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Review

The importance of circulating tumor products as `liquid biopsies` in colorectal cancer

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Abstract

Liquid biopsies represent an array of plasma analysis tests that are studied to evaluate and identify circulating tumor products, especially circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA). Examining such biomarkers in the plasma of colorectal cancer patients has attracted attention due to its clinical significance in the treatment of malignant diseases. Given that tissue samples are sometimes challenging to procure or unsatisfactory for genomic profiling from patients with colorectal cancer, trustworthy biomarkers are mandatory for guiding treatment, monitoring therapeutic response, and detecting recurrence.

This review considers the relevance of flowing tumor products like circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), circulating messenger RNA (mRNA), circulating micro RNA (miRNA), circulating exosomes, and tumor educated platelets (TEPs) for patients with colorectal cancer.

Keywords : liquid biopsies, circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), colorectal cancer (CRC), circulating exosomes, tumor educated platelets (TEPs)

Highlights

- ✓ Circulating tumor cells and circulating tumor DNA serve as a promising step forward in the detection and monitoring of colorectal cancer.
- ✓ More extensive research is required to determine the extent to which liquid biopsies would be able to impact the patient diagnosis and therapy

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Introduction

Colorectal cancer represents one of the dominant causes of mortality globally, and through precision medicine it has become one of the most promising areas for cancer therapy (1). Colorectal cancer appears in the colon or rectum, part of the large intestine. The preponderance of colorectal cancers are adenocarcinomas. Most of these cancers develop as a result of aging and lifestyle, with only a negligible proportion determined by genetic factors (2). Included among the risk factors are sedentary lifestyle, smoking, and obesity. Dietary factors, inflammatory bowel disease, and several genetic disorders also play a significant part in the evolution of colorectal cancer (3). Bowel cancer can be diagnosed by medical imaging and biopsy, but these procedures usually detect tumors only in the latter stages of development (2, 3).

In recent years, increasing attention has been given to liquid biopsy, centering mainly on the examination and determination of circulating tumor cells (CTC) and circulating cell-free tumor DNA (ctDNA) because of its relevance to precision medicine. The study of circulating tumor products has opened a new, important and precise pathway to diagnostic medicine. Traditional serum biomarkers are becoming inadequate for therapeutic guidance; because of its non-invasive nature and the amount of information obtained about the tumor, liquid biopsy has been increasingly considered for colorectal cancer diagnosis and therapeutic and recurrence monitoring (4).

For patients with colorectal malignancies, it is always beneficial to diagnose and genotype tumors using blood-based biopsies, but sometimes tumor tissue samples may be insufficient or difficult to procure due to the low quantity of tumor content and given the invasive nature for collecting samples. Liquid biopsies offer substantial information about inter- and intra-tumoral heterogeneity, in addition to revealing dominant mutation and relevant subclones of the tumor burden (5, 6).

This review evaluates the potential of this circulating tumor product to help identify new cancers, reveal recurrences, classify sensitivity, determine resistance, and predict prognosis.

Discussion

- *Accepted detection methods of circulating tumor products*

- **CTCs**

CTC presence was first confirmed 140 years ago (7). CTCs are rare, often as low as one cell in 10.000.000 leukocytes in the blood of cancer patients, so detection technologies are adopted in conjunction with enrichment procedures. The exclusive medical device cleared by the Food and Drug Administration (FDA) for CTC enumeration and selection is the CellSearch System (Veridex, New Jersey, USA).

To use this detection method effectively, CTCs must have the following properties: the staining pattern must be cytokeratin phycoerythrin and the fluorescent stain DAPI (4',6-diamidino-2-phenylindole) needs to be positive; also the leukocyte common antigen CD45 has to be negative, the shape of the cell has to be round with a minimum size of 4um, and lastly, the nucleus ought to be visible (8).

This system also detects a specific protein called antibodies against epithelial cell-adhesion molecule (EpCAM). The EpCAM protein is found exclusively on the exterior surface of the circulating tumor cells. CellSearch System pulls the CTCs out, using a magnetic field, after the EpCam antibodies are previously attached to a magnetic bead (9). But even after taking such effects into consideration, CTCs are rarely used in CRC because of their low numbers (approximately 2 cells per 7,5ml of blood) (10) (Figure 1).

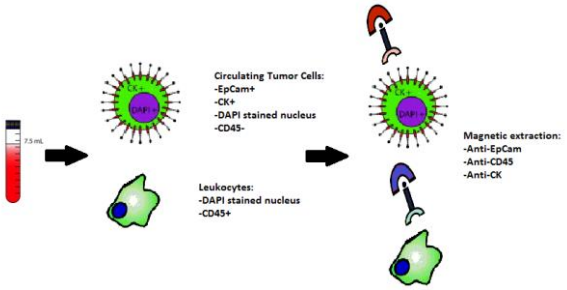

Figure 1. The CellSearch technology

- **ctDNA**

In patients with CRC, ctDNA can be detected in 48–73% of patients (11), and in the case of advanced metastatic gastrointestinal cancers, the detection rate increases to almost 100%, so for this reason ctDNA is more useful than CTCs. In order to detect ctDNA, a gene mutation has to exist in the tumor genome, with the genes most probably mutated being APC, TP53, and KRAS. In recent years many sensitive methods for detecting ctDNA have been developed, such as: BEAMing (12), Safe-SeqS, TamSeq (13), and digital PCR (14, 15).

The BEAMing technique (beads, emulsify-cation, amplification and magnetics) is very precise, detecting PIK3CA mutation from the ctDNA in the peripheral blood (16). Over the past several years there has been an increesed need for oncological medication that focuses directly on mutated genes (17, 18). The first stage of the BEAMing technique purifies the ctDNA. In the second stage, the ctDNA is amplified with the help of the PCR

technique. In the third step, specific ctDNA sequences undergo an emulsion process, so that in the fourth and final stages the sequences attach themselves to magnetic microbeads and thus can be easily isolated (19, 20).

In conclusion, the BEAMing technique is important because of its ability to isolate and quantify different gene mutations, even when in very low quantity.

- **mRNAs and miRNA**

Circulating messenger RNA and circulating micro RNA were first detected and analyzed in the late 1990s using the quantitative reverse transcriptase (qRT)-PCR method. Later, mRNAs were identified in the peripheral blood of colorectal cancer patients (21, 22). Some methods determine hTERT mRNA as a marker for colorectal cancer through telomerase activity since it is generally suppressed in healthy tissues. miRNAs are stable in blood and plasma and can be measured using the qRT-PCR method, followed by the TaqMan PCR method. The TaqMan technique, initially developed in 1991 by Cetus Corporation, is constructed in such a way that, using a fluorogenic probe, it can isolate a specific RNA gene sequence. TaqMan method is especially used to maximize the precision of the PCR technique (23, 24).

- **Exosomes**

Recently it had been discovered that circulating exosomes could be identified in the plasma of cancer patients. These exosomes transport genetic material that can be utilized to monitor the tumor. This type of micro and nano circulating tumor product is extremely important in the detection and monitoring of the early stages of cancer. Conventional techniques for isolation are ultracentrifugation and density gradient separation, but these methods are impractical and time consuming (25). In recent years, a number of scientific techniques have been perfected that can detect and isolate exosomes. Plasmonic exosome detection, also known as SPR (Surface Plasmon Resonance), is a highly sensitive method able to detect as low as 1 molecule. Weissleder/Lee group established a sensor named nPLEX (nano plasmonic exosome) that has multiple nanohole arrays that are able to detect all types of exosomes (26, 27).

- **TEPs (Tumor Educated Platelets)**

Tumor educated platelets (TEPs) are the result of the synergy between tumor cells and blood platelets. TEPs play an important role in tumor proliferation by modifying the RNA genetic profile. Of all liquid biopsies, TEPs are probably the most important in detection. Best et al. (2015) initially discovered their value in diagnostic oncology. Their research used the peripheral blood of 283 patients, of whom 228 were later diagnosed with cancer and 55 were healthy individuals. The study correctly diagnosed the cancer patients using TEPs in 96% of cases, demonstrating that TEPs modify mainly according to the type of cancer and in a very low percentage by the gravity of the disease or metastases. In summary, the power of TEPs to positively show the location of the tumor could potentially advance the usage of liquid biopsies in diagnostic oncology (28, 29).

- *Biomarkers role in the screening of patients with CRC*

Current screening methods include imaging, blood protein tests, histologic diagnostic, and direct visualization, but many of these tests cause discomfort (colonoscopies) or are unpleasant (stool studies) for the patient, and they may expose the patient to radiation (CT scans). Liquid biopsy is a non-invasive procedure having minimal risk for the patient. Furthermore, liquid biopsy offers the potential for studying the genomic evolution of the disease. In 2014, the US Food and Drug Administration approved the Cologuard test which detects abnormal fecal DNA and fecal hemoglobin (30). This test assesses DNA alterations which are distinct for the primary tumor, including abnormal methylated Bone morphogenetic protein 3, also known as osteogenin, the NDRG4 gene that is an alteration of the KRAS gene, the human beta-actin gene, and hemoglobin metalloprotein. In 2016, the FDA approved Epi proColon (Epigenomics, Berlin, Germany), a test that detects and examines the differential blood biomarker, methylated Septin 9 DNA. This marker is methylated in colorectal cancer. This test is possible with the use of the Real-Time PCR method. In 2014, Perrone et al. (31) directed a study in a high-risk population for colorectal cancer assessing circulating tumor DNA adopting the PCR method. The research revealed that ctDNA was prognosticative for colorectal cancer; however, it was not predictive for premalignant lesions, indicating that ctDNA represents an important complementary screening method in addition to traditional explorations (32, 33).

- *Pronostic value*

In colorectal cancer there is demand for supplementary prognostic markers to improve the managment of affected patients. Because circulating tumor products like CTCs and ctDNA are directly associated with the tumor burden, they can represent valuable independent prognostic biomarkers (Figure 2). Both CTCs and ctDNA are shown to correspond with survival in the case of colorectal cancer patients. A recent study supervised by Spindler et al. that included 229 patients with chemotherapy refractory mCRC and 100 healthy individuals showed that patients with low concentrations of ctDNA had a signifficantly higher rate

of survival than those with higher levels of ctDNA (19). However, in a study directed by Cohen et al. in 2009, 430 patients with metastatic colorectal cancer were divided into 2 separate groups based on the levels of CTCs, those that had <3CTCs per 7,5ml of plasma, and those that had >3CTCs per 7,5ml of plasma. Their results showed that patients having lower quantities of CTCs have almost twice the survival rate of those with higher quantities of CTCs (34). The amount of CTCs in the plasma of patients diagnosed with colorectal cancer is lower than in other types of cancer. A study by Deneve et al. on 75 patients diagnosed with CRC in situ and metastatic colorectal cancer, that studied the count of CTCs in both peripheral and mesenteric blood, showed a significant discrepancy between the two, therefore supporting the idea that the liver acts as a filter for CTCs in colorectal cancer (35).

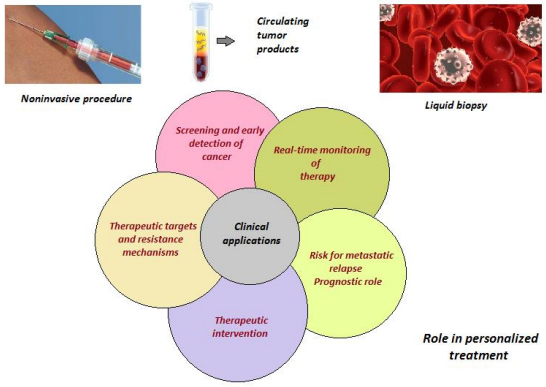

Figure 2. Clinical value of circulating tumor cells and circulating tumor DNA for cancer patients.

➢ *Detection of resistance to therapies*

The objective of personalized cancer therapy is to uncover a cancer's particular genes and proteins that permit the cancer cells to multiply, expand, and withstand aggressive therapy (36). KRAS and NRAS gene alterations are currently accepted as biomarkers that foretell resistance to anti-epidermal growth factor receptor (anti-EGFR) (37) (Figure 3). In a recent study organized by Spindler et al. (38) that included 105 patients previously diagnosed with methastatic colorectal cancer (mCRC) indicated that KRAS mutations occured in 38% of patients that initially reacted to anti-EGFR treatment therapy medication.

The patients were prescribed irinotecan 350 mg/m2 q3w and weekly cetuximab (250 mg/m2) and continued with the treatment until improvement or improper toxicity. Then ctDNA and mutated KRAS alleles were analyzed. The study tesified to the development of KRAS mutations in the plasma during treatment even before radiological indication of progression. More recent applications have now accepted the ctDNA system of attained resistance in the evolution of KRAS mutations (39, 40).

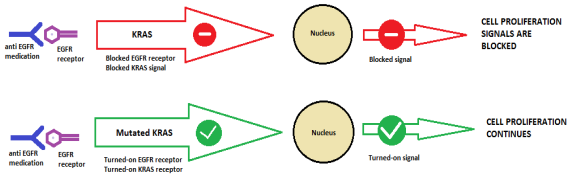

Figure 3. EGFR signaling in both Wild Type KRAS and Mutated KRAS.

Conclusions

Given that cancers are associated with mutated genes, it is only normal that genetic analysis should be included in recent years for the diagnosis, prognosis, and treatment of patients. Tumors discharge CTCs in the peripheral blood and these cells release circulating tumor DNA; thus, researchers have developed a number of detection technologies for the analysis of such mutations. More extensive research is required to determine the extent to which liquid biopsies impact patient care, but we can now conclude that circulating tumor cells (CTC) and circulating tumor DNA (ctDNA) serve as a promising step forward in the detection and monitoring of colorectal cancer.

The power of liquid biopsies from the peripheral blood to diagnose and monitor cancers could eventually simplify the management of oncological diseases, lower costs by focusing treatment, and eliminate unpleasant diagnostic tests for patients.

Liquid biopsy has the capacity to manage the complete array of colorectal cancer diseases. The study of circulating tumor material in the plasma has demonstrated the ability to diagnose the disease from early stages of development and, more importantly, to enable oncology caretakers the possibility of highly targeted drug therapy. Studying circulating tumor products gives researchers a chance to better understand the process of recurrence and metastasation in colorectal cancer.

In conclusion, the understanding of circulating tumor products is likely to transform the way clinicians approach colorectal cancer disease in the near future.

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All authors participated equally in this work.

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