Role of circulating free DNA in colorectal cancer

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Author contributions: Matikas A conceptualized and designed the review together with Georgoulias V; Matikas A, Voutsina A and Trypaki M drafted the initial manuscript; all authors reviewed and approved the final manuscript as submitted.

Conflict-of-interest statement: The authors declare no conflict of interest.

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Manuscript source: Invited manuscript

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Received: June 17, 2016
Peer-review started: June 18, 2016
First decision: July 27, 2016
Revised: September 6, 2016
Accepted: October 5, 2016
Article in press: October 9, 2016
Published online: December 15, 2016

Abstract
The gradual elucidation of the underlying biology of colorectal cancer has provided new insights and therapeutic options for patients with metastatic disease which are selected according to predictive biomarkers. This precision medicine paradigm, however, is incomplete since not all eligible patients respond to these agents and prognostic stratification is largely based on clinicopathologic variants. Importantly, no robust data exist to help properly select patients with localized disease at high risk for recurrence and most likely to benefit from adjuvant chemotherapy. There is a rapidly expanding body of literature regarding the role of the qualitative and quantitative analysis of circulating free DNA in various neoplasms, which consistently outperforms traditional tumor markers both as a predictive and as a prognostic marker. Several lines of evidence suggest that circulating free DNA may exhibit a complementary role to existing modalities for the early diagnosis of colorectal cancer, the selection of patients for adjuvant chemotherapy, for the follow-up of treated patients, for the selection of treatment for advanced disease and the assessment of response and for determining the prognosis of patients. These data, which are reviewed here, illustrate the important role that circulating biomarkers may soon have at the daily clinical practice.

Key words: Cell-free DNA; Circulating tumor DNA; Colorectal cancer; Biomarker; KRAS

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Core tip: Published studies clearly indicate that cell-free DNA levels and the detection of specific molecular events in the plasma of colorectal cancer patients is a relevant prognostic and predictive biomarker, with clinically meaningful value at various disease settings such as asymptomatic screening, follow-up after curative surgery and metastatic disease. Further randomized studies are needed before these techniques are implemented at the daily practice.

Matikas A, Voutsina A, Trypaki M, Georgoulias V. Role of circulating free DNA in colorectal cancer. World J Gastrointest Oncol 2016; 8(12): 810-818

ISSN 1948-5204 (online)

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4251/wjgo.v8.i12.810

World J Gastrointest Oncol 2016 December 15; 8(12): 810-818
INTRODUCTION

Globally, colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females; there is a significant regional variation in incidence and mortality rates in Western countries, especially the United States, where both are decreasing as a result of the widespread adoption of effective screening policies and of the evolution of treatment strategies at the adjuvant setting. Approximately 8% of cancer deaths are caused by CRC\(^{[1,2]}\). Twenty percent of newly diagnosed patients have de novo clinically overt metastases; moreover, 10% of patients diagnosed with local and 30% with regional disease will eventually relapse, most commonly with disseminated disease\(^{[3]}\). These patients presumably already harbor occult micrometastases, thus identifying them and administering systemic treatment following local excision may improve their chance for cure. Moreover, despite significant advances in the understanding of underlying molecular mechanisms and in the development and regulatory approval of several active agents during the past 15 years, 5-year survival rates of patients with metastatic CRC (mCRC) remain poor at 13%\(^{[3]}\), with the majority of these patients receiving palliative systemic treatment without a curative intent. Thus, it is clear that earlier diagnosis when interventions may be curable and also better predictive and prognostic biomarkers both for localized and advanced disease are highly needed.

Liquid biopsy is a minimally invasive process based on a simple venipuncture that potentially addresses several issues, since it can be safely implemented on a wide scale basis and can be repeated with minimal risks for the patient. Moreover, liquid biopsy may illustrate the molecular diversity of the underlying disease process and serial testing facilitates the monitoring of its spatial and temporal genomic evolution and at the same time it circumvents the need for re-biopsy, which is invasive, cumbersome and not always feasible\(^{[4]}\). Moreover, re-biopsy is subject to sampling bias and it may not be representative of the intratumoral heterogeneity. These biomarkers may be protein-based, such as cancer antigens [carcinoembryonic antigen (CEA)], cell-based, such as circulating tumor cells (CTC) and disseminated tumor cells and nucleic acid-based, such as circulating cell-free DNA (cfDNA) and micro RNAs. CEA has been the only circulating biomarker in clinical use for decades, but its usefulness is limited by suboptimal sensitivity and specificity\(^{[5]}\).

CIRCULATING cfDNA

cfDNA may originate from normal or from tumor cells and it can be detected in healthy subjects, with increased levels noted in benign conditions such as inflammatory processes and infections\(^{[6]}\). Necrotic and apoptotic cells may release DNA fragments passively, depending on the tumor burden, its growth kinetics and the effects of antineoplastic treatment, but it is also believed that cfDNA may be actively shed by tumor cells with the goal to transform cells in distant sites\(^{[7]}\). Finally, CTCs and micrometastases may also be the source of cfDNA, along with the primary tumor.

Several technical challenges hamper the ability to standardize the identification and measurement of cfDNA in oncology patients. First of all, the low concentration of highly fragmented DNA molecules renders their identification, amplification and quantification rather challenging\(^{[8]}\). Several DNA extraction methodologies have been developed with no one appearing to clearly improve yields and pre-analytical sample processing and storage could potentially influence results. Moreover, the abundance of available methodologies such as digital polymerase chain reaction (PCR), real-time quantitative PCR (qPCR) and emerging next-generation sequencing technologies result in a lack of comparability between results reported in various translational and clinical studies\(^{[9,10]}\). Despite these challenges, an exponentially increasing body of literature has clearly demonstrated the promise that the measurement of cfDNA holds in various clinical settings and in multiple different neoplasms and advances in technology aim to tackle both the problems of detecting diluted tumor DNA and determining its origin based on the presence of known aberrations found on the primary tumor.

EARLY DIAGNOSIS OF COLORECTAL CANCER

Screening for CRC has been consistently shown to reduce disease specific mortality\(^{[11]}\). An abundance of screening modalities is available and recommended by clinical practice guidelines, which implies that no one is clearly superior to the others since no randomized comparisons have been performed\(^{[11,12]}\). Colonoscopy is regarded as the preferred technique\(^{[13]}\). However, it is costly and invasive. Thus, enriching the population that undergoes colonoscopy with subjects at the highest risk for the development of CRC with a minimally invasive selection procedure is a matter of active research.

A large number of studies, reviewed elsewhere\(^{[14]}\), have evaluated the efficacy as screening tools of the detection of several molecular events, such as Kirsten rat sarcoma viral oncogene homolog (KRAS), adenomatosis polyposis coli (APC), TP53 and mismatch repair genes (MMR) mutations, DNA methylation and miRNA signatures in body fluids, mainly stool but also plasma and serum. In summary, the reported sensitivity and specificity rates vary widely, owing to the small quantity of hyperfragmented DNA, often of low quality, retrieved from body fluids (especially stool) and the different
techniques and biomarkers tested. Regarding plasma cfDNA, preliminary results are promising. In a high risk population with positive fecal occult blood test that subsequently underwent colonoscopy, Perrone et al\textsuperscript{[15]} demonstrated that the quantification of cfDNA by qPCR was predictive for CRC but not premalignant lesions (area under curve, 0.709; 95%CI: 0.508-0.909). This important finding clearly illustrates that cfDNA can have a complementary role to traditional screening modalities for CRC. In the same study, the detection rate of KRAS mutations in the plasma by mutant-enriched PCR was low at 3%, compared to tissue-based analysis (45%). However, KRAS mutations can also be detected in patients with inflammatory bowel disease, complicating the interpretation of its significance\textsuperscript{[16]}. Significant barriers to the implementation of cfDNA-based strategies include the relatively low sensitivity especially for premalignant lesions and the probability of over-diagnosis.

**MONITORING MINIMAL RESIDUAL DISEASE**

Following curative surgery for localized CRC, approximately 50% of stage III patients according to the American Joint Committee on Cancer (node-positive disease) and 20% of stage II patients (T3N0 and T4N0) are expected to experience disease relapse without adjuvant chemotherapy, possibly due to the presence of occult micrometastases. Therefore, identifying these high risk patients could optimize adjuvant treatment strategies. The benefit derived from chemotherapy is well established for stage III patients, with the results of large, well-conducted randomized trials demonstrating a benefit for overall survival (OS) for patients treated with the combination of a fluoropyrimidine (5-fluorouracil or capecitabine) and oxaliplatin (FOLFOX and XELOX, respectively)\textsuperscript{[17,18]}. The management of stage II patients is much more controversial, as clinical trials and meta-analyses indicate that the absolute risk reduction is marginal and sometimes non-significant\textsuperscript{[19-21]}. Clinical practice guidelines recommend the use of clinicopathological risk factors for the selection of eligible patients for adjuvant chemotherapy, such as T4 stage, perforation, obstruction, number of lymph nodes resected, presence of lymphovascular or perineural invasion, poor grade, preoperative CEA levels and positive or indeterminate margins\textsuperscript{[22-24]}. Additionally, molecular markers such as the presence of microsatellite instability\textsuperscript{[25]} and gene signatures\textsuperscript{[26]} have also been shown to have prognostic and/or predictive value. These data clearly underscore that robust decision making tools are needed for the selection of patients that will enjoy improved outcomes from additional chemotherapy, thus sparing from its toxic effects those not likely to benefit. Consequently, cfDNA has been evaluated in this setting. Reinert et al\textsuperscript{[27]} recently showed that using droplet digital PCR-based (ddPCR) personalized assays, the quantification of plasma cfDNA had almost 100% sensitivity and specificity for the prediction of relapse after surgery, with a mean lead time of 10 mo. Furthermore, the value of cfDNA measurement specifically in stage II CRC patients is being prospectively evaluated, with preliminary results showing that 7.7% of patients who had detectable cfDNA after curative surgery had higher relapse rates, 5/6 patients with detectable and 5/72 of those with undetectable cfDNA\textsuperscript{[28]}. The selection of the proper mutation markers to be monitored in cfDNA is as yet unresolved. Sato et al\textsuperscript{[29]} monitored preoperative and postoperative cfDNA levels based on a panel of 50 genes, using ddPCR; only markers with an allele frequency above 0.1% in plasma DNA correlated with the clinical course. Interestingly, cfDNA has also been shown to be useful in the early detection of relapse after metastasectomy of liver metastases, significantly outperforming both CEA and imaging in one study\textsuperscript{[30]}.

**PREDICTION OF RESPONSE TO TREATMENT**

The demonstration of the efficacy of monoclonal antibodies (moAbs) targeting the epidermal growth factor receptor (EGFR), such as cetuximab and panitumumab, in specific molecular subtypes of mCRC, marked a significant breakthrough towards the delivery of precision medicine. V-Ki-ras2 KRAS has a critical role in EGFR signaling transduction. Activating KRAS mutations at exon 2 are detected in approximately 40%-45% of patients with CRC and have been shown to confer resistance to treatment with cetuximab\textsuperscript{[31,32]} and panitumumab\textsuperscript{[33]}. Moreover, less common KRAS mutations at exons 3 and 4 and NRAS mutations at exons 2, 3, 4 identified at a further 17% of patients have been shown to also correlate with resistance to anti-EGFR moAbs\textsuperscript{[34]}. On the other hand, mutations at the B-Raf proto-oncogene, serine/threonine kinase (BRAF), which is further downstream at the mitogen-activated protein kinase (MAPK) pathway have been shown to have significant prognostic, but not predictive value as a marker of poor response to anti-EGFR antibodies when combined with chemotherapy, although evidence for the latter is not compelling due to the small number of patients and low statistical power of these analyses\textsuperscript{[35,36]}. To further complicate matters regarding the role of BRAF mutations, several lines of evidence suggest that their presence is a marker of resistance to monotherapy with anti-EGFR moAbs\textsuperscript{[37-39]}.

Availability of adequate tissue is rarely a problem in CRC patients. Additionally, there is a high rate of concordance between the primary tumor and metastases regarding the mutational status of mCRC but, importantly, approximately 10% of patients with KRAS wild type (WT) primary tumors have KRAS mutated metastases and vice versa\textsuperscript{[40]}. Qualitative analysis of cfDNA for the presence of activating mutations is an
emerging method which has been consistently shown to closely correlate with the primary tumor status. For example, Morgan et al\cite{41} utilized a commercial PCR kit and demonstrated that the detection of KRAS mutations in cfDNA is highly concordant with their presence at the primary tumor and they could also be detected in the plasma of a patient with KRAS WT primary. Accordingly, Thierry et al\cite{42} showed in a prospectively evaluated patient cohort that qPCR-based detection of KRAS and BRAF mutations in mCRC patients is exquisitely sensitive and specific when using the primary tumor status as a reference standard, results that have been confirmed by others\cite{30}. Taken together, these data clearly imply that the detection of predictive molecular events in mCRC patients is an excellent surrogate for their presence at the primary tumor and could be used in cases where archival tumor is not available or when the acquisition of fresh tissue is not feasible.

**MONITORING RESPONSE TO THERAPY**

The quantitative measurement of a known aberration in the plasma may be used for disease monitoring of mCRC patients while under treatment and data show that cfDNA levels robustly correlate with tumor burden and response to treatment, raising the possibility of its use at the near future as an adjunct to anatomical imaging. Several investigators have established the correlation between tumor burden, cfDNA and levels of specific mutations such as KRAS in circulation\cite{42-44}. Moreover, high levels of cfDNA were shown to be predictive for diminished disease control rates when mCRC patients were treated with irinotecan and cetuximab in a study by Spindler et al\cite{43}. In a prospective trial of mCRC patients undergoing first-line chemotherapy, a 10-fold or higher reduction in circulating tumor DNA (ctDNA) before cycle 2 correlated well with CT imaging at 8-10 wk while lesser degrees of reduction were associated with a trend for improved progression free survival (PFS)\cite{45}. Although the quantification of cfDNA levels and their temporal changes seem appealing for clinical use, two issues need to be considered: First of all, benign conditions such as infections may also lead to raised cfDNA levels. Also, the genomic evolution of the tumor could potentially lead to alterations in cfDNA levels regardless of tumor burden. Until more specific and sensitive methods that measure ctDNA are available, it is prudent that cfDNA levels are used in combination with imaging scans for response assessment.

**DETECTION OF RESISTANCE**

Despite appropriate patient selection according to molecular testing, approximately 10% of treatment naïve patients experience disease progression as best response when receiving first line treatment with a chemotherapy doublet and an anti-EGFR antibody\cite{46}.

Importantly, virtually all patients will eventually develop disease progression while receiving such treatment. The resistance mechanisms of mCRC to anti-EGFR moAbs can be broadly categorized in three categories and it should be mentioned that there is significant overlapping between primary and secondary resistance, with a few notable exceptions: (1) events that disrupt the binding of cetuximab or panitumumab at the EGFR. These events may be point mutations, with the best characterized being the S492R mutation which interferes with the binding of cetuximab but not panitumumab\cite{47}, a decrease of EGFR copy number or differential expression of the EGFR ligands epiregulin and amphiregulin\cite{48}; (2) activation of downstream kinases, effectively bypassing the inhibition of EGFR. The best described events include the KRAS, NRAS and BRAF mutations whose role at the primary resistance was previously described. Although less common, KRAS gene amplification has also been implicated in the development of resistance to cetuximab and panitumumab\cite{49}, and (3) activation of parallel, bypassing pathways. The most commonly described mechanisms are phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha exon 20 mutations in approximately 10%-15% of patients and phosphatase and tensin homolog (PTEN) mutations in 18%, both leading to activation of the PI3K/AKT/mTOR pathway\cite{39,50}. However, since these mutations frequently coexist with other resistance mechanisms such as KRAS mutations, it is difficult to establish a potentially direct causative role. Other genomic events that have been implicated in the development of secondary resistance are HER2 amplification\cite{51}, Igf-1R activation\cite{52} and MET amplification\cite{53}.

It is clear that CRC exhibits significant spatial and temporal heterogeneity while under the selective pressure of treatment, which is further complicated by the extensive crosstalk, shared downstream pathways and bypass signaling that is activated during treatment with anti-EGFR moAbs which allows pre-existing resistant clones to emerge. The early recognition of these molecular mechanisms, before disease progression is clinically or radiologically apparent, may offer a better chance at intercepting them, thus improving patient outcomes.

The best studied in cfDNA mechanism of acquired resistance is the emergence of KRAS mutations, which has been shown to occur while under treatment both with cetuximab\cite{44} and panitumumab\cite{55}. Notably, in a series of CRC patients receiving treatment it was shown that KRAS mutations developed in 38% of patients previously responding to anti-EGFR moAbs, 96% had newly acquired activation of the MAPK pathway and 70 new somatic mutations were described in total\cite{56}. Interestingly, in a series of 108 mCRC patients pretreated with a fluoropyrimidine, irinotecan and oxaliplatin, who received irinotecan and cetuximab, Spindler et al\cite{57} demonstrated that the emergence of detectable KRAS mutations in the plasma may precede...
radiological progression and that patients with a KRAS mutant primary but no detectable mutations in the plasma could benefit from treatment despite previous exposure to and progression after irinotecan.

Apart from KRAS mutations, other molecular events that emerge under the selective pressure of anti-EGFR treatment and that drive resistance to these agents have been detected in cfDNA, such as EGFR mutations\[58\] and MET amplification\[53\]. The relative importance of each specific aberration, in light of the frequent co-existence of several mechanisms in a single patient\[90\] needs to be elucidated in further studies.

CELL-FREE DNA AS A PROGNOSTIC MARKER

The prediction of survival in mCRC patients relies heavily on clinic-pathologic characteristics, such as hepatic tumor burden, node positive primary, CEA levels, microsatellite stability status, BRAF mutation status, resectability of metastatic disease and tumor grade\[60\]. Objective and reproducible biomarkers are needed in order to optimize risk stratification and guide treatment decisions. The prognostic capacity of quantitative and qualitative cfDNA characteristics have been investigated in several studies. cfDNA levels were indeed shown to correlate with survival in mCRC\[59\], including at the second line setting where increased cfDNA levels predicted shorter PFS and OS compared to lower levels, with a hazard ratio (HR) of 1.4 (95%CI: 1.1-1.7, \( P = 0.03 \)) for PFS and 1.6 (95%CI: 1.3-2.0, \( P < 0.0001 \)) for OS for each quartile of increase\[61\]. Qualitative characteristics, such as methylated cfDNA levels have also been shown to independently predict OS in mCRC\[62\]. In the largest published prospective mCRC patient cohort (\( n = 97 \)), El Messaoudi et al\[63\] used qPCR and showed that cfDNA levels, higher specific mutation loads and the level of cfDNA fragmentation are strong prognostic factors; cfDNA levels were independent prognostic factors for the entire patient cohort and the level of fragmentation only for the KRAS/BRAF mutated subset. Specifically, a difference in OS of 10 mo was reported between the groups of high vs low cfDNA levels (18.07 mo vs 28.5 mo respectively, \( P = 0.0087 \))\[63\]. Finally, Spindler et al\[64\] compared the prognostic value of the detection of KRAS mutations in the plasma compared to the primary tumor with the use of qPCR, with the former being an independent predictor for OS (HR = 2.98, 95%CI: 1.53-5.80, \( P = 0.001 \)) and PFS (HR = 2.84, 95%CI: 1.46-5.53, \( P = 0.002 \)), whereas the latter had no correlation with outcomes, which underscores the value of cfDNA qualitative testing.

DISCUSSION

There is enormous interest for the discovery, development, clinical evaluation and standardization of circulating biomarkers in oncology, since liquid biopsy offers the possibility of real-time monitoring of the disease trajectory. Due to the absence of large scale comparisons of the relative efficacy of cfDNA and CTCs and the lack of standardization of clinical assays, no clear recommendations can be made on which is the superior biomarker and published literature suggests that they may be complementary\[65,66\]. For example, CTCs have been shown to be useful in determining the level of heterogeneity of the disease, with significant differences compared to the primary tumor demonstrated in single cell whole genome analysis. Moreover, the phenotypic and genotypic characterization of CTCs could be a powerful tool aiding treatment planning and determining the likelihood of drug resistance\[67\]. However, the large number of CTC capture and enrichment techniques, which are based on antibody selection or on the physical properties of CTCs and the often times low quantity and quality of DNA extracted from these rare cells represent significant drawbacks\[68\].

Contrary, cfDNA exhibits several important advantages: Its extraction is less cumbersome compared to CTCs, but the preanalytical process is equally non-standardized. Also, multiple studies across various neoplasms have shown that its qualitative and quantitative measurement is representative of the overall tumor burden and of the mutational load of the disease and its heterogeneity. Therefore, its future potential role as a complement in asymptomatic screening and disease staging, as a tool for disease monitoring even without the use of anatomic imaging, as a prognostic marker and for the long term follow-up of disease free patients with CRC is exciting\[69\]. Nevertheless, the aforementioned lack of standardization is a significant obstacle for the commercialization and widespread adoption of cfDNA in oncology. Moreover, cfDNA, in contrast with CTCs, does not provide information regarding RNA and protein profiling of the tumor. However, sensitivity may be improved since cfDNA has been detected both in patients with and without detectable CTCs, but CTCs were not detected in the absence of cfDNA\[56\]. These observations clearly hint towards a combinatorial approach to circulating biomarkers.

It is conceivable that in the near future serial cfDNA testing will become a component of routine clinical practice regarding the management of CRC. Its already established prognostic power may be integrated in novel staging schemes and its predictive capacity may influence treatment decisions. Importantly, its use may spare patients from unnecessary toxicity caused by ineffective treatments. It is imperative, however, that cost-effectiveness analyses be conducted since these costly techniques require specific equipment and are often labor-intensive.

CONCLUSION

cfDNA has already shown promise in CRC in multiple
clinical settings. Its prospective evaluation in randomized trials is of paramount importance before it can be considered as standard practice and cost-effectiveness analyses are also needed. Until then, translational studies continue to underscore its clinical utility and offer insights on the continuously evolving understanding of the disease complexity.

ACKNOWLEDGMENTS
We acknowledge the volunteer assistance of the scientific secretary Vasso Athanasaki in the preparation of this manuscript.

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