As the prognosis of colorectal cancer (CRC) patients is mostly determined by the occurrence of distant metastases, circulating tumor cells (CTC) may be an attractive therapeutic target. Despite the rarity of CRC-derived CTC, considerable progress has recently been made in the investigation of their molecular characteristics. In this review article we present recent clinical and molecular data on CRC-derived CTC, discuss their implications and propose a new phenotypic state of CTC, the immune-evasive state (IES).


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Introduction

Colorectal cancer (CRC) is the third most common malignancy and among the leading causes of cancer-related death in western countries [1, 2]. While the primary tumor can in most cases be easily controlled, it is usually distant metastasis which ultimately limits the patients’ prognosis [3, 4]. The cellular correlate of the process of metastasis are circulating tumor cells (CTC), which actively leave the tumor, survive in circulation and then actively invade into distant organs, where they proliferate and form new tumors [5]. The phenotypic requirements for this multistep process are striking: In order to successfully metastasize, bulk tumor cells initially need to undergo epithelial-mesenchymal transition (EMT) to acquire invasive capabilities [6]. Once they have entered the blood stream, CTC must evade identification and elimination. The final steps of metastasis are attachment to the endothelium, invasion of the parenchyma of the target organ, mesenchymal-epithelial transition (MET) and, ultimately, proliferation.

During the past months, several groups have published data about genomic, genetic and transcriptomic analyses of CRC-derived CTC. In the following, we will review current CTC detection methods, introduce the new data and subsequently discuss their implications. Also, in addition to EMT and MET, we will propose a potential third phenotypic state of CTC, the immune-evasive state (IES).

Methods of CTC detection

CTC detection, enrichment and isolation can be achieved by exploitation of CTC-specific traits; either biological properties such as surface markers or physical properties such as size, density or stiffness. Negative selection alone (e.g., CD45 depletion) is suitable for CTC enrichment; however, in tumor entities with only few CTC such as CRC, negative depletion results in CTC numbers
that are still much smaller than the contaminating leukocyte fraction.

Prior to the availability of the CellSearch System, CTC detection was mainly achieved by CK20 PCR \cite{7,8}; however, this method is unable to distinguish between intact and viable tumor cells and tumor cell fragments. The current gold standard and only FDA-approved method of CTC detection in CRC is the CellSearch System, which has been shown to reliably detect CTC with prognostic relevance with a very low rate of false-positive results \cite{9}. This detection system is based on an initial step of immunomagnetic enrichment of EpCAM$^+$ cells; thus missing detection of EpCAM$^-$ CTC. In subsequent steps, cells are stained for other epithelial markers (cytokeratin 8, 18, 19) and CD45 as a leukocyte marker. Only cells with a DAPI$^+$ nucleus are considered CTC. The CellSearch System is highly standardized and reproducible; also, it has been extensively validated and has repeatedly shown its predictive and prognostic value in colorectal cancer \cite{10-12}. However, as tumor cells presumably undergo EMT during the metastatic cascade, they may lose their EpCAM expression \cite{13}. Therefore, EpCAM$^+$ CTC measured by the CellSearch System may only represent bystander CTC which coincide with the actual metastasis-inducing cells that have lost their EpCAM expression during EMT. Another disadvantage of the CellSearch System is a result of the cytokeratin staining, which requires permeabilization of the putative CTC; downstream analyses requiring intact or even viable cells (e.g. expression profiling, culture, retransplantation) are not possible with CellSearch-isolated CTC.

Other EpCAM-based methods include microfluidic devices \cite{14-16}, a magnetic sweeper device \cite{17}, three-dimensional nano structures \cite{18} or manual isolation \cite{19}; however, all EpCAM-based methods share the common disadvantage to be unable to detect EpCAM$^-$ CTC.

Distinct physical properties of CTC such as larger size and decreased deformability as compared to circulating leukocytes \cite{20} may allow label-free CTC isolation and are exploited in numerous novel CTC isolation technologies. Excellent overviews about those technologies have already been published \cite{21, 22}. Generally, such methods yield higher numbers of CTC than EpCAM-based methods \cite{21}; however, the clinical and prognostic relevance of such CTC remains unclear.

Figure 1. Putative mechanism of tumor cell dissemination. Bulk epithelial tumor cells undergo EMT to acquire a mesenchymal phenotype in order to invade blood vessels. Upon intravasation, the tumor cells shift their phenotype to an immune-evasive state (IES) to be able to cloak from immune cells. The tumor cells attach to the epithelium of the target organ and again acquire an invasive phenotype to enter the target organ. After extravasation, the cells undergo MET and proliferate as epithelial tumor cells.
Clinical significance of CTC in CRC

Many CTC studies showed controversial results due to the lack of standardized detection methods. With the introduction of the CellSearch System, a standardized and FDA-approved detection method became available, and most subsequent studies employed this technique to detect and enumerate CTC in CRC studies.

The clinical relevance of Cellsearch-detected CTC in patients with metastatic CRC has initially been demonstrated by Cohen and colleagues \[10, 11\]. In these analyses, patients with metastatic CRC were divided into patients with <3 CTC / 7.5 mL of blood and patients with ≥3 CTC / 7.5 mL. The authors clearly demonstrated that patients with favorable baseline CTC numbers showed significantly longer PFS and OS than patients with unfavorable (≥3 CTC / 7.5 mL) CTC numbers. In addition, patients who showed a decrease in CTC counts during chemotherapy also had a significantly longer PFS and OS than patients whose CTC counts did not respond to chemotherapy. These data demonstrate that CTC have both prognostic and predictive value in metastatic CRC. The predictive value of CRC-derived CTC has subsequently been validated by several follow-up studies; stable or increasing CTC numbers during systemic therapy are generally associated with poor response to therapy \[23, 24\].

In a recent meta-analysis, we definitively demonstrated the prognostic relevance of CRC-derived CTC: CTC detection is associated with poor recurrence free [HR = 3.24 (95%CI: 2.06-5.1)] and overall survival [HR = 2.28 (95%CI: 1.55-3.38)] \[25\]. In a prospective study including 200 patients we also demonstrated significantly higher CTC counts in the mesenteric venous blood compartment as compared to the central venous blood compartment \[26\]. This finding strongly supports the theory of continuous CTC shedding from the primary tumor into the bloodstream as well as the theory of the liver acting as a filter for CTC, a putative reason for the liver as the most common site for CRC metastases \[27\].

Molecular characteristics of CRC-derived CTC

Due to their rarity and resulting technical difficulties in molecular examination methods, the biology of CRC-derived CTC is still largely unknown.

We recently performed a study which demonstrated a genomic and mutational profile of CTC which is well in line with the profile of colorectal tumor cells and thus proved the CRC origin of the isolated cells. Interestingly, the genetic profile of CTC in comparison with corresponding tumor tissue differed in a few details which may be clinically relevant: We observed mutational differences in the KRAS gene, in particular KRAS mutant CTC in patients with KRAS wild-type tumors. These findings concur with findings from other groups, which have also observed mutational discrepancies between primary tumor and CTC \[28, 29\]. However, upon ultra-deep sequencing of the tumor, the mutations which were initially detected in CTC were also found in the tumor, thereby indicating genetic heterogeneity within the tumor bulk. CTC genotyping may thus be a valuable addendum to tumor genotyping prior to clinical decision making as CTC genotyping may increase the sensitivity and specificity of tumor genotyping.

As CTC require migratory and invasive capacities in order to actively leave the tumor, they presumably acquire mesenchymal traits; a process called epithelial-mesenchymal transition (EMT) \[5\]. During EMT, the phenotype of tumor cells shifts from epithelial to mesenchymal; a process which is reversed during mesenchymal-epithelial transition (MET) \[30, 31\]. MET happens after invasion of target organs such as the liver and results in metastases which are phenotypically quite similar to the primary tumor \[32\]. As the phenotypic changes required for successful metastasis must therefore be reversible and based on the RNA level, we have conducted experiments in which we isolated EpCAM+ CTC from CRC patients and performed an array of RT-qPCR tests to determine the expression profile of CTC \[13\].

Interestingly, we observed CTC to downregulate the expression of epithelial markers such as EPCAM (P = 0.027), CK19 (P = 0.012) and CEA (P = 0.002). This is well in line with the EMT theory as epithelial markers are supposedly downregulated during the mesenchymal phase of tumor cell dissemination. Surprisingly, migration-associated genes such as fibronectin (FN1, P = 0.047), CD44v6 (P = 0.027), CD151 (P = 0.062) and TSPAN8 (P = 0.064) were also downregulated in CTC. This may be explained by the time point in the metastatic cascade at which the cells were isolated: once CTC have left the tumor and entered the circulation, they do not require migratory capabilities before they attach to the epithelium in the target organ. As the RNA expression profile is very volatile, the putative overexpression of migration-regulated genes during invasion into the blood stream may be lost quickly after entering the circulation.

A question of continuous debate is why the immune system fails to detect and clear all putative circulating tumor cells from the blood stream. Generally, the blood is a hostile environment for CTC. From the millions of CTC shed into the blood stream every day \[33\], only few cells will survive and remain detectable. The expression profile data we generated from CRC-derived CTC may improve the understanding of the immune-escape techniques of CTC: We observed a significant upregulation of CD47 (P = 0.039), a “don’t-eat-me” signal preventing immune cells such as NK cells or monocytes/macrophages from killing...
CTC [34]. Concurrently, calreticulin (CALR), a “chaperone” protein expressed on damaged cells which stimulates immune cells to kill the damaged cells, was significantly downregulated on CTC (P = 0.001) [35]. Of note, CD47 was the only upregulated gene in CTC, which generally exhibited down regulation of all other examined genes including Ki-67 and c-Myc. This largely dormant state with low expressions of both epithelial and mesenchymal markers may represent the prevalent phenotypic state between EMT and MET which we call immune-evasive state (IES, Fig. 1).

Conclusion and future perspectives

Although the biology of CRC-derived CTC has become clearer during the past months, the exact mechanisms enabling CTC to eventually form distant metastases are still unclear. On the DNA level, it has been shown that CTC genotyping is feasible and may add valuable information to classical tumor genotyping. As the tissue sample used for tumor genotyping can only depict the genome of the biopsied region of the tumor, the genotyping results do not necessarily represent the clinically most relevant cell populations. As CTC are the direct correlate of metastatic activity, CTC genotyping may increase the sensitivity of tumor genotyping in the search for therapy-changing mutations. For example, it may be futile to treat a patient with a KRAS wild-type tumor but KRAS mutant CTC with EGFR inhibitors as the clinically most relevant tumor cell subpopulation has a constitutively active RAS signaling pathway and is thus resistant to the treatment. However, the information acquired by CTC genotyping needs to be further validated in large clinical trials before triggering any therapeutic and clinical decisions.

Expression profiling data have shown that CTC are a rather heterogeneous population of cells; only a fraction of cells will undergo the phenotypic changes depicted in Fig. 1. The phenotype of the actual metastasis-inducing cell population has yet to be determined; a recent publication about breast cancer-derived CTC indicates that the phenotype still needs to be narrowed down drastically [36]. In addition, the time point of CTC isolation may define the phenotype of the tumor cells. Cells that have newly invaded the blood stream may still exhibit a mesenchymal phenotype as they were just required to migrate towards and break into blood vessels. After some time in circulation, we speculate the cells exhibit an immune-evasive state (IES), whereas shortly prior to extravasation and invasion into the target organ, the cells will again acquire mesenchymal capabilities.

The role of EpCAM CTC remains unclear. Our data clearly indicate a loss of epithelial markers including EpCAM in CTC. However, as the detection method was based on EpCAM, EpCAM CTC were not analyzed and their phenotype remains therefore unknown. As EpCAM CTC have undergone EMT to the largest extent, they may exhibit an increased phenotypic plasticity, thus being able to better adapt to the environment in the blood stream and the new host organ. The metastasis-inducing capacity of EpCAM CTC may therefore be higher than that of EpCAM+ CTC; however, further studies about this highly interesting CTC subpopulation are required.

The ultimate goal of CTC phenotyping efforts remains the identification of a therapeutic target, enabling oncologists to effectively prevent the occurrence of distant metastases and tumor recurrence in early-stage patients.

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Conflict of interests

The authors declare no possible conflicts of interest.

References


