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Isolation and Enumeration of Circulating Tumor Cells (CTC) as Prognostic and Predictive Biomarkers in Post-Operative m-CRC

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ABSTRACT

Circulating Tumor Cells (CTCs) presents non-invasive, repeatable investigation of patient's disease. In metastatic Colorectal Cancer (m-CRC) patients, CTC enumerations have been comprehensively studied in evaluating metastatic disease. CTC analysis has been shifting from enumeration to more sophisticated molecular depiction of tumor cells, which is used for liquid biopsy of the tumor, reflecting cytological and molecular changes in metastatic patients over time. In this study, CTC enumeration in advanced and localized metastatic colorectal cancer, highlights the vital gains as well as the challenges posed by various approaches, and their implications for advancing disease management.

Detection of circulating tumor cells (CTC's) or circulating free tumor DNA (ctDNA) to conduct chemotherapy and reporting prognosis is extremely important, In view of the detail CTC has the potential to offer multiple samples by way of sequential minimally-invasive liquid biopsies. In fastidious, there is escalating evidence for the efficacy of CTC's in the clinical management of metastatic colorectal cancer (CRC). With most studies confirming the association of elevated CTC counts with worse prognosis.

CTC's were first identified by Ashworth in 1869. CTC research has been vulnerable by the failure to constantly detect these typical cells. While the normal range of WBCs in human blood is 4.5-119/L, there may only be a few CTC's. The most widely used CTC enumeration platform, Cell-Search (Veridex LLC, NJ, USA) was approved for clinical use. As treatment options expand for metastatic colorectal cancer (mCRC), a blood marker with a prognostic and predictive role could guide treatment.

Investigations were carried out that Circulating Tumor Cells (CTCs) could predict clinical prognosis in patients with mCRC. This pilot study, demonstrates that CTCs can serve as both prognostic and predictive factor for patients with mCRC. The presence of at least three CTCs at baseline and follow-up is a strong independent prognostic factor for inferior PFS and OS. When utilized in combination with imaging studies, CTCs provide additional prognostic information. There are several studies for which CTCs could have efficacy inmetastatic colorectal cancer.

The statistics suggests that CTCs may be used as a stratification feature in metastatic disease treatment trials. The current list of validated prognostic factors is short, with only routine status being universally recognized. Further study should prospectively deal with modification of regimens based on unfavorable CTCs early in the course of treatment will result in enhancement in PFS or OS. As treatment has become more effective for metastatic disease, decision making has become more complicated. Five classes of drugs are on hand for treatment. The most common initial chemotherapy is a fluoropyrimidine with oxaliplatin or irinotecan. CTC levels drawn at 3 to 5 weeks and 6 to 12 weeks, before PET imaging, may lead to prospective regimen choices and standby patients from unnecessary drug toxicity by suggesting that an early change in treatment is defensible.

Keywords:

Circulating Tumor Cells (CTC), Metastic Colorectal Cancer (m-CRC), Overall Survival (OS).

Introduction

Metastasis is a progression, involving cellular changes that separate from the primary tumor origin, intra-vasation into circulation and proliferation leading to secondaries. CTCs, cells in pheripheral blood to facilitate are shed from the primary tumor play a important function in the hematogenous spread disease. CTC's are atypical, with more or less one CTC found in every billion normal cells in a subject with metastatic cancer. The best part of CTCs are cleared from circulation; though, some can deposits in the bone marrow, termed spread tumor cells or seed other sites of metastasis. Fascinatingly, CTCs can reseed the primary tumor, expressing factors that guide to

increase tumor proliferation and angiogenesis. CTCs are in form of clusters, potentially represents tumor or product of intravascular proliferation [1-5]. Significance of CTC clusters is imprecise, although trials have shown gene expression profiles when compared to individual CTCs. Histopathology of metastasis is unclear, but promising data of Epithelial Mesenchymal Transition (EMT) being concerned. EMT is the process in which adherent epithelial cells achieve migratory and invasive properties. Through this procedure, cells are competent to rupture through the membrane, separate from the site of tumor, and endure in circulation. The significance of EMT is bolstered by means of information that CTCs demonstrate gains in mesenchymal markers. This course of action can be reversed, termed as mesenchymal epithelial transition. CTCs may bind and proliferate at a metastatic origin. The tumorigenic potential of CTCs has been examined [6-9].

Materials and Methods

CTC Isolation

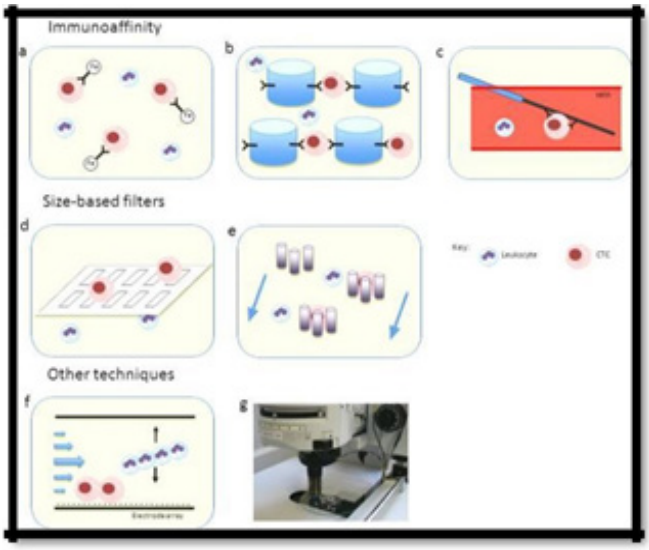


Figure 1: CTC isolation techniques.

Immunoaffinity utilizes EpCAM antibodies

Clinical importance of CTCs are highly dependent upon the procedures used for isolation (Figure 1). Each modality has its advantages and limitations in terms of the sensitivity, purity and ability to carry out further investigations on the cells. Furthermore, as there isheterogeneity among CTCs, different enrichment techniques impact molecular characterization [10-11].

Immunoaffinity is the most frequent method for isolation, utilizes expression of surface markers such as Epithelial Cell Adhesionmolecule (EpCAM). The CellSearch stage labels magnetic beads with antibodies to EpCAM, drawing out cells with a magnet and staining them with 4',6-Diamidino-2-Phenylindole (DAPI) nuclear stain and antibodies to cytokeratins and CD 45. The cells are scanned and sent to a reviewer who categorizes CTCs as cells that are nucleated, positive for cytoplasmic cytokeratin, and negative for CD 45. A recent trial demonstrated the feasibility of an automated scanning algorithm for CTC detection using CellSearch®, an approach which may in time serve as an alternative to human reviewers who can contribute to variability. Immunoaffinity techniques are commonly utilized, allow for reliable enrichment of specific subpopulations and have extensive preclinical and clinical data to support their use. CellSearch is the only method for detecting CTCs in metastatic breast, colon that has been cleared by the Food and Drug Administration (FDA). The major limitation of these techniques is their reliance on cell surface markers, which can have variable or no expression in some malignancies. EpCAM expression, for example, has been shown to be reduced in 20% of m-CRC samples. This limitation is more pronounced in cells that have undergone EMT, which can allow CTCs to escape detection [12-16].

CTC-chip is an immunaffinity technique with improved yield of CTCs. Utilizing a microfluidic platform, blood flows through a chip with 78,000 microposts positioned to maximize exposure to cells while minimizing shear forces on the cells. Using CTC-chip with posts coated with antibodies to EpCAM, CTCs were identified in over 99% of patients with various metastatic cancers, including m-CRC. The CTC-chip proved sensitive in m-CRC, identifying CTCs in seven out of seven men with localized disease. Size-based filters do not rely on cell surface markers but rather on the fact that CTCs are typically larger than other blood cells. Morphometric analyses have demonstrated a spectrum of CTC size depending on the cell line of origin with breast and prostate CTCs typically smaller than CTCs from cervical and liver cancer. All CTCs, however, were significantly larger than white blood cells. Described techniques include filtration through pores, slots or microcrescents. These techniques capture a broader spectrum of cells irrespective of cell surface markers and the cells are not bound by antibodies, helping with further analysis. On the other hand, size-based enrichment has potential drawbacks. CTCs may escape capture due to smaller size; conversely, cells that are captured still require subsequent positive identification using various immunofluorescent stains (e.g., cytokeratins). There are a multitude of other techniques to isolate CTCs. Dielectrophoresis separates cells through an electromagnetic method, taking advantage of cells having different polarisable properties. It has been combined with immunoaffinity assays to work synergistically. High speed laser scanning identifies CTCs after staining and is based upon a cell's morphologic and fluorescence patterns. Another technology, termed NanoVelcro-Chip, utilizes EpCAM antibody-coated silicon wires and has been shown to be easily replicated between different facilities. The addition of laser capture microdissection to this platform allows for pure CTC isolation and sequencing. Still another group utilized a three dimensional tumor cell culture on a microfluidic platform has been shown to successfully culture prostate cancer cells spiked into blood.Lastly, an in vivo EpCAM antibody-coated wire placed in a patient's vein for 30 minutes has shown promising results with regard to CTC capture in m-CRC [17-20].

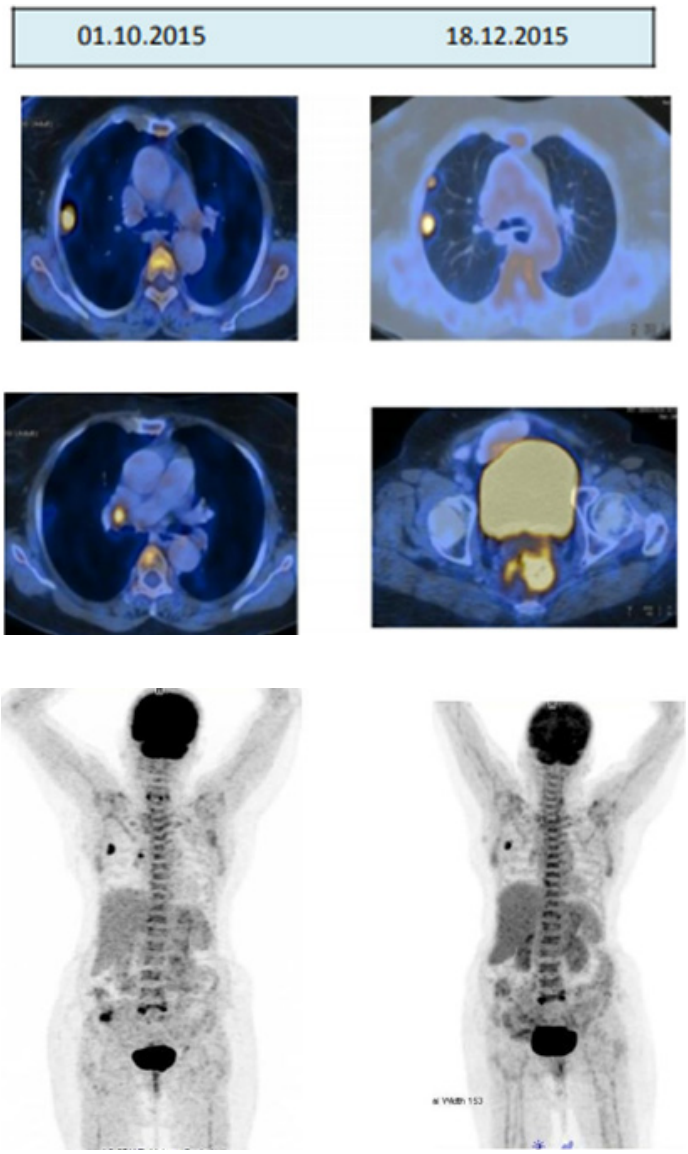
Chemotherapy Infusion

IFL Saltz + Bevacizumab regimen was administered Inj Irinotecan 200 mg, Inj Leucovorin Calcium 35 mg , Inj 5FU 850 mg, Inj Bevacizumab 400mg.

Study Type	Treatment	Detectin Technique	Samples	Prognostic or Predictive Utility	Conclusion
Meta Analysis	Palliative Chemotherapy IFL Saltz Regimen	IHC	Peripheral Blood, Central Venous Blood	Prognostic with PET CT imaging	CTC's associated with worse OS
Prospective Study	Bevacizumab	Cell Search	Peripheral Blood, Central Venous Blood	Prognostic & Predictive in combination with CT Imaging	High Base CTC's

Results

Studies addressing prognostic and predictive value of CTC's m-CRC



Protocol: With the patient fasting for 6 hours, 10mci of FDG was injected intravenously and 3D PET CT scan was performed. Physiological concentration is seen in the heart, gut, brain, kidneys and bladder.

Comparative FDG concentrations (: SUV: as per BW)

- Wall thickening at anastomotic site : SUV: 10.7 Previously 24.4
- Pleural based right lung nodules : SUV: 7.2 Previously 9.8
- Right hilar lymph node : SUV: Nil Previously 8.6
- Nonspecific FDG uptake in L 5 vertebra- due to collapse vertebra.

Discussion

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 favourable 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 [21-22]. 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)] [25-26]. 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].

Our initial report of this clinical trial with 47% of patient deaths recorded and a median follow-up for living patients of 11.0 months demonstrated that CTCs at baseline and follow-up time

points are a strong independent predictor of PFS and OS [6].

The current report extends these analyses with additional follow-up, with 71% of patients having died and a median follow-up for living patients of 25.8 months. The results are remarkably consistent, with a near-doubling in PFS and OS for patients with favorable compared with unfavorable baseline CTCs.

We also conducted an analysis of the impact of CTC baseline levels within patient and clinical subgroups and demonstrated that elevated baseline CTC count is associated with poorer OS in all patient subgroups. PFS was also generally inferior in patients with elevated baseline CTCs, but this finding did not reach statistical significance in all subgroups. However, each subgroup demonstrated at least a trend toward inferior PFS with unfavorable baseline CTCs. An updated multivariate analysis confirmed that baseline CTC count remains an independent predictor of PFS and OS.

These data indicate that CTCs should be considered as a stratification factor for OS infuture mCRC clinical trials regardless of line of therapy. In addition, choice of a particular drug does not affect the impact of CTCs for predicting OS. For all treatment- and patient-related characteristics evaluated, the percent of patients with baseline unfavorable CTCs was similar, and the impact on OS statistically significant. Given the large difference in survival noted between unfavorable and favorable CTC groups for all patient subgroups, it is critical that this factor be balanced when evaluating patient outcome in subsequent clinical trials.

The number of useful stratification factors in advanced colorectal cancer is limited. While an elevated Carcinoembryonic Antigen (CEA) may be a poor prognostic factor for resectable colorectal cancer [9], no data support its prognostic value for patients initiating chemotherapy for metastatic disease. In the last reported gastrointestinal intergroup mCRC study, CEA was not utilized as a stratification factor [10]. Other stratification factors included age (<65 versus ‡65), prior therapy, PS, and treatment location [10]. Our analysis clearly shows that CTCs remain a prognostic factor regardless of age, prior therapy, or PS. Thus, one can argue that the next large mCRC trial evaluating a new systemic therapy should utilize CTCs as a stratification factor for OS. While OS was statistically inferior in all subgroups with unfavorable CTCs, PFS was statistically inferior in many but not all subgroups with unfavorable CTCs. However, the trend in all subgroups was in the expected direction. This is not surprising when considering the utility of baseline CTCs as a prognostic versus a predictive marker. Prognostic markers will yield information about clinical outcome regardless of therapy.

Predictive markers will yield information about the differential impact of a selected therapy on clinical outcome [11]. At the current time, the weight of evidence supports the utility of CTCs as a prognostic marker. Thus, baseline CTCs may reveal relatively less information regarding PFS, depending on which regimen is initially selected. However, over a longer period of clinical follow-up, they are ultimately a strong predictor of survival. In support of this are data from our prior publication demonstrating that among those patients beginning with unfavourable CTCs, conversion to favorable CTCs on treatment results in significantly longer PFS and OS than patients who remain with unfavorable CTCs on treatment [28-30].

Conclusion

In conclusion, the current report with additional follow-up demonstrates that baseline CTC number remains an important prognostic factor for OS regardless of treatment and patient characteristics. PFS was also shorter for patients with unfavorable CTCs in all patient subgroups, reaching statistical significance in many. This can be used as supportive evidence for future evaluations of CTCs in specific patient subgroups as a marker of outcome and treatment effect. Baseline CTC levels should be considered as a stratification factor in future large mCRC clinical trials [31-33].

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 [34-35]. 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 Figure 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 be 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 andV 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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