

REVIEW ARTICLE

Methodological, biological and clinical aspects of circulating free DNA in metastatic colorectal cancer

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ABSTRACT

Background: Circulating DNA can be used to measure the total cell-free DNA (cfDNA) and for detection and quantification of tumor-specific genetic alterations in the peripheral blood, and the broad clinical potential of circulating DNA has attracted increasing focus over the past decade. Concentrations of circulating DNA are high in metastatic colorectal cancer (CRC), and the total levels of cfDNA have been reported to hold strong prognostic value. Colorectal tumors are characterized by a high frequency of well known, clinically relevant genetic alteration, which is readily detected in the cfDNA and holds potential for tailoring of palliative therapy and for monitoring during treatment. This review aims to present the current literature which has specifically reported data on the potential utility of cfDNA and on tumor-specific mutations in metastatic colorectal cancer (mCRC).

Method: Methodological, biological and clinical aspects are discussed based on the most recent development in this specific setting, and eligible studies were identified by systematic literature searched from Pubmed and EMBASE in addition to conference papers and communications.

Results: The literature regarding cfDNA in CRC is broad and heterogeneous concerning aims, nomenclature, methods, cohorts and clinical endpoints and consequently difficult to include in a single systematic search. However, the available data underline a strong clinical value of measuring both total cfDNA levels and tumor-specific mutations in the plasma of patients with mCRC, pre- and during systemic therapy.

Conclusion: This paper had gathered the most recent literature on several aspects of cfDNA in mCRC, including methodological, biological and clinical aspects, and discussed the large clinical potential in this specific setting, which needs to be validated in carefully designed prospective studies in statistically relevant cohorts.

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Metastatic colorectal cancer

Colorectal cancer (CRC) is a major cause of cancer death in the western world. The median overall survival from metastatic disease is approximately two years in clinical trials despite the development of new biological agents and substantial progress within the field of surgery for metastatic lesions [1–4]. Standard chemotherapy implies substantial toxicity, and only a fraction of the patients will benefit from treatment because reliable criteria allowing for the right drug, to the right patient, at the right time have not been identified. Predictive as well as prognostic markers would improve results and help avoid unnecessary side effects. Furthermore, agents targeting various downstream signaling pathways are being developed and tested in clinical trials. However, results are highly dependent on the molecular characterization of the tumor, which is challenged by intra-tumoral heterogeneity and heterogeneity between the tumor and metastatic lesions. Theoretically, heterogeneity can also develop over time due to, for example clonal selection or the appearance of new mutations contributing to development of resistance to therapy [5]. These aspects could have a major

influence on the success of both existing therapies and new targeted drugs. However, the development has been limited by ethical and practical issues related to repeated drawing of biopsies from malignant lesions. Liquid biopsies, i.e., analysis of tumor-specific characteristics in a peripheral blood sample, have the advantage of providing a less-invasive, instant measure of the molecular status of the disease, which could contribute to the development of precision medicine by timely, consecutive monitoring of efficacy, resistance and treatment failure [6]. Compared with repeated tissue sampling, liquid biopsies are feasible and they provide information about tumor cells as well as the biological mechanisms which accompany malignant growth and development of resistance. Studies emerging in this field are encouraging, and bear evidence of a true clinical potential which is discussed in this review.

Circulating nucleic acids in plasma and serum

Small fragments of circulating free DNA are present in the circulation under both healthy and pathological conditions,

including cancer [7–9]. The cell-free DNA (cfDNA) enters the circulation by direct secretion from the tumor cells, by apoptosis, necrosis, from circulating tumor cells, and from the normal surrounding tissues [10–13]. cfDNA seems to play an important role in the theory of genometastases, but the biological mechanisms underlying the dynamics of the cfDNA are far from fully understood, and substantial efforts are being made to explore these aspects. Another stand of research explores the potential clinical value of measuring the cfDNA and the emergence of recent technologies has enabled investigation of relevant cohorts of cancer patients in clinical settings. Several studies indicate a potential prognostic, predictive and diagnostic value of measuring the cfDNA, which calls for reliable validation [14–23].

Different methodological approaches have been used to explore the potential of cfDNA analysis which potentially provides: 1) a quantitative measure of total cfDNA; 2) identification of tumor-specific genetic alterations of the DNA in the sample, i.e., circulating tumor DNA (ctDNA) and 3) quantification of the number of mutated/altered alleles in the sample. These parameters can be correlated to the clinical information, which could become an important future tool in patient selection for chemotherapy, assessment of prognosis and early detection of resistance, as well as in the monitoring during treatment and follow-up for various malignant diseases.

Intense interest has been devoted to the methodological challenges and biological aspects in this field, whereas clinically relevant approaches still need to be addressed. This paper discusses some of the methodological, biological and clinical aspects of cfDNA with a special focus on metastatic colorectal cancer (mCRC).

Pre-analytical considerations and quantification of total DNA

Methodological aspects, including standardization, are of utmost importance for the comparison of data and the translation of cfDNA analysis into clinical practice. These aspects include a number of pre-analytical parameters that can affect the concentration and fragmentation of cfDNA as recently discussed [24–28]. It seems that a reasonable level of consensus exists regarding the optimal source for cfDNA measurement as most studies suggest that quantification is more reliable when determined from plasma than from serum [24,25]. The same applies to the optimal collecting tube, where the use of anti-coagulants is favored, and it is largely agreed that the sampling procedure must be performed with great care to avoid cell lysis and contamination with DNA from normal blood cells [29]. In addition, there seems to be some consensus that a two-hour delay between sampling and blood processing is acceptable, whereas storage temperature seems less important [24,30,31]. In contrast, disagreement prevails regarding the centrifugation procedures in terms of the level of gravity (g) and regarding the use of one- or two-step procedures to ensure adequate separation and to avoid contamination and degradation prior to DNA purification and quantification procedures [24,31].

The majority of recent studies have successfully used commercially available kits for DNA purification. Notably, potentially major bias may arise in the quantification of the total cfDNA due to contamination from normal, lysed lymphocytes or loss of DNA during pre-analytical procedures, but controls for both factors can be applied in the form of a quality check on the use of a sample in clinical materials, as recently published [29]. Another major challenge is the lack of consensus regarding the optimal approach to total cfDNA quantification, an aspect that urgently needs standardization. The lack of direct comparison between studies and the numerous different quantification approaches renders progress in terms of clinical relevance difficult. Among others, the different non-tumor-specific reference genes comprise cyclophilin, B2M, and albumin [26–28,31–33]; and other strategies include methylation assays or quantification of tumor-relevant genetic alterations [34]. Provided that all the above-mentioned steps are carefully considered, a reliable quantitative measure of total cfDNA is achievable and samples can be further analyzed to detect tumor-specific mutations and quantify the mutated alleles in the sample.

Analytical methods

In brief, there are two overall analytical approaches, for instance targeting a specific, well known genetic or epigenetic alteration or performing a broad search for alterations in major genetic sequences. *qPCR*. Quantitative real-time polymerase chain reaction (PCR) methods have been used to measure signals from labeled probes during amplification of different target genes, such as albumin, hTERT, B-globin, cyclophilin and many others for total cfDNA measurement, or quantification of tumor-specific mutations in CRC, primarily the RAS/RAF mutations [32]. The method is simple and reproducible, and can be designed with a high sensitivity. However, it has the disadvantage of being subject to the limitations of the single assay, which implies a need for a high number of separate assays to cover the increasing number of relevant mutations.

Digital droplet PCR. By digital droplet PCR (ddPCR), the DNA is diluted into isolated single molecules and each molecule is then individually analyzed for the target genes as reviewed by Taly et al. [34]. ddPCR enables detection of multiple mutations (multiplexing) from the same sample. ddPCR has a high sensitivity [34–37], but it also has limitations in terms of small input DNA applicable for the individual assays. Aspects such as level of sensitivity, specificity and background remain subject to optimization in the development of specific assays.

BEAMing. The BEAMing (beads, emulsions, amplification, and magnetics) technology, which was first described by Diehl et al., is based on single-molecule PCR on micro particles in water-in-oil emulsions [38]. Each compartment contains a bead that is coated with thousands of copies of the single DNA molecule originally present, and a high number of beads are then analyzed with flow cytometry. This methodology can be multiplexed for simultaneous detection of specific gene mutations, and can be considered an advanced

form of ddPCR with a high sensitivity suitable for analysis of mutated DNA at very low concentrations. Although complex and time-consuming, it has currently been refined for analysis of ctDNA in blood samples and applied in various clinical studies [39,40].

Next generation sequencing. Next generation sequencing (NGS) is based on the principle of sequencing in a massively parallel fashion, with the ability of providing an in-depth genome-wide search for genetic alterations. The method can detect unknown mutations in a specific gene or can be designed to focus on specific genes; or used for broad genomic analysis. NGS was recently applied for molecular characterization of cfDNA in cancer, and its clinical utility was recently described in large clinical cohorts [41–45].

Origin of circulating-free DNA

cfDNA can be detected in the blood, urine, and various bodily fluids [46]. cfDNA is comprised of small fractions of double-stranded DNA consisting of less than 200 base pairs [11]. The origin of cfDNA is still being debated, but the literature bears evidence of a mixture of DNA from both tumor and normal surrounding cells in cancer patients [11,47–52]. In healthy individuals, cfDNA seems to derive mainly from apoptotic hematopoietic cells [50], and there is evidence of a very short half-life in the circulation which indicates continuous and rapid release, degradation and filtration [51]. It has also become known that some of the cfDNA is circulating in nucleosomes, which can be analyzed for cell-type-specific

genetic signatures, and hereby be used to classify the cellular origin of the cfDNA [52]. Current knowledge suggests that cfDNA enters the blood by various mechanisms, mainly necrosis and apoptosis, but there is also increasing evidence of active release into the circulation. The size of DNA fragments seems to hold important biological information. This has been explored by several groups advocating that a cfDNA integrity index should be used in clinical investigations [15,20,53]. Although the potential that lies in detection and measurement of the tumor-specific DNA has been established, the clinical value of total cfDNA quantification is still being debated. Only few studies take the total levels of cfDNA into consideration as a picture of the overall complex biology, but recently a series of studies reported a clear correlation between total cfDNA levels and mutated DNA [45,54–63].

Cell-free DNA in metastatic colorectal cancer and healthy controls

cfDNA levels have been analyzed in relation to different cancer diseases. In general, the levels are higher in mCRC patients than in healthy controls, as shown in Table 1 [15,20,47,58,63–72]; and the levels have some correlation to stage as reported in a limited number of studies [15,41,68,73] in primary CRC. These findings give important biological information, and researchers suggest that cfDNA has potential as a diagnostic or screening tool; a potential that is currently being investigated by several groups. The possible

Table 1. Studies which have included a comparison of total cell-free DNA levels in colorectal cancer and controls.

Reference	Patients	Normal cohort	Total cfDNA analyzed	Plasma mutations analyzed	Results
El-Gayar et al. [15] 2016	50	20 Healthy 10 Polyps	+	(+)	Higher in CRC AUC 0.90
Hao et al. [20] 2014	204	110 Healthy 61 Polyps	+	–	Higher in CRC AUC 0.89
Spindler et al. [58] 2014	86 Stage IV	93 Healthy 53 With comorbidity	+	+	Higher in CRC AUC 0.86
Spindler et al. [63] 2014	229 Stage IV	93 Healthy	+	+	Higher in CRC AUC 0.94
da Silva et al. [63] 2013	50	30 Healthy	+	–	Higher in CRC
Perkins et al. [64] 2013	25 Stage IV	20 Healthy	+	+	Higher in CRC
Qi et al. [65] 2012	31	92 Healthy 30 Polyps	+	–	Higher in CRC AUC 0.904
Mead et al. [66] 2011	24	35 Healthy 26 Polyps	+	–	Higher in CRC than benign GI disease and healthy controls AUC 0.76
Czeiger et al. [67] 2011	38	34 Healthy	+	–	Higher in CRC AUC 0.84
Danese et al. [68] 2010	118 (28 Stage IV)	26 Healthy 49 Polyps	+	–	Higher in CRC than benign GI disease and healthy controls AUC 0.93
Frattini et al. [69] 2008	70 (6 Dukes D)	20 Healthy	+	+	Higher in CRC
Boni et al. [70] 2007	67	67 Healthy	+	–	Higher in CRC
Flamini et al. [71] 2006	75 (12 Dukes D)	75 Healthy	+	–	Higher in CRC AUC 0.86
Diehl et al. [47] 2005	33 + 56	10 Healthy	+	–	Higher in Dukes C-D than healthy, not significantly higher in polyps or Dukes A
Shapiro et al. [72] 1983	29	88 Healthy	+	–	Higher in CRC than benign GI disease and healthy controls

AUC: area under the curve; cfDNA: cell-free DNA; CRC: colorectal cancer; GI: gastrointestinal.

implications for screening purposes are, however, currently only based on theory.

Correlation to disease burden, carcinoembryonic antigen, and standard biochemical parameters

In general, a large disease burden is associated with a high risk of a poor outcome, which could imply an immediate need for intensified systemic therapy [4]. However, even small-volume disease can express extremely aggressive behavior. Response evaluation criteria in solid tumors, for instance RECIST is the standard evaluation of disease extent and response during chemotherapy courses for metastatic disease and serves to assist in clinical decision making during therapy and to facilitate comparison of results from clinical trials [74]. There are, however, shortcomings in single-modality evaluation by RECIST, and alternative ways of addressing response evaluation were recently proposed. Both 'deepness' of response, early tumor shrinkage, and optimal morphological response are potential future aspect for consideration in clinical evaluations [75]. A more nuanced evaluation of efficacy could also include alternative measures of tumor volume, morphological changes, and biological parameters. Analysis of cfDNA in relation to tumor volume as well as clinical outcome is therefore highly relevant, but a reliable measure of tumor volume or total disease burden is not easily obtained.

Pre-clinical studies show a strong correlation between concentrations and fragmentations of tumor-specific mutated DNA in plasma from mice, on the one hand, and tumor weight, on the other [68,76]. Diaz et al. used the aggregate cross sectional diameter of index lesions as well as carcinoembryonic antigen (CEA) as a measure for tumor volume and reported parallel levels with cfDNA, similar to the pioneering work by Dawson et al. in breast cancer patients [39,77]. Diaz et al. used mathematical modeling for evaluation of tumor growth based on ctDNA analysis [39]. The purpose was to demonstrate the presence of pre-existing mutated clones as mechanism of resistance to panitumumab. In contrast, Morelli et al. recently measured the sums of lesion volumes measured (manually on each image, by three independent readers in a total of 23 patients), but found no significant correlation between tumor volume and percentage of acquired mutant reads in the plasma [78]. In a Danish group of patients with mCRC, both total cfDNA and mutated DNA were analyzed in relation to positron emission tomography-computed tomography (PET-CT) parameters similar to a study published in lung cancer [79]. Data revealed a weak correlation to quantitative measures of both total and mutated DNA [33]. It is reasonable to speculate that the concentration of tumor-specific DNA directly correlates to tumor volume which is not readily measured, whereas the total cfDNA both reflects disease biology and tumor burden, mirroring the overall complex disease biology. Each parameter can be measured for specific clinical purposes, and further investigations could provide important biological and clinical information.

The only biomarker which has entered clinical application in CRC is CEA although its potential to aid in clinical decision

making is still being debated. CEA is traditionally regarded as a measure of tumor volume with prognostic value and potential for detection of tumor recurrence in various settings. It is therefore relevant to analyze the relation between cfDNA and CEA. As illustrated from the summary in Table 2, there is considerable variation in the measures, methods and parameter used to compare or combine the information from CEA and cfDNA analysis [15,20,32,33,39,41,65–72,81–83]. The table includes studies identified by a systematic literature search of circulating DNA in CRC (all stages) as illustrated by Supplementary Figure 1. Twelve studies investigated ctDNA, whereas a total of 13 included information of total cfDNA; and most of the studies were performed on sample sizes below 100 patients. The majority of these studies showed limited correlation, as analyzed by cross tabulation, correlation to tumor burden as a surrogate marker, or direct correlation analysis. In general, cfDNA seemed to perform better than CEA alone in relation to the clinical data, primarily prognosis; and the combination of CEA and cfDNA improved the prognostic value in some studies, as illustrated by Qi et al. [65]. The potential direct correlation between CEA and total cfDNA was analyzed in two Danish cohorts [32,33], $r = 0.37$; but when CEA was entered into the multivariate analysis, it failed to provide a significant contribution to progression-free survival (PFS) or overall survival. Overall, some correlation between CEA and cfDNA is reported, but cfDNA performed better in the majority of studies. Whether or not there is valuable clinical information contained in combining CEA and cfDNA remains to be analyzed prospectively, but data have suggested that cfDNA used independently provides strong prognostic information.

Standard laboratory parameters such as lactate dehydrogenase (LHD), platelets and neutrophil count are considered measures of biologically active disease with poor prognostic information, but the literature on correlation between cfDNA and these parameters is sparse [84]. Remarkably, small reports have combined the information about these parameters with cfDNA measures, and confirmed a correlation between the levels of cfDNA and LHD which showed a prognostic value in univariate analysis, but failed to do so in multivariate testing [33]. These results illustrate the generally unspecific value of common blood parameters in comparison with the tumor-specific biological influence that cfDNA seems to hold on patients' general outcome.

Clinical utility of total cell-free DNA

A high level of a biomarker in cancer patients compared with healthy controls naturally triggers investigation to establish any diagnostic or even screening value. In the metastatic setting, however, the most important aspects are that levels are proven to be comparable between cohorts, that a predictive and prognostic potential is established, and that a definition of an upper normal level is established for further investigations. Different research groups have used different methods which hampers a direct comparison between the studies. Still, in a series of seven independent and consecutively conducted Danish phase II trials in mCRC, the levels of cfDNA

Table 2. Studies investigating the correlation between circulating DNA and carcinoembryonic antigen.

Reference	cfDNA measure	Number of patients	Results of direct correlation between cfDNA and CEA	Comparison of cfDNA and CEA
El-Gayar et al. [15] 2016	DNA II and cfDNA (Alu repeats)	50	$r = 0.45$, $p < 0.001$	NA in relation to outcome Better diagnostic performance AUC 0.90 (DNA II) vs. 0.86 (CEA) Performed better than CEA
Tie et al. [82] 2015	ctDNA (various)	53	NA	Correlation to tumor burden: $r = 0.05$ (ctDNA) and 0.03 (CEA) Correlation to response: AUC 0.73 (ctDNA) and 0.52 (CEA)
Siravegna et al. [83] 2015 Spindler et al. [33] 2015	ctDNA (various) cfDNA (gCYG) ctDNA (KRAS)	10 134	NA $r = 0.37$, $p > 0.05$	Case studies, no visual correlation Performed better than CEA ctDNA strong prognostic marker CEA failed in uni- and multivariate analysis CEA used as measure of tumor burden. ctDNA less likely to be detectable in low CEA patients. Positive correlation between ctDNA and CEA levels in those with detectable KRAS mutations. Association in univariate analysis 0.439 (95% CI 0.22–0.658), $p < 0.001$ Performed better than CEA
Bettegowda et al. [41] 2014	ctDNA (KRAS)	69	NA	NA in relation to outcome Better diagnostic performance AUC 0.89 (DNA II) vs. 0.78 (CEA) Case studies, visual correlation ctDNA mirrors CEA in relation to tumor burden
Hao et al. [20] 2014	cfDNA and DNA II (Alu repeats)	204	NA	Better diagnostic performance (intestinal polyps vs. CRC) AUC 0.848 (cfDNA) vs. 0.68 (CEA) Perform better than CEA in uni- and multivariate analysis* (unpublished data)
Diaz et al. [39] 2012	cfDNA ctDNA (KRAS)	29	NA Case studies, visual correlation ctDNA mirrors CEA, but no statistical analysis performed $r = 0.232$, $p > 0.05$	Better diagnostic performance (intestinal polyps vs. CRC) AUC 0.756/0.772 (cfDNA/DNA II) vs. 0.0.596 (CEA) Diagnostic performance AUC 0.84 (cfDNA) NA for CEA Significantly higher cfDNA levels in patient who died from CRC than cured, performed better in combination with CEA
Qi et al. [65] 2012	cfDNA (Alubased)	31	$r = 0.29$, $p > 0.05$	Better diagnostic performance (intestinal polyps vs. CRC) AUC 0.756/0.772 (cfDNA/DNA II) vs. 0.0.596 (CEA) Diagnostic performance AUC 0.84 (cfDNA) NA for CEA Significantly higher cfDNA levels in patient who died from CRC than cured, performed better in combination with CEA
Spindler et al. [32] 2012, 2014 Mead et al. [66] 2011	cfDNA (gCYG) ctDNA (KRAS) cfDNA (Line1) ctDNA (Alu repeat II)	105 24	$r = 0.29$, $p > 0.05$	Better diagnostic performance (intestinal polyps vs. CRC) AUC 0.756/0.772 (cfDNA/DNA II) vs. 0.0.596 (CEA) Diagnostic performance AUC 0.84 (cfDNA) NA for CEA Significantly higher cfDNA levels in patient who died from CRC than cured, performed better in combination with CEA
Czeiger et al. [67] 2011	(cfDNA) fluorescent analysis	38	$p = 0.18$, $p = 0.29$	Better diagnostic performance (intestinal polyps vs. CRC) AUC 0.756/0.772 (cfDNA/DNA II) vs. 0.0.596 (CEA) Diagnostic performance AUC 0.84 (cfDNA) NA for CEA Significantly higher cfDNA levels in patient who died from CRC than cured, performed better in combination with CEA
Danese et al. [68] 2010	cfDNA (GADPH)	118	$r = 0.028$, $p = 0.78$	Better diagnostic performance AUC 0.93 (cfDNA) vs. 0.68 (CEA) No direct comparison of performed in correlation to clinical outcome after surgery for CRC
Frattoni et al. [69] 2008	cfDNA ctDNA (KRAS and p16 promotor hypermethylation Kit)	70	NA Some correlation by 2x2 cross tabulation	No direct comparison of performed in correlation to clinical outcome after surgery for CRC
Diehl et al. [81] 2008	ctDNA (various) (BEAMing)	18	$r^2 = 0.20$, $p < 0.001$	Performed better than CEA in relation to prediction of recurrence-free survival after surgery ($p = 0.03$)
Boni et al. [70] 2007	cfDNA (RNAse P gene) cfDNA (GADPH)	67 75	NA $rs = 0.16$, $p = 0.17$	Better diagnostic performance (mean values compared in CRC and healthy) Better diagnostic performance AUC 0.86 (cfDNA) vs. 0.82 (CEA), optimal combined (AUC 0.92)
Flamini et al. [71] 2006				Better diagnostic performance (cross tabulation)
Ryan et al. [80] 2002	ctDNA (KRAS2)	78	NA	Better diagnostic performance (cross tabulation)
Shapiro et al. [72] 1983	cfDNA (radioimmunoassay)	63	$r = 0.14$	Better diagnostic performance (cross tabulation, χ^2 -tests)

AUC: area under the curve; BEAMing: beads, emulsion, amplification, and magnetism; CEA: carcinoembryonic antigen; cfDNA: cell-free DNA; CRC: colorectal cancer; ctDNA: circulating tumor DNA; DNA II: DNA integrity index; GADPH: glyceraldehyde 3-phosphate dehydrogenase; KRAS: Kirsten ras; NA: not addressed; RNAse: P ribonuclease P gene.

were measured immediately prior to initiation of therapy. Identical laboratory methods were used, and the analyses showed similar baseline levels in all cohorts of mCRC patients [32,33,54–59]. Furthermore, analysis of samples from a large normal cohort enabled definition of an upper normal limit [57]. In addition, the levels of cfDNA seem stable between different cohorts of CRC patients, (whereas some differences seem to exist between different tumor types as illustrated in the data from Perkins et al. [64]). A systematic review and meta analysis, including more than 1000 patients, indicates a strong and consistent prognostic value of total cfDNA in favor of the patients with the lowest levels of the marker [85]. This opens up for reconsideration of the role of total cfDNA quantification as a prognostic marker in mCRC and a potential predictor of outcome from therapy.

Clinical utility of plasma mutation analysis

A high fraction of colorectal tumors harbors detectable tumor-specific mutations among which KRAS, NRAS and BRAF are the most frequently investigated. The RAS mutations are negative predictors of effect of anti-epidermal growth factor receptor (EGFR) therapy [86]. The question if plasma is acceptable for RAS mutation analysis in the currently used settings seems to be answered by the increasing data showing high concordance between tumor and plasma mutations.

Studies have compared mutation status in tumor and blood, using different methods, varying sample sizes, different clinical settings, and also different time intervals between tumor samples and peripheral blood analyzed. This evidently implies that studies report a broad range of agreement between tumor and blood mutation detection rates [87–93]. A negative discrepancy is generally described as a ‘false’ negative result due to methodological limitations. However, it is well known that tumor heterogeneity is a significant aspect which complicates molecular characterization of the disease. Removal of the primary tumor could theoretically result in removal of a RAS-mutated tumor load. The differences between primary tumor, local lymph nodes and distant metastasis could be of clinical importance, and heterogeneity over time has also been demonstrated for the RAS mutations in metastatic lesions. It has yet to be established whether or not the appearance of new mutations is an overgrowth by clonal selection or de novo appearance.

Consequently, there is a strong rationale for suggesting that the molecular characteristics of circulating DNA is a timely and clinically relevant biological feature of mCRC, and that discrepancies between tumor and plasma status are not only a methodological issue, but carry important information which could prove to have considerable clinical relevance. Yen et al. demonstrated a high concordance between tumor and plasma KRAS status [89], and showed a clinical utility of the plasma KRAS analysis for prediction of cetuximab-irinotecan therapy, in terms of non-inferiority to tumor testing. Xu et al. presented results from 242 patients prior to first-line chemotherapy alone. The concordance was high and data revealed that KRAS mutational disease was associated with a

poor prognosis, but no difference according to source of mutational detection was seen [90]. Three recently published studies in second- [59] and third-line settings [32,33], indicate a non-significant prognostic value of tumor KRAS mutations status, but a clearly enhanced prognostic value when analyzed in plasma samples by qPCR methods. Data also showed that none of the patients with detectable mutations in plasma responded to therapy with or without EGFR inhibition, indicating also a clinical effect in non-EGFR-targeted therapy. This observation was supported by Wong et al. who demonstrated shorter PFS during regorafenib treatment in patients with detectable KRAS mutations in plasma compared with those without such mutations [60]. Finally, results from the phase III randomized CORRECT trial have been published [45], comprising 553 patients who received placebo (166) or regorafenib (337) included in the additional biomarker analysis. Data showed high feasibility in using cfDNA as pre-treatment source for mutation detection. In brief, data support the use of cfDNA to establish tumor genotype at initiation of therapy.

Advanced technology increasingly improves the sensitivity and specificity of mutation analysis, and low-frequency clones have therefore now become detectable in both tumor and plasma samples. This adds further complexity to the investigation of the clinical relevance, for instance the definition of an optimal threshold for the number of mutated alleles in the plasma in relation to clinical outcome. These aspects should be taken into consideration in future investigations, but the current data indicate that cfDNA could provide a more timely and clinically relevant molecular characterization of the disease in the metastatic setting than archival tumor mutational status.

Cell-free DNA and importance of tumor dynamics during therapy

In 2012, two proof of principal studies reported that tumor mutations appear at the time of progression on anti-EGFR treatment. Diaz et al. analyzed tumor and serum from 24 patients with chemotherapy-refractory mCRC prior to and during therapy with monotherapy panitumumab [39]. Nine (38%) patients developed KRAS mutations during the course of therapy, and a significant lead time was seen between radiologically and molecularly determined resistance. In addition, mathematic modeling indicated a likelihood of pre-existing KRAS mutant tumor clones rather than de novo mutations. Similarly, in a small study based on tumor/plasma samples from 10 patients, Misale et al. showed that resistance to EGFR inhibition could be detected several months before radiological evidence of progression was observed [40]. The authors concluded that emergence of KRAS mutations suggested an acquired resistance to EGFR inhibition. These pivotal studies naturally facilitated scientific interest, and a list of studies that have analyzed circulating DNA in serial plasma samples from patients with mCRC is presented in Table 3 [39–41,60,82,83,93,94]. In a Danish cohort [94], results confirmed that mutations not detectable in the primary tumor or pre-treatment blood sample emerged in the

Table 3 List of studies having investigated cfDNA and/or tumor-specific mutations in serial blood samples in metastatic colorectal cancer patients.

Reference	Patients with mCRC	Therapy	Markers	Methods	Results
Misale et al. [40] 2012	3.	EGFR inh.	KRAS	BEAMing	Emergence prior to progression
Diaz et al. [39] 2012	28.	EGFR inh.	KRAS	BEAMing	9/24 developed mutations at progression
Spindler et al. [94] 2014	105	EGFR inh.	KRAS, BRAF cfDNA	qPCR	Emergence of mutations prior to progression Decrease of cfDNA and plasma mutations in responders, no decrease in non-responders
Bettegowda et al. [41] 2014	24 (20 from ref. Diaz)	EGFR inh.	Multiple alterations	BEAMing	A total of 70 mutations emerged in the 24 patients
Mohan et al. [93] 2014	10	EGFR inh.	Multiple alterations	Plasma-seq	Gain of KRAS mutations in 4 patients Acquired KRAS copy number changes 4/10
Tie et al. [82] 2015	53	First-line chemotherapy	KRAS	Digital genomic assay Safe-SeqS	Spike Day 3 in 3 responders 10-fold drop correlated to good response
Wong et al. [60] 2015	35	Regorafenib	Multiple alterations	BEAMing	Decline of ctDNA in responders, and those with long PFS Emergence of new mutations?
Siravegna et al. [83] 2015	16 2 3	EGFR inh.	Multiple alterations	ddPCR NGS	Emergence of mutations prior to progression Decline after withdrawal Potential for re-challenge illustrated

BEAMing: beads, emulsion, amplification, and magnetics; BRAF: proto-oncogene B-Raf; cfDNA: cell-free DNA; ctDNA: circulating tumor DNA; EGFR inhibitor: epidermal growth factor receptor inhibitor; KRAS: Kirsten ras; qPCR: quantitative PCR.

plasma samples prior to radiologically detected evidence of progression in 5/45 primary wild type (wt) patients treated with third-line irinotecan-cetuximab. In addition, patients with primary tumor mutations but absence of mutations in pretreatment plasma samples showed benefit from anti-EGFR inhibition in terms of long stabilization of their disease. These studies focused on the mechanisms of resistance to EGFR inhibition as provided by acquisition/growth of specific mutations. Interestingly, an Italian group demonstrated that the KRAS mutant clones fluctuated according to therapy with a relative increase in altered KRAS alleles at the time of progression and a subsequent decline upon withdrawal of the EGFR blockade. This was interpreted as dynamic clonal competition and molecular evidence that re-challenge with EGFR inhibitors after a withdrawal period could be relevant for some patients, which was confirmed in a few presented case stories [83].

Lately, several other downstream alterations have been added to the panel of detectable mutations. Bettegowda et al. published data from a large cohort of patients with different solid tumors of late and early stages, including a subset of 24 patients with mCRC treated with anti-EGFR monoclonal antibodies [41]. The study confirmed that monitoring patients for resistance-conferring mutations in ctDNA, including the KRAS, BRAF, NRAS, EGFR, PIK3CA genes, was feasible in 96% of patients. Another study showed emergence of a BRAF V600 mutations in the plasma during cetuximab-based combination chemotherapy [93], similar to previous observations [94].

Along with the search for relevant genetic alterations methodological development has been introduced, including the use of deep sequencing of plasma samples from 10 patients with primary wt KRAS disease also treated with Pmab/Cmab [95]. In this study, KRAS amplifications rather than mutations were detected at the time of progression and

were suggested to be a previously under-investigated mechanism of EGFR resistance. In a recent report, newly detectable mutations were demonstrated with highly sensitive BEAMing analysis in plasma from a total of 32/62 patients, including 11 with multiple new mutations [78]. When 20 tumor samples from the 27 cases with new detected plasma KRAS mutations were re-analyzed, seven (36%) tested positive for low-frequency sub-clones, indicating that the developed mutations derive from rare pre-existing clones in the primary tumor tissue, and that the relative clonal burden may develop over time.

Whether these findings apply to EGFR inhibitor treatment only or are a more general biological phenomenon in CRC remains to be determined. Only a few studies have investigated cfDNA dynamics in relation to non-EGFR therapy for mCRC. The dynamics of tumor pharmacodynamics and cfDNA was investigated in 35 patients who were treated with regorafenib for chemotherapy-refractory disease [61]. Patients with a longer PFS showed an earlier decline in mutant DNA fraction than did patients with a shorter PFS. This finding was in line with the Danish observation of a significant decline of total cfDNA and pKRAS alleles in responding patients, but not in non-responders [94]. A recent study investigated the quantitative levels of ctDNA during standard first-line chemotherapy prior to the first cycle, three days after treatment, and prior to the second cycle. The significant reduction in ctDNA prior to the second cycle correlated with radiological responses at 8–10 weeks, suggesting that measurement of ctDNA can be used for early tumor response to first-line chemotherapy [82]. In addition, four patients had a spike in ctDNA at Day 3, which rapidly declined. Remarkably, all three patients showed an excellent response to therapy.

In conclusion, detection of new mutations in circulating DNA and increasing levels of mutated alleles seem to reflect disease progression, whereas a rapid decline after initiation

of therapy could reflect an excellent response to treatment. Furthermore, a decline post-EGFR treatment may indicate a potential benefit from re-induction of therapy. The above studies represent some of the most important reports within this field and all harbor evidence of considerable clinical potential. However, most of the presented studies are limited by a retrospective single design based on small sample sizes, and their findings need to be investigated further in prospective clinical studies with adequate samples sizes.

Conclusions and future perspectives

Analyzing circulating nucleic acids in plasma and serum has great clinical potential in mCRC, and methodological development will continue to advance the field towards ever more sensitive, feasible and reliable quantification of total cfDNA, for detection of mutations and quantification of the mutated fraction of alleles. Recent data suggest that the total cfDNA levels hold overall prognostic importance in mCRC are related to disease burden, and moreover reflect important biological mechanisms in advanced cancer disease. Plasma can be used prospectively as a source for selection of EGFR inhibitor therapy, and monitoring patients during therapy with EGFR inhibitors could become an important clinical tool in achieving better palliative treatment. However, sufficient evidence of true primary resistance or de novo acquired mutations has yet to be established. More importantly, the true clinical threshold for cfDNA needs to be established. The role of cfDNA and tumor-specific mutations in relation to chemotherapy alone should be reconsidered and investigated further as it has not yet been established if the tumor-specific mutations confer resistance to a specific therapy or constitute a more general biological feature of heterogeneous disease behavior.

In future, international cooperation to validate and standardize methods for DNA purification and cfDNA analysis is strongly encouraged. Furthermore, prospective clinical trials investigating pre-defined clinical aspects such as selection criteria for EGFR inhibitor therapy, palliative chemotherapy and tools for monitoring during therapy should be designed with great care.

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