* analysis or recovery of an enriched cell fraction for downstream molecular studies. Moreover, microfluidics provides many other significant advantages, enabling a cost-effective, simple, and automated operation, while reducing sample and reagent demands to carry out highly sensitive cell isolation as well as allowing miniaturization and portability. Many microfluidic devices recently developed are currently undergoing validation with promising results, and actually some of them have already entered clinical trials.

The study of CTCs in Portugal is very recent. From the over 22,000 original research articles published on the topic worldwide since the year 2000 (according to the Web of Science), only 11 had authors from Portuguese institutions. Of those, only 9 indeed studied CTCs from patients, and the rest where proof-of-concept studies using animal models or cell lines. From these 9 articles, 4 focused in molecular analysis of the genetic material extracted from the white fraction and another one used standard flow cytometry to study CK+ cells in blood with very poor efficiency[7]. The remaining 4 publications are from the last 4 years and coming from our groups, and demonstrate novel techniques for the isolation and analysis of circulating tumour cells.

These recent efforts in Portugal were devoted to the development of a microfluidic device that allows efficient isolation of viable CTCs based on their size and deformability from an unprocessed blood sample, and posterior identification *in situ*, using

antibodies against CK and CD45[8]. Since the isolation is independent of surface markers, this methodology allows the incorporation of other biomarkers to identify CTC subpopulations, for example the ones undergoing EMT or expressing other phenotypes, such as altered glycosylation. Until know, using this technology, the authors were already able to identify CTCs in samples from bladder, colorectal, gastric, pancreatic, esophageal and head and neck cancer patients (Figure 1)[8,9,10]. The technology was also compared against the gold standard in a head-to-head study, demonstrating superior performance of the microfluidic system in a small cohort of metastatic colorectal cancer patients, detecting more CTCs and consequently predicting recurrence 6-months to 1-year earlier[8].

Several studies have already demonstrated the prognostic value of CTCs, as their presence and number has been correlated with disease stage, reduced progression-free survival (PFS) and overall survival (OS). A number of studies that enumerated CTCs at different time points during treatment in distinct types of cancers, showed that an increase in CTC counts predicted disease progression, while a reduction correlated with improved PFS and OS[11,12].

The potential of CTCs is, however, far beyond prognostic. CTCs have been found to be an efficient biomarker in (i) patient screening and stratification, (ii) guiding treatment decisions, (iii) predicting relapse, and (iv) predicting therapy resistance in individual patients based on their particular molecular signature much earlier than conventional technologies[10]. To enhance the clinical value of CTCs as biomarkers to identify therapeutic targets, downstream analysis of the CTCs may be required. Molecular and phenotypic characterization of CTCs will allow an earlier and more accurate interpretation of the patients' response to therapy, by providing a non-invasive approach for tumour profiling in real time. This procedure can be repeated regularly during treatment to monitor the acquisition of new genetic alterations in response to the pressure of treatment[8]. In contrast with ctDNA that originates from necrotic and apoptotic cells, CTCs provide mutational information from live resistant cells, and also allow multi-omics analysis (transcriptomics, proteomics, metabolomics, etc) and functional studies. Furthermore, single-cell analysis of CTCs also opens the possibility to find resistant clones that could be responsible of metastasis-initiation. The analysis of these clones would be an enormous step towards understanding the metastatic process and, at the same time, open a new opportunity for clinicians to use alternative treatment strategies that might prevent the expansion of resistant clones. Finally, and most importantly, designing personalised therapeutic strategies for each patient, based on CTC analysis, would minimise the exposure to inefficient therapies and their potentially harmful side effects, increasing patient compliance and quality of life.

In parallel, since CTCs are shed from different heterogeneous sites within the tumour, and/or from different metastases, their analysis provides real time information about the biology of the cancer. In this sense, the development of personalised CTC-derived 3D models holds great promise for the development and screening of drugs[11,14]. To be able to perform functional studies with the CTCs upon their isolation, it is imperative for the cells to be viable after the recovery process. In this line, several studies have been reported to successfully expand CTCs in culture, establishing cell lines and also implanting xenografts in animal models. This field opens new perspectives for future *in vitro* and *in vivo* drug testing studies, including the supervision of the dynamic patterns of a tumour's drug susceptibility. New therapeutic targets could also be screened in a patient-derived model, revealing the molecular basis of drug response or resistance. With the fast development of this field, in the near future, CTCs could be used as pharmacological markers allowing physicians to design rational combinational therapies, select optimal doses, schedule antineoplastic drug administration, and ultimately predict treatment outcomes[14,15,16].

Besides the validity of CTCs for the real time evaluation of metastatic lesions and the potential

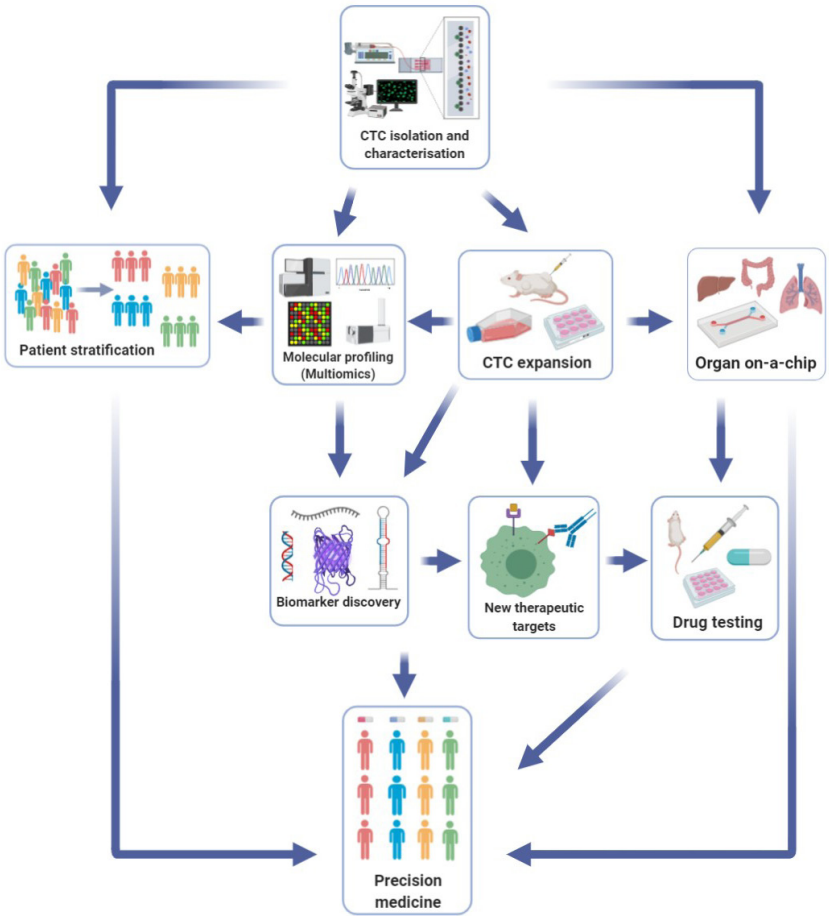

Figure 2 – ***CTC analysis is a multimodal diagnostic tool that enables precision medicine in cancer. This flow chart shows the combination of diagnostic tools and therapeutic strategies that derive from CTC isolation and characterisation and that will ultimately enable the realisation of precision medicine.***
Figures were created with BioRender.com

clinical utility as a monitoring tool during systemic therapy, liquid biopsy also shows potential in an early setting, as CTCs have been reported to be in circulation during primary disease[17]. In this setting, the analysis of CTCs could provide primary diagnosis in tumours where tissue biopsy is difficult, unfeasible or inaccessible[15]. Still, to undercover the full potential of CTCs and e include them into clinical routine, further technological advances are still required, important to achieve low-cost and high-throughput CTC isolation and characterisation systems that present minimal inter-user and inter-laboratory variability[18].

Real-time liquid biopsies have the potential to become, in the near future, an important landmark of precision medicine. The well-known cancer heterogeneity means that each patient is unique, and that two patients with the same condition, sharing the same tumour localization, histopathological classification and stage, can still present different outcomes and response to treatment. This is due to the fact that each cancer cell has different characteristics in terms of metabolism, mutational burden, gene expression and regulation and also protein translation[16]. Since each patient is different, the choice of treatment should be individual, with the ultimate aim of achieving an effective therapeutic outcome through the application of precision medicine (Figure 2).

Although there is extensive literature showing the prognostic value of CTCs and their clinical validity, their clinical utility has not yet been demonstrated and, consequently, its assessment is not yet recommended in cancer guidelines neither for diagnosis, nor to influence treatment decisions. There are many reasons for this lack of consensus, among them a lack of a standardization on sampling

frequency[11]. Generally, single or infrequent CTC sampling points are applied always assuming that changes in CTC counts are gradual, according to the success of the therapy applied, however CTC kinetics are not yet fully understood[11]. In order to elevate the knowledge in this area we are preparing several prospective studies in various cancer models, such as digestive tract (colorectal, gastric, esophageal and pancreatic cancer) and sarcomas.

Notwithstanding the limitations currently being addressed, CTCs appear as one of the most promising and versatile biomarkers in translational oncology. Once the scientific community is able to overcome the technical limitations associated to the CTC field, CTC-based liquid biopsy may emerge as an alternative to tissue biopsy with the ultimate goal to be incorporated into disease management strategies. In the meantime, perhaps we should benefit from liquid biopsy as a complementary approach to be used in conjunction with tissue biopsy, radiologic imaging, serum tumour markers, and clinical assessment, for real-time monitoring of disease status and therapeutic efficacy[11].

REFERENCES

(1) Chen, L., Bode, A. M., & Dong, Z. (2017). Circulating tumor cells: Moving biological insights into detection. Theranostics, 7(10), 2606–2619.

(2) Pantel, K., & Alix-Panabières, C. (2013). Real-time liquid biopsy in cancer patients: Fact or fiction? *Cancer Research*, *73*(21), 6384–6388.

(3) Zou D, Cui D. Advances in isolation and detection of circulating tumor cells based on microfluidics. *Cancer Biol Med*. 2018;15(4): 335-353. doi:10.20892/j.issn.2095-3941.2018.0256

(4) Alix-Panabières, C., & Pantel, K. (2013). Circulating tumor cells: Liquid biopsy of cancer. Clinical Chemistry, 59(1), 110–118.).

(5) Wisha, M. & Hayes, D. (2011). Circulating Tumour Cells: not all detected cells are bad and not all bad cells are detected. Journal of Clinical Oncology, 29(12), 1508-1511.

(6) Andree, K. C., van Dalum, G., & Terstappen, L. W. M. M. (2016). Challenges in circulating tumor cell detection by the CellSearch system. Molecular Oncology, 10(3), 395–407.

(7) Tralhao, J. G.; Hoti, E.; Serodio, M.; Laranjeiro, P.; Paiva, A.; Abrantes, A. M.; Pais, M. L.; Botelho, M. F.; Sousa, F. Castro. (2010). Perioperative tumor cell dissemination in patients with primary or metastatic colorectal cancer. 36 (2), 125-129. 10.1016/j.ejso.2009.07.003.

(8) Fast and efficient microfluidic cell filter for isolation of circulating tumor cells from unprocessed whole blood of colorectal cancer patients. S. Ribeiro-Samy, M. Oliveira, T. Pereira, L. Muinelo, J. Gaspar, P. Freitas, R. López, C. Costa, L. Diéguez. Scientific Reports 9, 1-12 (2019)

(9) Lima, L., Neves, M., Oliveira, M. I., Dieguez, L., Freitas, R., Azevedo, R., … Ferreira, J. A. (2017). Sialyl-Tn identifies muscle-invasive bladder cancer basal and luminal subtypes facing decreased survival, being expressed by circulating tumor cells and metastases. *Urologic Oncology: Seminars and Original Investigations*, *35*(12), 675.e1-675.e8. https://doi.org/10.1016/j.urolonc.2017.08.012

(10) Exploring sialyl-Tn expression in microfluidic-isolated circulating tumour cells: a novel biomarker for precision oncology applications. Neves, M.; Azevedo, R.; Lima, L.; Oliveira, M.; Peixoto, A.; Ferreira, D.; Soares, J.; Fernandes, E.; Gaiteiro, C.; Palmeira, C.; Cotton, S.; Santos, M.; Mereiter, S.; Campos, D.; Afonso, L.; Ribeiro, R.; Fraga, A.; Tavares, A.; Mansinho, H.; Monteiro, E.; Videira, P.; Freitas, P.; Celso, R.G.; Santos, L.; Diéguez, L.; Ferreira, J. A. New Biotechnology, 2019, 49, 77-87.

(11) Leong, S. M., Tan, K. M. L., Chua, H. W., Tan, D., Fareda, D., Osmany, S., … Koay, E. S. C. (2015). Sampling circulating tumor cells for clinical benefits: How frequent? *Journal of Hematology and Oncology*, *8*(1), 1–8.

(12) Abalde-Cela S, Piairo P, Diéguez L: The Significance of Circulating Tumour Cells in the Clinic. Acta Cytologica 2019;63:466-478. doi: 10.1159/000495417

(13) Huang, M. Y., Tsai, H. L., Huang, J. J., & Wang, J. Y. (2016). Clinical implications and future perspectives of circulating tumor cells and biomarkers in clinical outcomes of colorectal cancer. Translational Oncology, 9(4), 340–347.

(14) Yang, C., Xia, B. R., Jin, W. L., & Lou, G. (2019). Circulating tumor cells in precision oncology: Clinical applications in liquid biopsy and 3D organoid model. *Cancer Cell International, 19(1), 1-13.*

(15) Mader, S., & Pantel, K. (2017). Liquid biopsy: Current status and future perspectives. *Oncology Research and Treatment*, *40*(7–8), 404–408.

(16) Werner, S., Stenzl, A., Pantel, K., & Todenh, T. (2017). Isolation and Molecular Characterization of Circulating Tumor Cells. In *Advances in Experimental Medicine and Biology* (Vol. 994).
(17) Tanaka F, Yoneda K, Kondo N, et al. Circulating tumor cell as a diagnostic marker in primary lung cancer. Clin Cancer Res 2009;15:6980-6.
(18) Alix-Panabières, C., & Pantel, K. (2016). Clinical applications of circulating tumor cells and circulating tumor DNA as liquid biopsy. *Cancer Discovery*, *6*(5), 479–491.

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Data de recepção do artigo:
15/06/2020

Data de aceitação do artigo:
26/06/2020