IMPACT OF CIRCULATING TUMOR DNA (CTDNA) IN PATIENTS WITH GASTROINTESTINAL MALIGNANCIES

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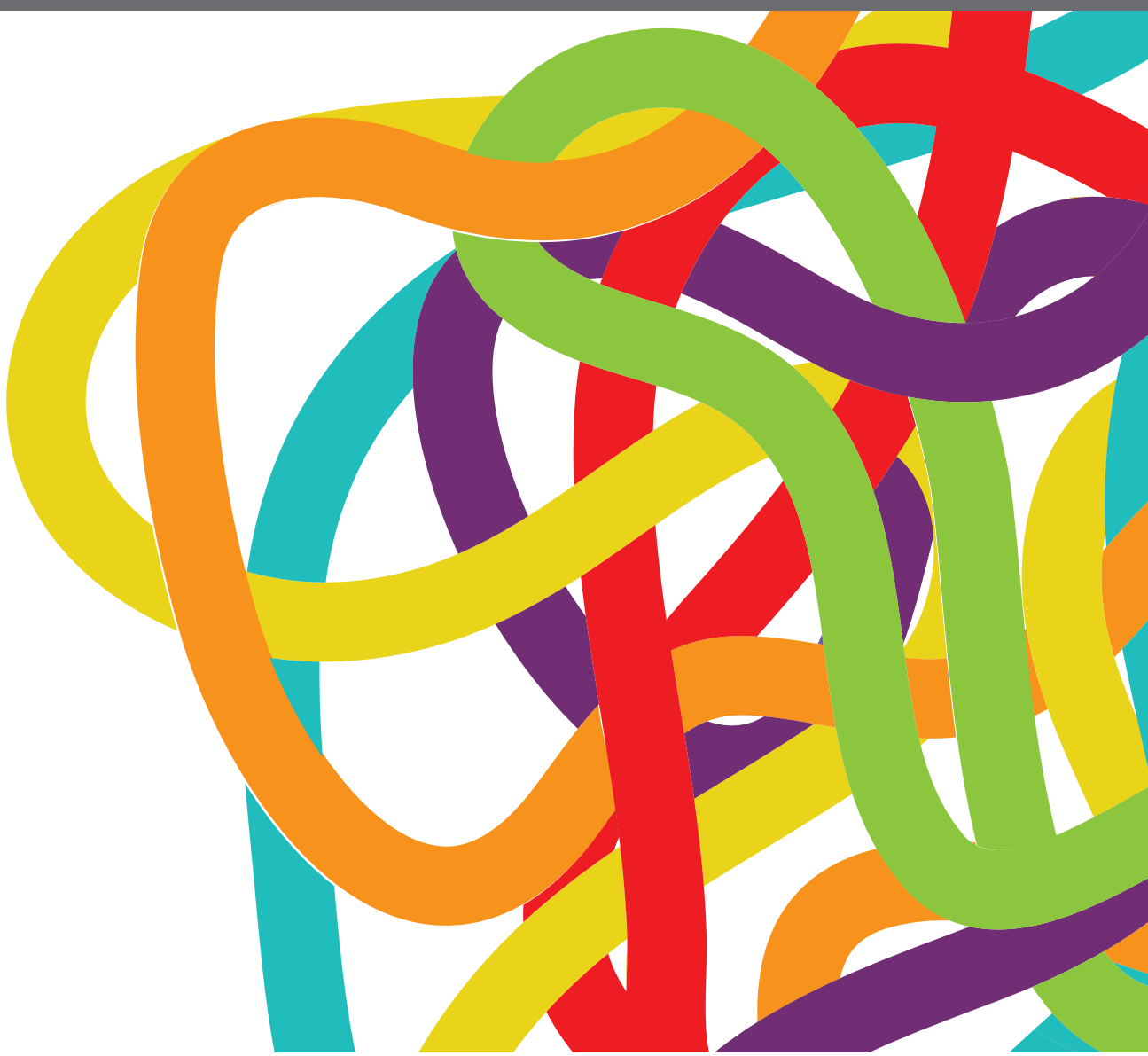

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IMPACT OF CIRCULATING TUMOR DNA (CTDNA) IN PATIENTS WITH GASTROINTESTINAL MALIGNANCIES

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REVIEW
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A Review of Circulating Tumor DNA in Hepatobiliary Malignancies

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Circulating tumor DNA (ctDNA) is released into circulation (blood) specifically from tumor cells undergoing metabolic secretion, apoptosis, or necrosis, carries tumor-specific genetic or epigenetic alterations. Technologies enabling clinical evaluation of ctDNA continue to advance rapidly and allow for the assessment of patient-specific tumoral genetic and epigenetic alterations. This holds great potential for earlier detection of disease, serial monitoring of tumor heterogeneity, identification of therapeutic targets, and evaluation of treatment response and mechanisms of resistance. Hepatobiliary malignancies are often diagnosed late, recur commonly, yield limited available tumor on biopsy, and harbor several genomic alterations with potential therapeutic impacts. Patients suffering from or at risk for these diseases thus stand to benefit immensely from this technology. Herein, we review the limited literature pertaining to the potential for ctDNA technologies in such patients. Patients with these cancers stand to benefit greatly from the application of ctDNA technologies, and concerted efforts at further investigation of such are ongoing and greatly needed.

Keywords: cholangiocarcinoma, hepatocellular carcinoma, circulating tumor DNA, liver neoplasms/blood, liver neoplasms/genetics

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INTRODUCTION

Circulating genetic material is made up of exosomes, tumor-educated platelets, circulating tumor cells, microRNA, and cell-free DNA (cfDNA) (1, 2). The content of cfDNA is predominately short, double-stranded fragments of nuclear and mitochondrial DNA. While healthy individuals have cfDNA detectable in their serum that is released from normal cellular processes, the cfDNA in cancer patients is composed of DNA fragments released from normal and cancer cells. Circulating tumor DNA (ctDNA) is a component of cfDNA found in cancer patients composed of DNA released into circulation specifically from tumor cells that undergo metabolic secretion, apoptosis, or necrosis (**Figure 1**). Serum samples generally yield more cfDNA, but the additional material above and beyond ctDNA is derived from, for example, leukocyte lysis during clotting, which thus dilutes the ctDNA content. There are various techniques available to extract ctDNA from the plasma of cancer patients, and these methods vary in their ability to purify fragments of different sizes, thus changing the total quantity of cfDNA isolated and the fraction of ctDNA captured (3). Discriminating ctDNA from normal cfDNA is aided by the fact that tumor DNA is defined by the presence of mutations. These mutations are present only in the genomes of cancer cells or precancerous cells and are not present in the DNA of normal cells. This affords ctDNA significant biologic specificity as a biomarker (4, 5). The ability to detect and characterize ctDNA enables a wide array of practical clinical applications that are not possible with routine sequencing of tumor tissue or with other circulating biomarkers (4).

Circulating tumor DNA carries tumor-specific genetic or epigenetic alterations, such as point mutations, copy number variations, chromosomal rearrangements, and DNA methylation patterns.

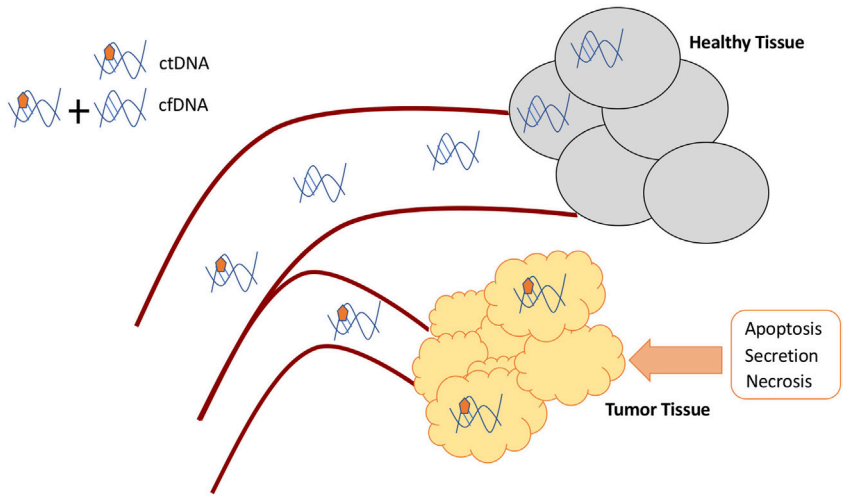

FIGURE 1 | Circulating DNA generation and differences.

PCR-based (digital PCR) and next-generation sequencing (NGS)-based methods are two dominant approaches in this field for analysis of ctDNA (5). Digital PCR approaches are highly sensitive but can only examine a single or a few mutations of interest at any one time. Sequencing-based approaches have the ability to look at a number of genes at a whole-genome or whole-exome level; however, these techniques are currently limited due to detection rates that approach error rates of PCR and sequencing technology. Capture-based NGS has the ability to enrich genomic regions of interest by hybridizing target genes/regions to antisense oligonucleotides before sequencing; this approach allows for agnostic analysis of large portions of the genome and can identify multiple mutations with increased sensitivity (6).

The evaluation of ctDNA enables assessment of patient specific tumoral genetic and epigenetic alterations and offers a unique opportunity for serial monitoring of tumor genomes in a non-invasive, convenient, and accurate manner. Potential applications of ctDNA testing in patients with cancer include (a) early detection of disease, (b) monitoring of tumor heterogeneity, (c) identification of therapeutic targets, (d) real-time evaluation of treatment response and tumor relapse, and (e) real-time assessment of evolution of drug resistance (4). Along with significant advancements of sequencing technology in recent years, an equal effort and investment are underway to optimize ctDNA use for routine clinical practice.

Hepatobiliary (HPB) malignancies including hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) stand to gain immensely from the use of ctDNA given that (a) diagnosis currently is more often made at advanced stages of disease, (b) recurrences are common despite pursuit of potentially curable treatments such as surgery, (c) biopsies are not always obtained or often yield suboptimal quantities of tumor cells and thus insufficient tumor DNA for tissue-based genomic profiling, and (d) multiple genomic alterations which are targetable with therapeutics currently in the clinic demonstrating significant efficacy are known to occur in disease such as CCA.

HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma is a lethal liver malignancy with an exceptionally high incidence in Asia and Africa. The number of new cases in many countries is rapidly increasing, making HCC a worldwide health problem (7).

The diagnosis of HCC can often be made using non-invasive imaging such as ultrasonography, computed tomography (CT), and magnetic resonance tomography (MRI), aided by the use of LiRADS criteria, along with the measurement of alpha-fetoprotein (AFP) level, a predictive biomarker for HCC (8). Given the ability to use non-invasive techniques to make a diagnosis, invasive biopsy is less commonly pursued and considered to make a diagnosis of HCC only when imaging tests are less confident in a particular case. As a result, pre-treatment tissue sampling is rarely available for genomic profile analysis. However, imaging tests can only determine HCC with confidence when nodules grow to over 1 cm in size. In addition, the use of AFP to aid in a confident diagnosis is not always possible given that not all HCC can produce elevated levels of AFP (9).

Early-stage HCC are currently difficult to diagnose and characterize, but can be effectively treated by surgical resection with a 5-year survival rate of 90% (10). Other than surgical resection, several options exist for definitive management of disease including liver transplantation, transarterial chemoembolization, radiofrequency or microwave ablation, or radioembolization. Unfortunately, however, a considerable proportion of patients are still diagnosed with advanced disease for which treatment options have been limited and prognosis remains poor.

Early Detection

A large part of the potential of ctDNA use in cancer, is the possibility to use it for earlier detection of disease thus enabling institution of more effective, potentially curative treatment approaches. In the case of HCC, a few studies have evaluated ctDNA use for this purpose by evaluating the ability to detect ctDNA-specific genomic alterations linked with HCC (**Table 1**). Ser249 of *TP53*

TABLE 1 | Circulating DNA biomarkers explored in hepatocellular carcinoma.

Early detection	Diagnosis/prognosis
TP53 Ser249	Cell-free DNA levels
CTNNB1	GSTP1
	hTERT
	TP53

has been the most reported mutation hotspot in HCC patients, and mutation of this site leads to a defect in TP53-specific DNA-binding ability (11–13). Huang et al. demonstrated the ability to detect, in ctDNA, the presence of this mutation in patients residing in the Qidong area of China exposed to aflatoxin and with high prevalence of hepatitis B virus carriers. The mutation was found in 40% of HCC cases, 20% of cirrhotics, and 7% of healthy controls, with an adjusted odds ratio of 22.1 for HCC cases compared with controls. They suggested that the detection of this mutation in ctDNA testing was potentially a method for early diagnosis in this population (14). The presence of the same mutation was evaluated in ctDNA in a population of patients with similar exposure to aflatoxin and hepatitis B in Gambia, Africa. This group, in particular, compared the tissue and ctDNA detection rates of this particular mutation and noted a concordance between tumor tissue and matched plasma of 88.5% (15). A group in Egypt also examined the presence of the same TP53 mutation, in addition to mutations in CTNNB1, in cfDNA of patients with HCC or chronic liver disease. Circulating DNA concentrations were significantly higher in HCC patients compared with HBV and HCV carriers without cancer, and to seronegative individuals. However, their results regarding detection of Ser249 TP53 mutations did not parallel those from prior studies (16). Interestingly, this Ser249 mutation has also been detected in noncancerous hepatic tissues of HCC, in the plasma DNA of a minority of healthy individuals, and in patients with relatively more severe cirrhosis (17, 18). Importantly, these results highlight the potential of ctDNA as a part of early detection strategies for particular populations at higher risk for HCC, though clearly much work is necessary to identify sensitive genomic targets in particular high-risk populations, and to validate these alterations as highly sensitive targets for enabling early diagnosis.

Diagnosis and Prognosis

In addition to its potential in the setting of early diagnosis of disease, evaluation of ctDNA has potential as a tool to assist with the diagnosis and prognostication of HCC at other stages of disease too, including diagnosis of disease in particular higher risk populations. In addition, given the risk of HCC recurrence after potentially curative treatment strategies, such as surgery, there may be value to the use of ctDNA in post-treatment surveillance. The value of circulating DNA evaluation in regard to prognosis has also been evaluated. Both circulating DNA level and the presence of specific gene alterations have been shown to be potential prognostic markers, indicating higher risks of disease recurrence and shorter survival (**Table 1**).

First, in the realm of using circulating DNA as a diagnostic tool, one study evaluated a cohort of 96 patients with HCV-related HCC and in 100 HCV carriers without known HCC and validated the finding that serum cfDNA levels were significantly higher in HCC patients than in HCV carriers ($P < 0.0001$). To investigate the value of circulating DNA when combined with other blood-based biomarkers, another study evaluated the power of combined detection of circulating cfDNA, AFP, and α L-fucosidase (AFU) for diagnosis of HCC in serum samples from 39 HCC patients and 45 normal controls. cfDNA levels in HCC patients were significantly higher than that in normal controls ($P < 0.05$). Quantitative analysis of cfDNA was found to be sensitive and feasible, and the combined detection of cfDNA with AFP or AFU or both was found to improve the diagnostic sensitivity for HCC (19). A meta-analysis evaluating published results regarding qualitative and quantitative analyses of circulating cfDNA in HCC and the use of cfDNA values for HCC diagnosis investigated three subgroups: qualitative analysis of abnormal concentrations of cfDNA, qualitative analysis of single-gene methylation alterations, and multiple analyses combined with AFP. A total of 2,424 subjects included 1,280 HCC patients in 22 studies were included. The pooled sensitivity and specificity of quantitative analysis were 74 and 85%, respectively. For qualitative analysis, the sensitivity and specificity were 53.8 and 94.4%, respectively. After combining with AFP assay, capabilities improved, with the values being 81.8 and 96%, respectively (20).

As a diagnostic tool Iizuka et al. evaluated the use of a real-time PCR assay for levels of the glutathione *S*-transferase pi (GSTP1) gene in cfDNA in the blood of 52 patients with HCC associated with HCV, 30 HCV carriers without known HCC, and 16 HCV-negative non-cancer patients. cfDNA levels were significantly higher in HCC patients than in HCV carriers or the control subjects with a sensitivity of 69.2% and a specificity of 93.3% in discriminating HCC and HCV carriers (21). Another study sought to evaluate the use of cfDNA, focused on a particular gene, hTERT, as a diagnostic and prognostic tool in HCC. In 142 plasma samples obtained from 66 patients with HCC, 35 with cirrhosis, and 41 with advanced HCV-related chronic hepatitis, cfDNA was documented in the plasma of 22% of chronic hepatitis patients, 57% of those with cirrhosis, and 61% of HCC patients. Patients with multinodular HCC showed significantly higher levels of cfDNA ($P = 0.05$), and survival was significantly longer in patients with cfDNA below than in those above the cutoff value (37 versus 24 months, $P = 0.03$) (22).

In regard to the use of circulating DNA in the post-operative setting, another study evaluated cfDNA levels in 87 patients who had undergone curative-intent hepatectomy for HCC. They found that those with a high cfDNA level post-operatively had a significantly shorter overall survival (OS) time compared with those in whom the cfDNA level was not high. cfDNA level was determined to be an independent prognostic factor for OS and cancer recurrence in distant organs (23). Ono and colleagues enrolled 46 patients with HCC who underwent hepatectomy or liver transplantation and evaluated the cumulative incidence of recurrence and extrahepatic metastasis in the ctDNA-positive group, noting that it was statistically significantly worse than in the ctDNA-negative group ($P = 0.0102$ and 0.0386, respectively) (24). Another study evaluated a gene-specific approach, seeking to specifically detect p53 mutations in the cfDNA of transplanted HCC patients and to determine the utility of this method in the diagnosis of HCC tumor recurrence. In a group of 24 liver-transplanted HCC patients, compared with a group

of healthy controls, it was indeed (a) possible to detect mutated p53 genes in cfDNA and (b) this was noted to be useful as a biomarker of tumor recurrence during the clinical evolution of transplanted patients (25). In yet another study, Ren et al. sought to quantify the circulating DNA in pre-operative plasma from 79 patients with HCC before operation, 20 patients with liver cirrhosis, and 20 healthy volunteers, and assess for an association between circulating DNA level and prognosis of HCC patients. Circulating DNA level was closely associated with tumor size ($P = 0.008$) and TNM stage ($P = 0.040$) and was negatively associated with the 3-year DFS ($P = 0.017$) and OS ($P = 0.001$) (26).

Treatment

Perhaps one of the most exciting and explored areas of potential for ctDNA across cancer types has been as a more non-invasive, comprehensive tool to enable precision medicine as a therapeutic reality for some patients. As regards the use of circulating DNA for the purposes of treatment, work has been ongoing to optimize cfDNA/ctDNA's capabilities to provide comprehensive genomic profiling of potential therapeutic targets and also to monitor disease response on treatment. To enable its use in clinic on a routine basis, it is necessary to prove high concordance with the gold standard, tissue-based profiling, for one. Little has been reported in the literature with regard to tissue and circulating DNA mutation analysis concordance, unfortunately. In one study, from data in 105 patients with GI malignancies, some with HCC, overall concordance rates of 96, 94, 95, and 91%, respectively, were found between ctDNA and tissue biopsy in the four most common alterations (KRAS amplification, MYC amplification, KRAS G12V, and EGFR amplification) (27). One small study performed whole-exome sequencing and targeted deep sequencing (TDS) in 32 multiregional tumor samples from five patients. Matched cfDNA was sequenced accordingly. Although the genome profiling efficiency of cfDNA increased with sequencing depth, an average of 47.2% total mutations were identified using TDS, suggesting that tissue samples outperformed it. Optimistically, 38.6% of patients carried mutations that were considered potential therapeutic targets (28). Focusing on 574 cancer genes known to harbor actionable mutations, another small study in 3 patients identified the mutation repertoire of HCC tissues and monitored the corresponding ctDNA features in blood samples to evaluate its clinical significance. Analysis revealed that ctDNA could overcome tumor heterogeneity and also provided information regarding tumor burden and prognosis. Analysis on a fourth HCC case with multiple lesion samples and sequential plasma samples identified 160 subclonal SNVs in tumor tissues and matched peritumor tissues with PBMC as control. 97% of this patient's tissue mutations could be also detected in plasma ctDNA. Many mutations also showed circulating levels correlating to cancer progression (29).

In terms of evaluating the landscape of genomic alterations in HCC, through the eyes of ctDNA, again there are limited reported data. A notable recent study in 213 patients with advanced gastrointestinal cancers sought to assess the utility of ctDNA detection across a panel of 68 genes with a commercially available assay, with HCC patients representing 15% of the study's population. The majority of patients (58%) had >1 characterized alteration (excluding variants of unknown significance), with a median number of characterized alterations being 1 (range, 0–13). The number of detected alterations per patient varied between different cancer types: in HCC, 74% of patients had >1 characterized alteration, versus 24% of appendiceal adenocarcinoma patients. Of the 123 patients with characterized alterations, >99% had one or more alterations potentially actionable by experimental or approved drugs. These observations from this large study suggest that many patients with gastrointestinal tumors, including difficult-to-biopsy malignancies like hepatocellular cancers, frequently have discernible and pharmacologically treatable ctDNA alterations (27).

Overall, the existing literature is still quite limited but, with this caveat, thus far demonstrates that the use of ctDNA for genomic profiling in HCC is feasible and may provide a tissue biopsy-free alternative in these difficult-to-biopsy patients. That being said, further study of the clinical validity and utility is needed.

CHOLANGIOCARCINOMA

Cholangiocarcinomas are malignant tumors arising from cholangiocytes that form the epithelium of the biliary system (30). Tumors are traditionally classified by location as intrahepatic (iCC), perihilar (pCC), and extrahepatic (eCC) based on their presumed site of origin within the biliary ducts. While this anatomic classification seems simplistic, it is effective in differentiating biliary tumors in terms of epidemiology, etiology, clinical presentation, and treatment (30). As with HCC, early diagnosis is ideal given that surgical resection or liver transplantation, offers the patient the best chance at cure. However, the majority of patients diagnosed with this malignancy have advanced stage disease precluding surgical management.

While CCA is a rare malignancy accounting for approximately 3% of gastrointestinal cancers, its incidence has been rising steadily in the US (31–33). The disease is more prevalent in many countries of the Asian continent especially. Several risk factors for CCA have been described with most etiologies producing increased risk for cancer associated with long-standing inflammation (33). In Asia, long-standing biliary inflammation due to infection with biliary flukes *Opisthorchis viverrini* and *Clonorchis sinensis*, as well as chronic hepatolithiasis, are commonly associated with CCA. Chronic hepatitis C and B infection are also known to increase the risk for CCA. In Western countries, long-standing inflammation associated with primary sclerosing cholangitis (PSC), fatty liver disease, cholelithiasis, and smoking all are associated with increased risk (33).

Diagnosis and Prognosis

The diagnosis of CCA can be challenging. Cross-sectional imaging using a combination of ultrasound, CT, and magnetic resonance imaging (MRI) is often important for lesion identification and localization. Blood-based biomarkers, most commonly Ca19-9, may also be helpful though they are elevated in just 60–65% of CCA patients. The utility of this marker is also limited by the large number of CCA patients with normal CA19-9, as well as elevations seen in a number of benign conditions such

as PSC and biliary obstruction (34). Histologic confirmation of malignancy can be challenging, particularly in patients with PSC and biliary strictures. Brushings and biliary cytology can be occasionally obtained through endoscopic cholangiography, but its clinical yield can be low and insufficient especially for DNA extraction to enable genomic profiling. Pathologic interpretation of the cytology can be challenging particularly in the presence of inflammation. In addition, the desmoplastic nature of many CCA tumors also contributes to limitations of yield. For the above reasons, the potential is great for ctDNA as a means of diagnosis, in addition to prognosis.

The use of ctDNA in the diagnosis of CCA has particular interest due to the difficulty in diagnosing this malignancy in patients with inflammatory conditions and/or strictures. Obtaining sufficient cytologic material to confirm a cancer diagnosis is challenging, let alone acquiring enough additional material with which to perform genomic analyses. Andersen and Jakobsen utilized a multiplex digital PCR assay to screen for 31 mutations in *KRAS*, *NRAS*, *BRAF*, and *PIK3CA*. The accuracy of the assay was first confirmed in pooled normal serum and positive controls developed by site-directed mutagenesis (35). The authors then conducted the assay on serum of five CCA patients with known tumor mutations and 6 patients who were known to be wild type for the assayed mutations. The assay correctly identified the five known mutations while none of the six wild-type samples had mutations identified in cfDNA. While this multiplex mutation analysis appears to have good results for cfDNA, the applicability of this assay for CCA may be limited since the frequency of *KRAS, BRAF*, and *PIK3CA* are just 12, 4, and 6%, respectively, in The Cancer Genome Atlas (TCGA) analysis (36).

Investigations into the use of ctDNA in CC have been hampered by the rarity of the disease and the relatively incomplete understanding of the genetics of this cancer. The recent characterization of the CCA genome by several studies including that utilizing data from a commercially available tissue-based assay (37), in addition to TCGA analysis, has not only enhanced our understanding of the breadth of targetable somatic alterations in this cancer but also identified important target genes and subsets of tumors based on molecular profile (36 37). In fact, based on this work, a number of novel-targeted therapeutics have emerged and are in clinical trials for patients with CCA.

Genomic alterations in FGFR2 are found in up to 40% of CCAs. The most common form of alteration is a gene fusion products that join the 5′ exons containing the kinase domain to 3′ partner genes with fusions to *BICC1, AHCYL1, TACC3, MGEA5, KIAA1598, FRK, PPHLN1*, or *C10ORF118* (38). Goyal et al. analyzed ctDNA collected by serial sampling in patients enrolled in a Phase 1 study of BGJ398, a FGFR inhibitor (39). Among 32 patients screened, 9 (28%) had FGFR2 fusions detected and 4 patients were enrolled in the trial. Sequencing of the FGFR portion of the fusion genes were compared at enrollment and after progression in three cases. In all three cases, post progression sequencing of the FGFR2 gene demonstrated *de novo* point mutations that conferred resistance to BGJ298 (39). While certainly a small study, this publication provides insight into the significant potential of ctDNA analysis to monitor and predict treatment responses by evaluating the accumulation of mutations that confer treatment resistance.

The topic of ctDNA in CCA as a whole remains a vastly underexplored area, yet one with significant clinical potential. The difficulty in obtaining adequate tissue biopsies provides a challenge not only to obtain molecular characterization but also to confirm malignancy. With our recently enhanced understanding of the genomics of this disease and the real, emerging options of targeted therapies for a number of the genomic subtypes of CCA, ctDNA continues to be a tantalizing option for tumor characterization and monitoring, but significant study is necessary going forward to realize this potential.

CONCLUSION

Hepatobiliary malignancies are uncommon and devastating malignancies whose incidences are on the rise globally. Though the current literature is quite limited, ctDNA is a promising tool with great potential for application in the detection and management of these malignancies. This review provides a summary of our existing knowledge regarding circulating DNA in the realm of HPB malignancies and seeks to highlight the potential of this tool in these patients. Ongoing and future investigations are encouraged and should seek to prove ctDNA's capabilities in patients suffering from and those at risk for these devastating diseases.

AUTHOR CONTRIBUTIONS

Conceptualization; manuscript writing and editing: KM and SC.

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CASE REPORT
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Secondary Germline Finding in Liquid Biopsy of a Deceased Patient; Case Report and Review of the Literature

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Liquid biopsies are increasingly used in the care of patients with advanced cancers. These tests are used to find mutations and other genomic alterations, quantify these findings over time, and guide treatment. It is not unexpected that germline mutations contributing to the development of cancer can be identified in cell-free DNA. Consequently, increased use of liquid biopsies has resulted in subsequent rise of secondary identification of germline mutations. Clinicians need to be aware of this potential use of liquid biopsies and the need to evaluate the patient and family members for confirmation. Our case documents a deceased patient's liquid biopsy result that was confirmed as a germline mutation through a methodical work-up of the patient's family members. Here, we present the case and provide a brief review of pertinent literature.

Keywords: cell-free DNA, germline mutation, liquid biopsy, hereditary cancer syndromes, pancreaticobiliary neoplasms

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BACKGROUND

Tissue biopsies have been the gold standard for diagnosis in the field of oncology. Traditionally, a single biopsy was obtained to establish the cancer diagnosis and was primarily focused on understanding the site of cancer. With advancements in the understanding of the role of DNA alterations as a primary driver of tumorigenesis, the initial biopsy is now also used to assess the genomic alterations inherent to the tumor and ultimately to help guide therapeutic decision-making. However, the heterogeneous nature of cancer limits the ability to capture the spatial and temporal heterogeneity in a single baseline biopsy (1). Liquid biopsies, utilizing plasma derived cell-free circulating tumor DNA (cfDNA) have the ability to identify tumor derived somatic alterations with high concordance to tissue biopsy, similar patient outcomes as those with tumor identified somatic alterations and have the added advantage of being minimally invasive with the ability to capture evolving intra- and inter-tumoral mutations in patients with metastatic disease (2, 3). Given these features, cfDNA is increasingly being used to guide the use of targeted treatments in patients with newly diagnosed advanced cancers and those progressing on targeted therapies who may have developed resistance to therapy (4–7). Emerging areas of clinical use and active areas of research include: the utilization of cfDNA as an alternative cancer biomarker of tumor burden, to monitor disease progression, to detect metastasis, and to monitor response to therapy (8, 9).

Comprehensive cfDNA analysis utilizes next generation sequencing (NGS) to sequence both normal circulating leukocytic DNA, as well as the small proportion of cfDNA that is tumor derived.

The differentiation between somatic and germline mutations has been studied in tissue-based NGS (10, 11); however, there is limited published data on secondary germline findings of cfDNA by liquid biopsy.

Here, we describe the case of now deceased male with pancreatobiliary carcinoma with a secondary identified *BRCA2* alteration in cfDNA (Guardant360®). The finding led to subsequent familial testing and to the identification of a familial hereditary breast and ovarian cancer syndrome (HBOC).

CASE

A 39-year-old Hispanic male of Salvadoran ancestry and no significant past medical history and a nonspecific family history of cancer, presented to the hospital with epigastric abdominal pain, nausea, and vomiting. Abdominal ultrasound showed multiple hypoechoic hepatic masses measuring up to 4.5 centimeters (cm) and the appearance favored metastatic disease. A follow-up computed tomography scan of chest, abdomen, and pelvis showed bilateral pulmonary embolus, retroperitoneal lymphadenopathy, and re-demonstration of the hepatic lesions (**Figure 1**). The patient underwent an ultrasound-guided liver biopsy, with pathology showing moderately to poorly differentiated adenocarcinoma with immunohistochemical stains favoring pancreatobiliary origin. A subsequent esophagogastroduodenoscopy and colonoscopy identified no definite primary malignancy. Due to the small amount of tumor tissue obtained on biopsy, comprehensive cfDNA analysis (Guardant360) was ordered with the goal of finding a targetable therapeutic mutation.

Over the 2 weeks following his clinical evaluation, the patient's symptoms worsened and he was re-admitted to the hospital for intractable nausea and vomiting, abdominal pain, and subjective fever and chills. Further workup showed no evidence of bowel obstruction; however, the findings were highly suspicious for ischemic enteritis due to tumor obstruction of the portal vein. Given patient's extremely debilitated state and poor performance status with an ECOG of 3, he was deemed not to be a candidate for further systemic therapy. He was discharged to home on hospice care and died within a few days.

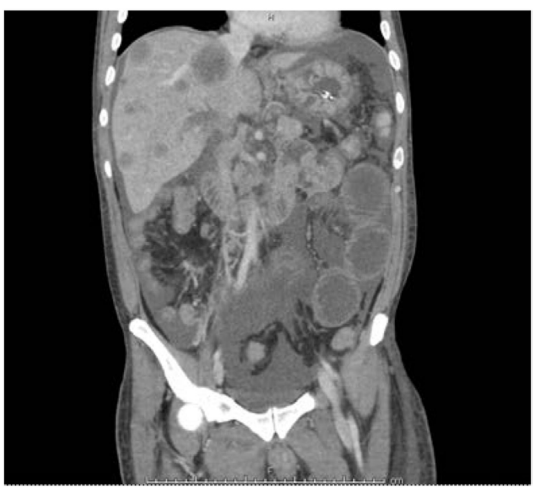

FIGURE 1 | Frontal view panel showing liver metastasis and thickening of deuodenum and jejunum.

Guardant360 is a New York State Department of Health-approved comprehensive cfDNA NGS assay that evaluates tumor derived genomic alterations in up to 73 genes and is performed at Guardant Health (Redwood City, CA, USA), a CLIA certified, College of American Pathologists (CAP) accredited laboratory. The gene list was selected to prioritize the identification of genomic alterations that are actionable—therapeutically targetable for an approved or late stage therapy, prognostic or predictive of therapeutic response, or informative of the presence of tumor-derived cfDNA. Point mutations in 73 genes, small insertions and/or deletions (indels) in 23 genes, copy number amplifications (CNAs) in 18 genes, and fusions in six genes are evaluated. single-nucleotide variants (SNVs), indels, and fusions are reported with a corresponding mutant allele fraction (MAF), calculated as the percentage of calls at a specific genomic position that are mutant over those that are wild type or mutant [mutant/(mutant + wild type)]. The reportable range for SNVs, indels, fusions, and CNAs is ≥0.04, ≥0.02, ≥0.04, and ≥2.12 copies, respectively (12). The median (or 50th percentile) MAF across more than 5,000 clinical samples tested on Guardant360 is 0.39%.

cfDNA was resulted after the patient's death and were notable for the following four alterations and their corresponding MAF: *BRCA2* R2520* (66.02%), *TP53* L344P (20.92%) and R337G (19.26%), and *MET* Y989fs (0.21%) (**Table 1**). The *BRCA2* MAF twofold higher than the *TP53* MAF and within the range suspicious for germline variants. This finding, in combination with the patient's young age at cancer diagnosis and nonspecific maternal family history of an early onset abdominal malignancy, raised the suspicion for a hereditary cancer syndrome.

The genetic counselor was contacted by the medical oncologist to discuss the identification of the potentially germline *BRCA2* alteration identified in cfDNA. ClinVar[1] and PubMed[2] were both searched to determine if this particular alteration had been previously identified in the literature as a pathogenic germline mutation. R2520* corresponds to dbSNP:rs80358981 and in the clinical literature is reported as c.7558C > T (p.Arg2520*) or as 7786 C > T (R2520X). This nonsense mutation is located in exon 15 of the *BRCA2* gene and creates a premature stop codon. It is classified as pathogenic in ClinVar by all reporting clinical laboratories as well as by ENIGMA curation (13). A literature review found multiple publications that included reports of families with this mutation (14–16) and confirmed clinical history of HBOC.

The patient's medical oncologist reviewed the cfDNA results with the deceased patient's wife and offered her a genetics consultation to further discuss the findings and their potential implications. The patient's wife was very interested in obtaining more information and a consult was scheduled with the medical oncologist and genetic counselor. During this visit, a discussion was held as to the role of somatic and germline mutations in cancer etiology and subsequently the parents contacted the counselor within 2 weeks of the initial

[1] www.ncbi.nlm.nih.gov/clinvar/ (Accessed: December 7, 2017).
[2] www.ncbi.nlm.nih.gov/pubmed/ (Accessed: December 7, 2017).

TABLE 1 | Alterations identified in cfDNA in the patient.

Alteration	% cfDNA
BRCA2 R2520*	66.02
TP53 L344P	20.92
TP53 R337G	19.26
MET Y989fs	0.21

**Represents a change from arginine to a stop codon at amino acid 2520.*

consultation, confirming that they would participate in a consultation. The genetic counselor met with both parents and expanded on the family history previously reported. The 62-year-old mother confirmed that her father (patient's maternal grandfather) was diagnosed with and died of stomach cancer at age 49 and her mother (patient's maternal grandmother) died at 85 with no personal history of cancer. The 70-year-old father reported a maternal uncle (patient's paternal great-uncle) with prostate cancer, diagnosed at an unknown age, and several first cousins with colorectal and uterine cancers, at unknown ages of diagnosis. After genetic counseling and a discussion of the limits and benefits of genetic testing, both parents underwent clinical genetic testing utilizing a multi-gene panel (Invitae Corporation, San Francisco, CA, USA). In addition, both consented to an USC IRB approved cancer genetics registry (0S-12-4). All family members agreed to publication or presentation of the results for scientific purposes and their agreement is noted in their medical records. No mutations were identified on the father's analysis, but the mother was found to carry the same *BRCA2* mutation (c.7558C > T; R2520*) identified on the patient's cfDNA analysis, confirming the diagnosis of HBOC within the family.

Both parents, as well as the deceased patient's wife, presented to review the results of the genetic testing. The patient's mother is 62, with one ovary intact; so, the personal implications for cancer risk management and prevention were discussed. In addition, she has other adult children who each has 50% probability of having inherited the *BRCA2* mutation. A family member letter was provided to facilitate communication of the patient's mother's genetic test results to her offspring. The deceased patient's wife was counseled that given her children's current age, no testing was indicated, as it would not impact their care. However, once they are adults they should discuss testing with their health-care providers, as breast cancer risk management begins at age 25 for mutation-positive women.

DISCUSSIONS AND REVIEW OF LITERATURE

The potential of liquid biopsies to identify a germline mutation is significant and the impact of such detection will extend beyond the patient to their family members to serve as a mechanism for cancer prevention. To our knowledge, this is the first case report of germline testing in the family members of a deceased individual, in whom a secondary *BRCA2* alteration was identified by liquid biopsy. Secondary unexpected genetic findings, regardless of the indication for ordering this test is an important and novel issue. We hereby will discuss how to trace and recognize such findings as germline on liquid biopsy and emphasize on their value even if the patient is deceased.

Discovery of secondary pathogenic germline variants in tumor tissue testing, confirmed by parallel normal DNA testing, have been reported. These secondary findings were found in 4.3% (19 out of 439) of patients in a study by Seifert et al. (17); and in 2.3% of 1,000 cancer patients in 19 cancer-related genes (18). Jones et al. analyzed matched tumor and normal DNA and identified germline alterations in cancer-predisposing genes in 3% of patients with apparently sporadic cancers (19). The frequencies of such findings have never been reported in liquid biopsies.

In tumor tissue sequencing, distinction between somatic and germline mutations can be challenging. Tumor-only sequencing approaches can not definitively identify germline alterations in cancer-predisposing genes and lead to an additional 31 and 65% false-positive findings in targeted and exome analyses, respectively, including in potentially actionable genes (19). The study by Mandelker et al. in a breast cancer population (101 patients) showed tumor-only testing identified *BRCA1/2* alterations in approximately 40% of the patients, with a majority of these patients not having germline mutations. Conversely "subtraction" of germline from tumor DNA sequence would have disguised 59 germline *BRCA1/2* cases (20). These data suggest that combined matched tumor-normal sequencing analyses are essential for precise identification and interpretation of somatic and germline alterations and have important implications for the diagnostic and therapeutic management of cancer patients. The same confirmatory principle with normal DNA testing should be applied to secondary germline findings discovered by NGS. This highlights the significance of our case, as the germline finding was confirmed by testing of the parent's normal DNA (as the patient was deceased by the time of this necessary investigation). However, there are often barriers to collect additional tissue, including cost, putting patients through additional invasive procedures, and potential ethical concerns (21).

Studies have shown that examining the MAF of a suspected germline variant identified on tumor tissue genomic testing can be helpful in differentiating germline versus somatic status in the absence of normal tissue sequencing (tumor only testing). Germline variants often occur at an MAF of around 50% (when heterozygous) or around 100% (when homozygous, or due to loss of heterozygosity). Somatic alterations are acquired after birth and usually have an MAF < 50% (22). While Funchain et al. did report a mean MAF of 51% in tumors for variants confirmed to be in the germline, the range of MAF was 35–72% (23). Meric-Bernstam et al. reported that the median MAF was higher for confirmed germline alterations compared to somatic alterations (46 versus 33%). However, the range of MAF for germline alterations was 13–94%, highlighting the importance of considering more than MAF when evaluating tumor alterations and their potential to be germline in origin.

Our literature search revealed several other studies on plasma cfDNA testing that also used MAF to identify secondary potentially germline findings. However, unlike our case, these studies used germline testing of the same individuals to confirm their findings (24, 25). Hu et al. reported a patient with metastatic lung adenocarcinoma and positive family history of lung cancer who had both *EGFR* L858R and *EGFR* T790M mutations on tissue NGS. Plasma NGS (Guardant360) detected initial MAF of *EGFR* L858R as 5.3%, fluctuating during the course of treatment; whereas initial MAF of T790M identified as 50.9%, stayed constant during therapy. The latter mutation's trend and a positive family history raised the suspicion of an underlying germline mutation, which was verified by germline testing. The investigators then tested their theory on a large cohort of cancer patients (n = 31,414), showing that the MAF of *EGFR* T790M in plasma NGS samples can aid in differentiating germline and somatic alterations (24). Shukuya et al. reported a case with lung adenocarcinoma and no personal or family history of breast or ovarian cancer, who had a *BRCA2* mutation with MAF of 50.7% identified on cfDNA. An underlying germline mutation was suspected due to the MAF being at a frequency suspicious for germline alterations. Germline status was confirmed after referral to genetic counseling and germline testing (25). In a third study examining samples from over 10,000 patients, 1.7% (n = 173) of patients had a putative germline alteration identified on cfDNA, with the majority of these alterations having an MAF ranging between 40 and 55% (26).

The MAF of the *BRCA2* alteration reported in our case was 66%, nearly twofold higher than the cooccurring *TP53* alterations identified on the same sample. This relatively high MAF increased our suspicion that the *BRCA2* alteration was of germline in origin. Other findings that can be suggestive of an alteration being germline include finding a well-characterized mutation in a hereditary cancer predisposing gene, such as one of the known *BRCA* founder mutations. Another characteristic of a germline alteration is a relatively consistent MAF over sequential tests. In contrast, somatic alterations tend to fluctuate, as illustrated in the other case report referenced above (25, 27). While the alteration identified in our patient's assay was not one of the *BRCA1/2* founder mutations, it is a mutation that is well-documented in the clinical literature as occurring in the germline setting and is known to be pathogenic. Our case is the only report that entertained MAF percentage and trend as a clue to track the same mutation in a patient's close relation, as the patient's own DNA was not available at that time to confirm this finding as germline. This was necessary to verify a hereditary malignancy present in this patient's family and would have significant implications for his family members.

Based on the ACMG (American College of Medical Genetics and Genomics) recommendation for secondary findings in exome and genome sequencing, only known pathogenic or expected pathogenic variants should be reported to patients. The ACMG recommends that laboratories performing clinical sequencing, report pathogenic variants in 59 genes, regardless of the indication for testing (28). Schrader et al. suggested that there is a potential value to a broad germline sequencing approach in the context of tumor-normal analysis. In their study of 1,566 cancer patients, 16 were found to carry potentially pathogenic variants in known Mendelian disease-associated genes and 59% of the individuals with a potentially pathogenic variant in a cancer-susceptibility gene had cancer not known to be associated with that gene (29). A Joint Consensus Recommendation from the Association for Molecular Pathology, American Society of Clinical Oncology, and CAP published in 2016 spoke to secondary germline findings identified in the course of tumor testing, recommending that germline variations with evidence of clinical impact be reported (22). We suggest the same principles applied to exome and genome sequencing, as well as other tumor sequencing, be translated to plasma genotyping as well. As outlined in Robson et al., oncologists ordering somatic genomic tumor testing should counsel their patients about the potential to identify secondary findings outside of the primary indication for testing (30). Our case highlights that patients undergoing cfDNA tumor analysis should be counseled similarly due to the potential to identify underlying germline alterations. Ordering clinicians should consider that it may not be possible for patients to "opt out" of learning germline mutation status (e.g., a *BRCA2* alteration may be germline, and also makes patient eligible for treatment with PARP inhibitors, making the genomic finding important for therapeutic decision-making—the primary indication for ordering somatic tumor testing). Also, given that most somatic tumor testing, utilizing tumor tissue or cfDNA, is being performed in patients with late stage cancers, ordering clinicians should have a discussion with their patients about alternative individuals to whom potential germline results can be returned, as evidenced by our experience. Finally, ordering clinicians should be aware of resources within or near their institution to help with interpretation of potential germline alterations identified on somatic tumor testing, and genetic counseling resources available for their patients (27).

Incorporating tumor genomic information into a patient's therapeutic decision-making is the premise of precision oncology (31). We expand on that premise and suggest that precision oncology can provide a mechanism for identification of families appropriate for genetic counseling and cancer prevention. Clinicians should be attentive to the potential to identify secondary germline alterations, as they can have great therapeutic and preventive implications for patients and their families. With increased use of liquid biopsies in clinical practice to help with treatment decisions and to offer targeted therapies, development of an algorithm for identification and confirmation of potential germline mutations identified through this testing is critical. Clinicians should consider the personal and family history of the patient, along with information in the cfDNA results including reported pathogenicity of the variant in the clinical literature, MAF of the variant, and MAF relative to other variants identified on the sample. Strategies should be put in place for genetic counseling referral in case of such discoveries. A standard method of analysis and interpretation of these test results is essential to prevent any lost opportunity for prevention in the patient and their at-risk family members.

AUTHOR CONTRIBUTIONS

Reviewed clinical data, performed literature review, and wrote majority of paper.

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SUPPLEMENTARY MATERIAL

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REVIEW
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The Clinical Landscape of Circulating Tumor DNA in Gastrointestinal Malignancies

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Technologies for genomic analyses have revealed more details in cancer biology and have changed standard treatments for cancer, including the introduction of targeted gene-specific therapy. Currently, liquid biopsies are increasingly being utilized in clinical trials and research settings to analyze circulating tumor DNA (ctDNA) from peripheral blood. Several studies have shown the potential of ctDNA in the screening, prognostication, molecular profiling, and monitoring of gastrointestinal malignancies. Although limitations continue to exist in the use of ctDNA, such as method standardization, the sensitivity, concordance with tumor tissue, and regulatory issues, this field offers promising benefits for cancer treatment. A deeper understanding of tumor biology *via* ctDNA analyses and ctDNA-guided clinical trials will lead to the increasing use of ctDNA in clinical practice in the near future; this development will result in the improvement of outcomes among patients with gastrointestinal malignancies.

Keywords: circulating tumor DNA, colorectal cancer, gastric cancer, esophageal cancer, gastrointestinal malignancies

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INTRODUCTION

Over several decades, the gold standard in the diagnosis and screening of tumors has been tissue biopsy (1). However, conventional tissue biopsies are invasive, painful, and carry a risk of complications such as bleeding, local infection, and damage to neighboring tissues. Moreover, a tissue biopsy cannot always reflect tumor dynamics or response to treatment. The recent era of precision medicine, which represents a paradigm shift in cancer, has challenged the gold standard in diagnosis by introducing a transition from tissue biopsy to liquid biopsy. Compared with tissue biopsies, liquid biopsies carry minimal potential risk and can be repeatedly performed in routine practice during cancer treatment by using peripheral blood. Furthermore, liquid biopsies have the potential to provide more complete information regarding the biology of whole tumors despite tumor heterogeneity. Liquid biopsies include the testing of soluble factors, such as circulating tumor DNA (ctDNA) and circulating cell-free DNA (cfDNA), as well as proteins and tumor markers (2). cfDNA is highly fragmented DNA that is released from necrotic or apoptotic cells into the bloodstream (3–5). cfDNA consists of DNA from healthy cells and tumor cells, whereas ctDNA is defined as DNA that is derived only from primary or metastatic tumor cells.

Since Mandel and Metais (6) reported fragmented DNA in blood for the first time in 1948, technologies for cfDNA quantification have changed over 70 years from quantitative polymerase chain reaction to complex BEAMing and deep next-generation sequencing (NGS), thus achieving improvements in the sensitivity and specificity of cfDNA detection. With the development of sensitive techniques that can detect rare mutations, the heterogeneous landscape of tumors can be determined using blood samples. In fact, National Comprehensive Cancer Network guideline for non-small cell lung cancer (version 4. 2018) states that plasma biopsy should be considered if repeat biopsy is not feasible (7).

Here, we review ctDNA in gastrointestinal malignancies by focusing on clinical utility and future perspectives.

ctDNA AND RELATED TECHNOLOGIES

The presence of cfDNA in the blood is a well-established fact, and DNA fragments are released from dying cells because of cellular turnover or other types of cell death (2). In cancer patients, a fraction of cfDNA is tumor derived and is termed ctDNA. ctDNA originates from primary tumors, metastatic tumor cells, or circulating tumor cells. ctDNA molecules are shorter than non-mutant cfDNA molecules in plasma, as demonstrated by PCR (8, 9) and sequencing (10, 11).

Representative approaches for analyzing ctDNA are summarized in **Table 1** (12). Mutation-specific real-time or endpoint PCR has been used for the detection of point mutations in ctDNA (13–17). More recently, digital PCR methods such as BEAMing and droplet digital PCR have been developed to improve the identification of genomic alterations in ctDNA (18–20). The recent implementation of NGS has allowed the direct sequence-based detection of chromosomal alterations in plasma DNA (21–23); however, it is necessary to distinguish the relatively few somatic alterations in ctDNA from the larger numbers of structural variants present in the germline cells of all individuals. Bioinformatics-based filters that enrich high-confidence somatic structural alterations while eliminating germline and artifactual changes have been developed (12). In addition, importantly, amplification in ctDNA can be depend on both the amount of ctDNA in the plasma due to high tumor burden and high copy number of specific gene. Commercially available kits for the NGS assays of ctDNA are summarized in **Table 2**.

EARLY DETECTION OF CANCER

The early detection of cancer is one of the most important issues in reducing cancer-related deaths. In many cases, gastrointestinal cancer is detected *via* endoscopy or CT scans conducted for symptoms such as anorexia, abdominal pain, or constipation. ctDNA may have a potential role in the noninvasive early diagnosis and screening of gastrointestinal cancer. Even localized cancers shed DNA into circulation; therefore, ctDNA can be detected in patients with localized cancers, in addition to patients with advanced or metastatic cancers.

In a study across several early and late-stage cancers, ctDNA was detected in 73, 57, and 48% of patients with colorectal cancer (CRC), gastroesophageal cancer, and pancreatic cancer, respectively (25). The use of several biomarkers in ctDNA including the levels of overall ctDNA, *ALU247* fragment concentration (26), *KRAS* mutations (27, 28), *TP53* mutations (29, 30), *BRAF* mutations (28), and *septin 9* (*SEPT9*) methylation (31–34) have been demonstrated for the diagnosis of CRC. Also, detection of methylated SEPT9 DNA in plasma is US FDA approved as a blood test for CRC screening. Compared with biomarkers for CRC, biomarkers for the diagnosis of gastric cancer (GC) and esophageal cancer have been assessed in a relatively small number of cohorts (25, 35). For the early detection or screening of cancers including CRC, GC, and esophageal cancer, the sensitivity of ctDNA analysis needs to be improved. Analysis that can be performed using a few milliliters of blood would be suitable for cancer screening; however, increasing the analytical sensitivity beyond 0.1% may not provide clinical benefits because it also leads to difficulties in distinguishing oncological mutations and sampling noise. In fact, cancer-associated genomic alterations have been found in plasma from healthy individuals (36). In addition, because many cancers share common gene mutations such as *TP53* mutations and *KRAS* mutations, ctDNA presents challenges in the detection of the specific organ sites of malignancies. To overcome these issues in ctDNA, the methylation profiling of cfDNA has been investigated in cancer diagnosis. Methylation haplotyping in plasma is a promising strategy for the early detection of a tumor and its primary growth site (37). Studies have reported the utility of methylation scores from over 9,000 CpG sites in cfDNA for cancer detection, with 76.3% accuracy for the prediction of cancer type (38).

Despite the above hurdles to the use of ctDNA in cancer screening, it is expected that the clinical use of ctDNA is less than a decade away because of its utility and convenience in cancer screening (**Figure 1**).

PROGNOSIS AND DETECTION OF RESIDUAL DISEASE

Following curative therapy for gastrointestinal malignancies, ctDNA may be a potential biomarker for minimal residual disease. The detection of ctDNA even in the absence of any other clinical

TABLE 1 | Available assays of detection of circulating tumor DNA (12, 24).

Characteristic	PCR assays		Next-generation sequencing (NGS) assays	
	Allele-specific PCR	Emulsion PCR	Amplicon-based targeted NGS	Capture-based targeted NGS
Variants potentially detected	Known recurring mutations	Known recurring mutations	Any exonic mutations, copy number gains	Exonic mutations, intronic gene fusions, copy number gains
Quantitation	Semiquantitative	Absolute or relative quantitation, wide dynamic range	Quantitation of relative AF, but vulnerable to PCR amplification bias	Quantitation of relative AF
Speed	Rapid	Rapid	Slower	Slower
Examples	Cobas (Roche), therascreen (Qiagen)	Droplet digital PCR (Biorad), BEAMing (Sysmex Inostics)	Tam-seq (Inivata)	Guardant360 (Guardant), cancerselect (personal genome diagnostics)

evidence of disease may mean that the patient has higher risk of relapse. In a cohort of 230 patients with stage II CRC, the assessment of ctDNA using the Safe-SeqS NGS method at the first visit after surgery indicated that recurrence-free survival at 3 years was 0% in a ctDNA-positive group and 90% in a ctDNA-negative group (39). Other studies have also demonstrated that the persistent detection of ctDNA after local therapy (surgery or radical radiotherapy) predicts a high risk of relapse in patients with colon cancer (40, 41). In addition, methylated *BCAT1*/*IKZF1* have been evaluated as biomarkers for CRC (42, 43). Of 397 patients with CRC who underwent primary tumor resection, odds ratio of a positive CEA test for recurrence was 6.9 (95% CI 2–22) compared to 14.4 (5–23, 25–40) for *BCAT1*/*IKZF1*.

In a meta-analysis of 16 studies including 1,193 patients with GC, the presence of ctDNA was significantly associated with the shorter disease-free survival (HR 4.36, 95% CI 3.08–6.16, $p < 0.001$) and overall survival (HR 1.77, 95% CI 1.38–2.28, $p < 0.001$) of GC patients, with high specificity (0.95, 95% CI 0.93–0.96) and relatively moderate sensitivity (0.62, 95% CI 0.59–0.65) (44). Another study demonstrated that the level of ctDNA was associated with tumor recurrence in patients who underwent curative surgery for GC (45). Similarly, several studies have reported the tumor-associated mutations in ctDNA and the prognosis of patients with esophageal cancer (46, 47); however, these studies included a limited number of patients, and further investigations are warranted. Almost all of these studies followed a retrospective design and provided limited validation for clinical use in gastrointestinal malignancies. One of the ideal applications of ctDNA is in the early detection of residual disease or recurrence compared with CT imaging and tumor markers. A more attractive idea is patient-specific ctDNA panels in patients who have undergone curative surgery (41, 48). Individual surgical tumor samples may provide a great opportunity to obtain tumor DNA from each patient to guide the design of patient-specific ctDNA panels from peripheral blood samples. Despite hurdles such as tumor heterogeneity, validation, regulatory issues, and quality control of individual panels, patient-specific ctDNA panels are potential biomarkers for postoperative monitoring.

TABLE 2 | Commercially available circulating tumor DNA next-generation sequencing assays.

Panel	Company	Gene number	Assays
Guardant360	Guardant Health	73	Capture-based
PlasmaSELECT-R64	Personal Genome Diagnostics	64 + MSI	Capture-based
FoundationACT	Foundation Medicine	62	Capture-based
Oncomine Colon cfDNA Assay	Thermo Fisher Scientific	14	Amplicon-based

BIOMARKERS OF CHEMOTHERAPY RESPONSE AND RESISTANCE IN METASTATIC DISEASE

Another clinical potential of ctDNA is in the determination of systemic chemotherapy regimens, the prediction of response to chemotherapy, and the identification of resistance mechanisms. The short half-life of ctDNA enables the real-time monitoring

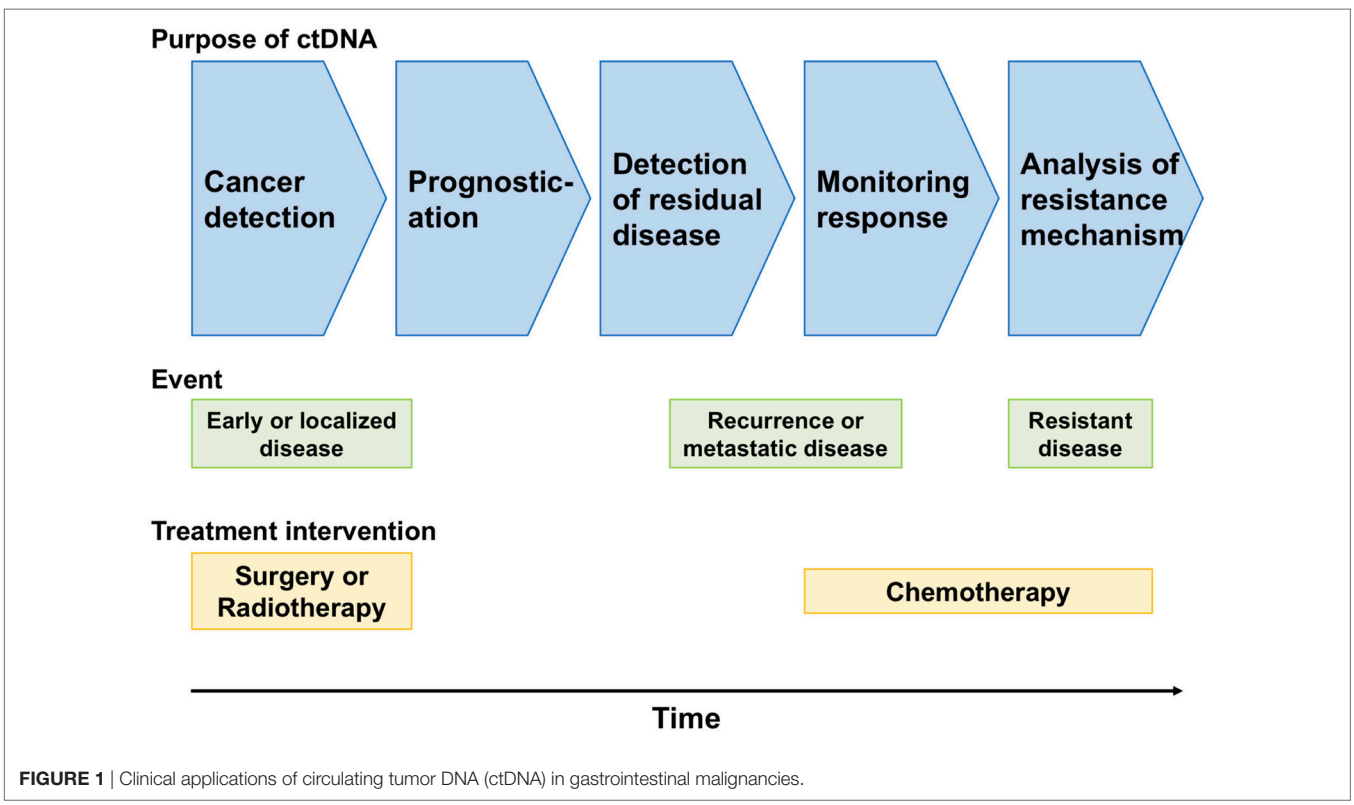

FIGURE 1 | Clinical applications of circulating tumor DNA (ctDNA) in gastrointestinal malignancies.

of cancer burden, in contrast to radiological imaging or tumor markers. Indeed, a prospective study of 53 patients with metastatic CRC demonstrated that early changes in ctDNA during first-line chemotherapy predicted later radiologic response. Significant reductions in ctDNA levels were observed before cycle 2 and were correlated with CT response at 8–10 weeks (odds ratio 5.25 with a 10-fold ctDNA reduction; $p = 0.016$) (49). Blood-based monitoring is an ideal strategy during cancer treatment because of its minimal invasiveness and avoidance of radiation exposure.

RAS Mutations in Metastatic CRC

The assessment of *RAS* status has been mandatory in patients with metastatic CRC to predict the response of cetuximab and panitumumab to anti-EGFR antibodies (50–55). A double-blinded prospective study of 106 patients with mCRC has been performed to compare the *KRAS* mutation status assessed using tumor tissue *via* routine gold-standard methods to that assessed using plasma DNA *via* qPCR-based methods; the resultant specificity and sensitivity for the detection of *KRAS* point mutations were 98 and 92%, respectively, resulting in 96% concordance (28). In addition, the retrospective exploratory analysis in a biomarker subgroup of the CORRECT trial, which was a phase III trial investigating the efficacy and safety of regorafenib in patients with mCRC, confirmed the utility of detecting *KRAS*, *BRAF*, and *PIK3CA* mutations in ctDNA. Plasma DNA detected with BEAMing in 503 patients demonstrated that mutation status in ctDNA changed dynamically during chemotherapy and differed from that in pretreatment archival tissue (56). Currently, the OncoBEAM RAS CRC assay is the only European committee *in vitro* diagnostic test for *RAS* mutations in ctDNA. This assay is a qualitative PCR-based test and allows for the detection of 34 mutations within exons 2, 3, and 4 of *KRAS* and *NRAS* genes from a single blood sample. Four large cohort studies have been reported to achieve high concordance of approximately >90% (range, 89.7–93.3%) between OncoBEAM using plasma ctDNA and tumor tissue analysis in patients with CRC (57–60).

Acquired resistance to anti-EGFR antibody therapy has also been found by using ctDNA analyses. ctDNA from 28 patients receiving panitumumab monotherapy was assessed using qPCR, and 9 out of 24 (38%) patients whose tumors were initially *KRAS* wild-type showed *KRAS* mutations in ctDNA after panitumumab treatment (61). This study suggested that the emergence of *KRAS* mutations is a mechanism of resistance to anti-EGFR therapy and that these mutations may be detected in ctDNA as a more sensitive monitoring tool than radiological imaging. More recently, other studies have also demonstrated mutations associated with the resistance and decline of mutant *KRAS* clones after the withdrawal of anti-EGFR therapy (62, 63).

BRAF Mutations in Metastatic CRC

BRAF mutations have been confirmed to be associated with poor prognosis in patients with metastatic CRC; moreover, the limited efficacy of anti-EGFR therapy in patients with *BRAF*-mutant metastatic CRC has been shown in several studies (64–66). The analysis of *BRAF* mutations in ctDNA by using qPCR-based methods has shown specificity and sensitivity of 100% (28). Based on preclinical studies (67, 68), the clinical trials of dual EGFR and MAPK signaling pathway inhibition in patients with *BRAF*-mutant metastatic CRC is ongoing. In a phase Ib study of a combination therapy of dabrafenib, trametinib, and panitumumab, *BRAF* V600E mutant burden in ctDNA was more markedly reduced in responders than in nonresponders, and the emergence of *RAS* mutations was seen with disease progression in 9 of 22 patients (41%) (69). This exploratory analysis suggested that the monitoring of *BRAF* V600E mutant fraction in ctDNA could effectively predict response to combination therapy including a BRAF inhibitor and that overcoming the emergence of *RAS*-mutant subclones is important in combating resistance to this combination therapy.

Other Alterations in Metastatic CRC

HER2 or *MET* amplification is also known as a mechanism of resistance to anti-EGFR therapy in patients with metastatic CRC. The patient-derived xenograft models of *HER2*-amplified CRC showed resistance to anti-EGFR therapy (70, 71). In addition, the frequency of *HER2* amplifications increased from approximately 3% in treatment-naïve patients to over 10% in patients who were administered anti-EGFR therapy (72). Although there are few studies regarding the concordance of *HER2* status between ctDNA and tissue samples, 4 of 18 (22%) patients exhibited *HER2* amplification in ctDNA by digital PCR after cetuximab therapy despite being negative for *HER2* amplification prior to anti-EGFR therapy (73). The promising results of trastuzumab and T-DM1 combination therapy in the HERACLES trial (71) have encouraged clinical trials in patients with HER2-positive metastatic CRC such as the MyPathway trial (74) and the TRIUMPH trial (75); notably, the TRIUMPH trial includes patients with *HER2* amplification detected using not only tissue samples but also ctDNA analysis using an NGS-based method.

Another important alteration that causes resistance to anti-EGFR therapy is *MET* amplification. A preclinical model of *MET*-amplified CRC also showed resistance to anti-EGFR therapy (76). In fact, *MET* amplification in ctDNA was detected using NGS in 12 of 53 (22.6%) patients who showed disease progression with anti-EGFR therapy; no such amplification was detected in patients before cetuximab therapy. Furthermore, *MET* amplification in ctDNA was not detected in patients with *RAS* mutations after cetuximab therapy, thus suggesting that *MET* amplification is one of the mechanisms (other than *RAS* mutations) that cause resistance to anti-EGFR therapy (77). In a phase Ib trial of cabozantinib and panitumumab combination therapy, the preliminary evidence of efficacy in patients with MET-amplified metastatic CRC was reported (78).

HER2 Amplification in GC

The amplification of the *HER2* gene or overexpression of the HER2 protein, which contributes to cancer progression, has been reported in approximately 20% of patients with advanced GC (79, 80). According to the results of the ToGA trial, HER2 is a key biomarker of HER2-targeted therapy using trastuzumab for advanced GC (79). The gold-standard diagnostic method for detecting HER2 positivity and suitability for trastuzumab therapy is an immunohistochemistry score of 3+ or 2+ with a positive

result in fluorescence *in situ* hybridization. A retrospective study of 52 patients with advanced GC and 40 healthy volunteers demonstrated that the plasma *HER2–RPPH1* ratio (with *RPPH1* as a reference gene) was significantly higher in patients with HER2-positive tumors than those with HER2-negative tumors (81). More recently, the droplet digital PCR of *HER2* copy number in ctDNA has been reported. In a study of 60 patients with GC, including 17 patients who developed recurrence and 30 healthy volunteers, preoperative plasma *HER2* ratio correlated with tumor HER2 status; postoperative plasma *HER2* ratios were high during the recurrence of tumors, which were diagnosed as HER2-negative tumors in surgery samples (82). Considering that HER2 status may be altered after recurrence, the *HER2* copy number analysis in ctDNA enables the real-time evaluation of HER2 status and leads to more effective treatment choices with HER2-targeted agents.

FUTURE PERSPECTIVES

Overall, the data generated in all studies discussed above support the potential role of ctDNA in the diagnosis and treatment of patients with gastrointestinal malignancies. Despite a few limitations, including the standardization of detection and ctDNA quantification, the sensitivity, and concordance between ctDNA and tissue biopsies that currently hinder the routine use of ctDNA in clinical trials and clinical practice, its use would allow a deeper understanding of cancer biology and enable better cancer treatment, thus leading to improvements in patient survival.

In the context of clinical trials for metastatic disease treatment, several studies are ongoing or have been conducted using eligibility criteria based on gene alterations in ctDNA. A prospective study on the comprehensive ctDNA-guided treatment of advanced GC and lung cancers is ongoing in Korea (83). Another trial called the Targeted Agent and Profiling Utilization Registry, which is a large basket/umbrella trial sponsored by the American Society of Clinical Oncology, is accepting patient selection on the basis of ctDNA analysis (NCT02693535). In addition, an umbrella trial in patients with mCRC based on the molecular profiling of ctDNA, including the status of *HER2*, *BRAF* V600E, *BRAF* non-V600E, *MET*, or high tumor mutation burden, is ongoing in Japan. If promising results are obtained in these clinical trials, ctDNA will be used in routine clinical practice and in clinical trials in the near future.

Economic and regulatory issues still hinder the practical use of ctDNA. Although most guidelines recommend that comprehensive molecular profiling should be conducted, the substantial costs of NGS assays lead many community oncologists to rely on PCR tissue tests and do not understand the added benefit of a comprehensive genomic test. In addition, emerging ctDNA-guided clinical trials are essential to obtain approval for the use of ctDNA in clinical practice. These barriers need to be challenged, perhaps initially in patients with CRC, which is one of the most prevalent gastrointestinal malignancies worldwide. Simultaneously, further studies are needed on other gastrointestinal malignancies such as esophageal cancer and GC to identify the best gene biomarkers that are detectable in ctDNA for the diagnosis, prognosis, and prediction of therapy response.

CONCLUDING REMARKS

The potential role of ctDNA in gastrointestinal malignancies has been shown in basic studies, retrospective studies, and limited prospective studies. A paradigm shift in cancer diagnosis and treatment in ctDNA-based clinical trials and clinical practice will occur in the near future, thus leading to the availability of more DNA sequence information compared with that in the past decade. Although some limitations continue to exist on the use of ctDNA in clinical practice and clinical trials, ctDNA-based personalized therapy promises to improve patient outcomes and quality of life.

AUTHOR CONTRIBUTIONS

This review was drafted by KS and DK and was revised by HB.

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REVIEW
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The Use of Circulating Tumor DNA for Prognosis of Gastrointestinal Cancers

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Gastrointestinal cancers, including oesophageal, gastric and colorectal cancers (CRC) have high rates of disease recurrence despite curative resection. There are a number of recent studies that have investigated the use of circulating tumor DNA (ctDNA) for prognostic value in these cancers. We reviewed studies that had been published prior to March 2018 that assessed the prognostic values of ctDNA in patients with oesophageal and gastric cancers, gastrointestinal stromal tumors (GIST) and CRC. We identified 63 eligible clinical studies that focussed on recurrence and survival. Studies assessed investigated various ctDNA biomarkers in patients with different stages of cancer undergoing surgical resection, chemotherapy and no treatment. For oesophageal squamous cell carcinoma and oesophageal adenocarcinoma, methylation of certain genes such as *APC* and *DAPK* have been highlighted as promising biomarkers for prognostication, but these studies are limited and more comprehensive research is needed. Studies focusing on gastric cancer patients showed that methylation of ctDNA in *SOX17* and *APC* were independently associated with poor survival. Two studies demonstrated an association between ctDNA and recurrence and survival in GIST patients, but more studies are needed for this type of gastrointestinal cancer. A large proportion of the literature was on CRC which identified both somatic mutations and DNA methylation biomarkers to determine prognosis. ctDNA biomarkers that identified somatic mutations were more effective if they were personalized based on mutations found in the primary tumor tissue, but ctDNA methylation studies identified various biomarkers that predicted increased risk of recurrence, poor disease free survival and overall survival. While the use of non-invasive ctDNA biomarkers for prognosis is promising, larger studies are needed to validate the clinical utility for optimizing treatment and surveillance strategies to reduce mortality from gastrointestinal cancers.

Keywords: circulating tumor DNA, colorectal cancer, oesophageal cancer, gastric cancer, gastrointestinal stromal tumor, cell free circulating DNA, survival, recurrence

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INTRODUCTION

Gastrointestinal cancers, in particular gastric (stomach) and colorectal cancer (CRC), have high incidence and mortality rate. CRC is one of the most prevalent cancers, with ∼1.4 million new cases diagnosed and 693,933 deaths worldwide per year in 2012 (1). The incidence and mortality rate of gastric cancer during the same period was 951,594 and 723,073 respectively, while oesophageal (adenocarcinoma or squamous cell carcinoma) cancer incidence was 455,784 with a mortality of 400,169. From this it can be seen that while the incidence of oesophageal cancer is less common than gastric cancer and CRC, it has a higher mortality rate which is due to the tumors being rarely detected before the disease has metastasized to lymph nodes and distant organs (2, 3). Gastrointestinal stromal tumors (GIST) are mesenchymal tumors that can originate anywhere in the gastrointestinal tract, but with a higher proportion in the stomach and small intestine. They are not common and are thought to make up <1% of all gastrointestinal tumors. Five year survival from this cancer is ∼50% (2, 4).

In recent years there have been large improvements in early detection, surgical resection and treatment of gastrointestinal cancers, especially colorectal and gastric. Despite this, the risk of recurrence of the cancer within 5 years is reported to be up to 50% (4–6). For oesophageal cancer and GIST approximately half of the patients undergoing curative resection develop recurrence (4, 5), with half of the oesophageal recurrences occurring within the first year post-resection (5). Despite deeming patients free of disease at the conclusion of initial therapy, gastric cancer has been reported to have a recurrence incidence of 26% (7), and following CRC resection, incidence of recurrence is ∼35%, with 80% occurring within the first 2 years of resection (6). Metastatic recurrence is most commonly detected too late for successful intervention (8, 9) although for CRC at least early detection of tumor progression and recurrence provides an additional effective way to improve clinical outcomes. Accurate prognostic assessment to allow for early and effective treatment is vital to improving patient outcomes.

CURRENT TOOLS FOR PROGNOSIS AND SURVEILLANCE

Assessment of prognosis and determining treatment and surveillance strategies is currently guided by the stage at diagnosis which is classified according to the T, N, M system, i.e., depth of tumor invasion (T stage), presence of lymph node metastasis (N stage), and presence of distance metastasis (M stage) (10). Staging of GIST is based on tumor size instead of depth of invasion, with mitotic rate combined with T, N, and M scores to give an overall stage (11). While the use of TNM stage is highly prognostic for stage I and IV, it is less predictive for stage II and III. For example, patients with stage II CRC are considered to have low risk for recurrence and therefore are not generally recommended adjuvant chemotherapy, but despite this, one study found that 23% of stage II patients had a recurrence within 5 years (12). Consequently, other clinicopathological factors have been sought to help predict who is at greatest risk for recurrence (examples provided in **Table 1**). For oesophageal cancer increased depth of tumor invasion correlates with an increased risk of disease recurrence (14), however it is not highly predictive of which patients are at low or high risk for disease recurrence. Similarly for CRC, T stage, vascular invasion, tumor grade, and number of examined lymph nodes have been associated with poor prognosis (**Table 1**), however, recurrence still occurs in patients without these risk factors (31).

Intensive surveillance after resection may be applied to detect asymptomatic recurrence early enough to enable curative therapy and improve survival. Current methods for surveillance post-resection are radiological techniques (e.g., CT) and endoscopy with biopsy, but these have disadvantages of radiation burden, lack of sensitivity, invasive nature, and cost as well as limited resources in some countries. Biopsy only samples a small area of the epithelium which might not be representative of the stage of disease (3). In addition the cost effectiveness of intensive surveillance has been questioned (32). Oesophageal cancers may also benefit from surveillance with cytological sampling obtained by brushing the oesophageal surface, or use of the capsule sponge, but these techniques are also limited by inadequate sensitivity and specificity, or limited validation (3). There remains a need for non-invasive and sensitive prognostic markers to establish who would benefit from adjuvant therapy and surveillance. This could be done through pre- or post-operative blood analysis.

ASSESSMENT OF BLOOD FOR BIOMARKERS OF CANCER PROGNOSIS

The use of blood biomarkers has the potential to provide further prognostic information of value for gastrointestinal cancers, however, current clinical use is limited. Blood testing for proteins are not routinely used for oesophageal cancers or GIST, but the proteins carcinoembryonic antigen (CEA), carbohydrate antigen-19-9 (CA 19-9), and carbohydrate antigen 72-4 (CA 72-4) have been used for monitoring disease progression of CRC and gastric cancer (33). For CEA, while it is upregulated in 90% of advanced CRC (34), it is not reliably used for prognosis, and studies have shown an unacceptably low sensitivity for recurrent CRC of 32–37% (35–37). The sensitivity of CEA for recurrence in gastric cancer has been reported to be between 30.8 and 34.3% (38, 39). There is a wide range of sensitivity of CA 19-9 of 30.8–57.1% (38, 39), with a similar average sensitivity of CA 72-4 of 48.4% (39). The low sensitivity supports the need for other blood biomarkers for clinical management to assess risk for recurrence.

Genetic markers arising from tumors and being released into blood might provide the solution. Most gastrointestinal cancers are thought to develop through a series of epigenetic changes or somatic (non-hereditary) lesions. The common mutations are in genes including *APC, TP53, KRAS* and *BRAF* for colorectal cancer [reviewed in Testa et al. (40)], *TP53* and *p16/CDKN2A* in oesophageal adenocarcinomas [reviewed in Testa et al. (41)], *CDH1, PIK3CA,* and *RHOA* in gastric cancers [reviewed in Ang et al. (42)], and *KIT* and *PDGFRA* with GIST [reviewed in Wozniak et al. (43)]. These alterations can contribute to

TABLE 1 | Significant predictive clinicopathological factors of recurrence for gastrointestinal cancers on multivariate analysis.

Patient group	Clinicopathological variable	Multivariate analysis findings
Oesophageal cancers (73.3% adenocarcinomas) (13)	Poor differentiation	HR 1.74; 95%CI 1.28–2.38
	Advanced clinical stage	HR 6.46; 95%CI 2.90–14.38
Oesophageal cancers (82.5% squamous cell carcinoma) (14)	Depth of tumor invasion	RR 1.9; 95%CI 1.3–2.7
Gastric cancer (15)	Age at diagnosis	OR 1.813; 95%CI 1.050–3.131
	T stage	OR 2.865; 95%CI 1.603–5.123
	N1 stage (vs. N0)	OR 4.029; 95%CI 1.708–9.500
	N2 stage (vs. N0)	OR 4.425; 95%CI 1.889–10.365
	N3 stage (vs. N0)	OR 9.860; 95%CI 4.314–22.536
	Lauren histotype	OR 3.492; 95%CI 1.810–6.736
	Lymphovascular invasion	OR 3.460; 95%CI 1.335–8.969
Gastric cancer (lymph node negative) (16)	T stage ≥3	SHR 2.7; 95%CI 1.5–5.2
Gastric cancer (lymph node negative) (17)	Diffuse + mixed histotype (vs. intestinal)	RR 2.11; 95%CI 1.25–2.95
	T3 stage (vs. T2)	RR 3.55; 95%CI 1.98–6.44
Gastrointestinal stromal tumor (18)	Mitotic index 6-10/50 HPF (vs. ≤5/50)	RR 0.282; 95%CI 0.121–0.660
	Platelet to lymphocyte ratio	RR 1.737; 95%CI 1.041–2.899
	Gastrointestinal bleeding	RR 0.457; 95%CI 0.254–0.823
Gastrointestinal stromal tumor (19)	High risk	HR 13.01; 95%CI 2.68–63.21
	Omental/colorectal site	HR 5.13; 95%CI 1.68–15.69
	Age at diagnosis	HR 0.96; 95%CI 0.92–0.99
Gastrointestinal stromal tumor (20)	Size ≥5 cm (vs. <5 cm)	HR 3.43; 95%CI 1.12–11.8
	Mitotic index ≥5 (vs. <5/50 HPF)	HR 3.28; 95%CI 1.25–8.59
Gastrointestinal stromal tumor (21)	Female	HR 0.469; 95%CI 0.257–0.854
	Size ≥10 cm (vs. <5 cm)	HR 20.989; 95%CI 3.560–125.673
	Epithelioid component	HR 5.315; 95%CI 1.402–20.149
	Mitotic index ≥10 (vs. <10)	HR 45.951; 95%CI 8.811–239.657
Gastrointestinal stromal tumor (22)	Size ≥10 cm (vs. <10 cm)	OR 4.715; 95%CI 1.142–19.471
Colon cancer (stage I-III) (23)	Stage II (vs. I)	HR 4.6; 95%CI 1.05–19.9
	Stage III (vs. I)	HR 10.8; 95%CI 2.6–45.8
	Clinical obstruction	HR 3.8; 95%CI 1.9–7.4
	Positive margin	HR 4.1; 95%CI 1.9–8.6
	Lymphovascular invasion	HR 1.9; 95%CI 1.06–3.5
	Local tumor invasion	HR 2.2; 95%CI 1.1–4.5
Colon cancer (stage III) (24)	Positive lymph node	HR 1.24; 95%CI 1.18–1.31
Colon cancer (stage I-III) (25)	4.0–7.9 cm (vs. <4 cm)	HR 0.45; 95%CI 0.293–0.696
	Venous invasion	HR 1.61; 95%CI 1.085–2.376
	Stage III (vs. stage I)	HR 3.80; 95%CI 1.482–9.744
Rectal cancer (stage I-III) (25)	Lower rectum (vs. rectosigmoid)	HR 2.20; 95%CI 1.408–3.424
	Anal canal (vs. rectosigmoid)	HR 7.19; 95%CI 3.052–16.950
	Serosal invasion	HR 1.63; 95%CI 1.130–2.343
	Venous invasion	HR 1.90; 95%CI 1.407–2.566
	Stage III (vs. stage I)	HR 3.64; 95%CI 1.993–6.634
	Questionable residual tumor	HR 1.84; 95%CI 1.281–2.634
Rectal cancer (stage III) (26)	tumor budding	HR 2.005; 95%CI 1.021–3.934
	N stage	HR 1.818; 95%CI 1.057–3.128
	Perineural invasion	HR 1.046; 95%CI 1.011–1.081
	T stage	HR 1.606; 95%CI 1.149–2.244
Colorectal cancer (stage I-III) (27)	Vascular invasion	HR 2.304; 95%CI 1.067–4.975
	Perineural invasion	HR 3.040; 95%CI 1.389–6.667
Colorectal cancer (stage I-III) (28)	Lymph node metastases	HR 7.652; 95%CI 4.162–14.827

(Continued)

TABLE 1 | Continued

	Clinicopathological variable	Multivariate analysis findings
	Vascular invasion	HR 4.360; 95%CI 2.793–10.847
Colorectal cancer (stage II) (29)	T4 stage (vs. T3)	HR 23.072; 95%CI 2.951–203.247
	Vascular invasion	HR 6.204; 95%CI 2.879–12.694
	≥12 lymph nodes retrieved	HR 2.656; 95%CI 1.319–6.127
Colorectal cancer (stage IV) (30)	High grade differentiation	HR 1.514; 95%CI 1.124–2.040
	Curative operation	HR 2.642; 95%CI 1.966–3.549
	Resection of primary tumor	HR 0.507; 95%CI 0.366–0.704
	Multiple metastatic lesions	HR 1.679; 95%CI 1.165–2.418

95% CI, confidence interval; HR, hazard ratio; OR, odds ratio; SHR, subhazard ratio.

aberrant cell behavior such as uncontrolled cell growth and proliferation, disordered apoptosis, increased angiogenesis, and promotion of invasion and metastasis (44). As primary and metastatic cancers shed cells, cell components and DNA into the blood, these genetic changes can be monitored in the circulation by assaying for circulating tumor cells (CTCs) or for circulating tumor DNA (ctDNA), with the process sometimes referred to as liquid biopsy. While CTCs show promise in early detection of recurrence [reviewed in Tan et al. (45)], clinical use is limited by low numbers of CTCs in the blood (one mL of whole blood generally contains less than one CTC, but 10^7 normal blood cells) (46). Furthermore, CTCs show heterogeneity such that extraction techniques might not be effective for all cell types; CTCs can be comprised of epithelial tumor cells, epithelial-to-mesenchymal transition (EMT) cells, and stem cells (46). The use of ctDNA which is more abundant and easier to extract, overcomes some of the technical issues associated with using CTCs in clinical practice and captures the genetic material released independent of cell structure. ctDNA remains in the circulation for a few hours before being metabolized, (47) which allows real-time monitoring of the tumor burden, with a comprehensive molecular profile of the heterogeneity of the disease, compared to what is provided by a single tumor tissue biopsy (48).

The release of ctDNA into the bloodstream as cell free DNA (cfDNA) is thought to be the result of apoptosis or necrosis of tumor cells (49). When DNA is released through necrosis of cells, the fragments can vary in size, whereas DNA released through apoptosis creates fragments 185–200 base pairs in length (50). As the main source of DNA from non-neoplastic healthy cells is apoptosis, assessment of the ratio of longer DNA to short fragments (through measuring ALU repeats) is able to indicate presence of ctDNA (51). Other common strategies involve assessing cell free DNA levels, tumor specific DNA mutations, and tumor specific epigenetic changes. The latter two can be assessed through targeted PCR-based ctDNA assays, detecting known somatic mutations or epigenetic changes. One such example is assessment of *RAS* mutations of colorectal cancer tissue which are of similar prevalence in plasma as in the tumor (51 and 53% respectively), demonstrating that blood-based testing for RAS mutation is a viable alternative to tissue-based testing (52). A growing number of studies have assessed DNA methylation as there is evidence that epigenetic alterations are more common and frequently precede mutational (somatic) changes (53). Also unlike mutations, promoter methylation can be consistently measured as it occurs in specific regions of the DNA (CpG islands).

CtDNA has been evaluated as a screening tool and for diagnostic purposes, but there has been limited effectiveness with early stage cancers and it does not appear useful in predicting the presence of colonic polyps (54). Instead the use of ctDNA for prognosis and treatment monitoring is more promising. The following sections of this review will describe the studies that have been performed in gastrointestinal cancers to assess the utility of ctDNA for their prognostic value, whether measured as cfDNA concentration, integrity (fragment lengths), copy number alterations, mutation or methylation status. These are comprehensively summarized in Supplementary Tables 1–4.

SEARCH STRATEGY

Identification of eligible studies was performed through searching the PubMed database until 1st March 2018. The following search criteria were applied: "(ctDNA OR "circulating tumor DNA" OR "tumor derived DNA" OR "circulating tumor DNA" OR "tumor derived DNA" OR "cell free DNA") AND (gastrointestinal OR GIT OR esophagus OR esophagus OR oesophageal OR esophageal OR gastroesophageal OR stomach OR gastric OR "large intestine" OR colon OR caecum OR rectum OR colorectal) AND (tumor OR tumor OR malignan* OR cancer OR neoplasm OR carcinoma OR carcinoid OR adenocarcinoma)." This resulted in 657 search results. Two independent reviewers (HS and ES) screened the available literature, and discrepancies were discussed and resolved. Included studies were those conducted in gastrointestinal cancers with a clinical outcome of survival or recurrence. Exclusions were review articles, biomarker studies that did not include blood analysis, studies in animal models or cell lines only, articles that were not in English, and those that analyzed circulating tumor cells (CTC) rather than circulating tumor DNA or cell free DNA. In the case of more than one report on the same cohort of patients, the study with the shorter follow-up time was excluded. In addition, studies were not included where the focus was on associations of biomarkers with pathology indicators of

poor prognosis, rather than an actual clinical outcome of poor prognosis. The final number of eligible studies for review were 63, including 7 on oesophageal cancers, 13 on gastric cancers, 2 on GIST, and 41 on CRC.

CTDNA BIOMARKERS FOR PROGNOSIS OF OESOPHAGEAL CANCER

Biomarkers for prognosis have been investigated for both adenocarcinomas and squamous cell carcinomas of the esophagus as summarized below.

DNA Levels, Integrity, and Copy Numbers

It was previously shown that cfDNA levels correlated with stage in oesophageal squamous cell carcinoma (SCC). Tomochika et al ($n = 91$) found that DNA levels were higher in advanced tumors vs. early stages, and significantly higher in patients with distant metastases ($p = 0.011$) (55). Correlation of DNA levels before oesophagectomy for stage I-III SCC were also observed with tumor lymphovascular invasion and relapse ($p = 0.018$), and a poor 5 year disease free survival rate in 81 oesophageal SCC patients ($p = 0.013$) (56).

DNA Mutations

Ueda et al conducted a longitudinal study to look at 53 cancer related genes in 13 oesophageal SCC patients undergoing surgery of all stages. Changes in allele frequency in ctDNA was associated with tumor burden, and the allelic frequency increased prior to radiographic detection of recurrence (6 months before radiological evidence) (57). Eisenberger et al assessed loss of heterozygosity (LOH) in pre-operative ctDNA of SCC ($n = 28$) and oesophageal adenocarcinoma ($n = 32$) patients of all stages in two separate studies. In both types of cancers, no relationship was found between recurrence and LOH; however, in SCC a trend toward shorter survival was observed for patients with LOH in tumor tissue and ctDNA (58, 59).

DNA Methylation

Of the few studies that have assessed prognostic value of methylated ctDNA biomarkers in oespophageal cancer, there have been mixed outcomes, which may be related to different cancer types studied. Presence of high pre-operative methylated ctDNA (*MSH2*) was predictive of lower disease free survival for 209 SCC patients of all stages (60), while in all stages of oesophageal adenocarcinomas pre-operative methylated ctDNA (*TAC1*) was not associated with survival ($n = 61$) (61). Hoffman et al assessed methylation of *DAPK* and *APC* promoter in 24 SCC and 35 adenocarcinoma patients of stage 0-III at pre- and post-operative stages. Presence of pre-operative *DAPK* methylation was associated with poorer survival ($p = 0.01$) and detection of post-operative methylation of *APC* promoter was correlated with residual tumor ($p = 0.03$) (62).

Summary

There have been a limited number of studies undertaken to develop prognostic biomarkers with oesophageal SCC and adenocarcinoma. Some of these studies are highlighted in **Table 2** [limiting the studies displayed to those with at least 20 events of interest (recurrence or death)], but there have been very limited accuracy data for each test. Only a test utilizing copy numbers was assessed for sensitivity for recurrence (61.2%), but specificity was not assessed (56).

CTDNA BIOMARKERS FOR PROGNOSIS OF GASTRIC CANCER

DNA Levels, Integrity and Copy Numbers

A number of studies have investigated the use of cfDNA levels to determine the clinical outcome following surgical resection of gastric cancer. Kim et al ($n = 30$) and Pu et al ($n = 73$) provided data that supported that advanced gastric cancer (stage III/IV) patients had higher levels of DNA compared with early gastric cancer patients ($p = 0.035$) (63, 64). Pu et al conducted a longitudinal study and found that DNA levels were elevated pre-operatively and at 21 days post-operatively; but they declined 3 months post-surgery and then increased again if the patient had tumor progression. However this study showed no significant association of DNA levels with survival (64). A large study of 428 gastric cancer patients by Lan et al found that persistently high DNA levels post-resection was an indicator of recurrence (65). In a study that focussed on 277 stage IV cases it was found that a high level of DNA with more mutations was present pre-operatively ($p < 0.0001$) and these patients had an increased risk of recurrence ($p = 0.037$) and lower overall survival ($p = 0.039$) over the 5-year follow-up period (8). Several studies have also assessed DNA copy numbers for prognostic purposes. A study by Shoda et al examined 61 stage I and II surgical resection patients and found that *HER2* to *RPPH1* ratio of ctDNA increased post-operatively with recurrence (66). In a separate study, this research group looked at the value of EBV (Epstein–Barr virus) DNA in 153 gastric cancer patients undergoing resection. In the 21 (13.7%) patients with EBV-associated gastric carcinoma, circulating EBV DNA levels reflected the clinical status of the patient as it was absent after surgery in all 9 cases assessed, and increased prior to clinical detection of recurrence in one patient with longitudinal follow-up over 2 years (67). While plasma EBV DNA may useful for monitoring clinical load in patients with EBV-associated gastric carcinomas, no significant difference was found between prognosis of recurrence-free survival of those with high pre-operative EBV copy numbers compared to those with low levels (67). Kinugasa et al (68) assessed the ctDNA *HER2* status in relation to survival of patients with non-resectable gastric cancer (2 stage III and 23 stage IV). They reported that patients with a positive pre-therapy *HER2* ctDNA status had significantly shorter survival than patients with a negative status ($p = 0.01$). However, as a poor concordance was found between tissue and serum *HER2* status, only 3 of the 7 patients that were ctDNA *HER2* positive were also positive with tissue biopsy and received directed therapy (trastuzumab). No difference in survival was found when comparing survival rates of patients with a positive or negative *HER2* status of the tissue. Caution must therefore be taken in interpreting the prognostic value of *HER2* ctDNA status.

TABLE 2 | Accuracy of circulating tumor biomarkers for oesophageal cancer recurrence and survival (excluding duplicate studies and those with unclear number or fewer than 20 recurrences or deaths).

Author, year	Biomarkers	Stage	Pre-op, post-op or time of recurrence	No. recurrence/ total	Sensitivity for recurrence	Specificity for recurrence	NPV for recurrence	PPV for recurrence	HR for recurrence (95% CI)	Adjusted HR (95% CI)	Mean/Median recurrence-free survival
RECURRENCE											
Eisenberger; 2006 (59)	12 microsatellite markers that indicate LOH	Adenocarcinoma; all stages	Pre-op	22/32	N/A	N/A	N/A	N/A	N/A	N/A	No association with recurrence.
Hsieh; 2016 (56)	DNA copy number (cyclophilin)	Squamous cell carcinoma; stage I-III	Post-op	49/81	61.2%	N/A	N/A	N/A	N/A	N/A	N/A
SURVIVAL											
Author, year	**Biomarkers**	**Stage**	**Pre-op or post-op**	**No. deaths/total**	**Sensitivity for death**	**Specificity for death**	**NPV for death**	**PPV for death**	**HR for survival (95% CI)**	**Adjusted HR (95% CI)**	**Mean/Median survival**
Eisenberger; 2006 (59)	12 microsatellite markers that indicate LOH	Adenocarcinoma; all stages	Pre-op	24/34	N/A	N/A	N/A	N/A	N/A	N/A	No association with survival.
Hsieh; 2016 (56)	DNA copy number (cyclophilin)	Squamous cell carcinoma; stage I–III	Post-op	43/81	N/A	N/A	N/A	N/A	N/A	N/A	24.3% (vs. 43.4%), $p = 0.164$

CI, Confidence interval; HR, hazard ratio; N/A, not applicable; NPV, negative predictive value; PPV, positive predictive value; post-op, post-operative; pre-op: pre-operative.

DNA Mutations

Very few studies have assessed DNA mutations for gastric cancer prognosis. One of the studies was a longitudinal study in 42 stage II gastric cancer patients undergoing surgical resection which evaluated concentration of *TP53* mutations. It was found that the change in ctDNA fraction corresponded with disease status of the patients i.e. the levels decreased post-operatively but increased in patients with recurrence (69). However, the authors did not perform statistical analyses on these results as there were only 3 cases with recurrence.

DNA Methylation

A few papers have studied methylation of ctDNA in gastric cancer and found a significantly worse clinical outcome in patients who have aberrant methylation of various genes in ctDNA. Pimson et al found 85 and 95% of 101 advanced gastric cancer patients had *PCDH10* and *RASSF1A* methylation which was associated with a reduction in median survival to ∼8 months ($p < 0.001$) (70). Balgkouranidou et al also studied *RASSF1A* methylation, along with *APC* methylation, in 73 operable gastric cancer patients of stage I-III and did not find a significant correlation with *RASSF1A* promoter methylation and clinical outcome; but showed that the group with pre-operative *APC* promoter methylation had a higher incidence of death (HR 4.6, $p = 0.008$). *APC* methylation levels were also associated with high levels of the conventional tumor biomarkers, CEA and CA19-9 (71). In a similar study, Balgkouranidou et al found that methylation of *SOX17* in pre-operative ctDNA of 73 patients with operable gastric cancer had decreased overall survival ($p = 0.049$) (72). Two studies investigated different ctDNA biomarkers, *MINT2* promoter and *TIMP-3* respectively, for disease-free progression and risk of recurrence in the same population of 92 gastric cancer patients of all stages undergoing surgical resection. Aberrant methylation of *MINT2* promoter in pre-operative ctDNA was associated with peritoneal dissemination and tumor progression ($p < 0.0001$); and methylation of *TIMP-3* was associated with poorer disease free survival rates ($p < 0.001$) (73, 74). A study by Ling et al assessed *XAF1* methylation in pre-operative and post-operative follow-up ctDNA of 202 gastric cancer patients of all stages and showed that negative to positive methylation change post-surgery was associated with a poorer disease-free survival ($p < 0.0001$) (75).

Summary

As with oesophageal cancers, there have been few thorough studies into ctDNA for prognosis of gastric cancer (**Table 3**) and none have shown to be an independent predictor for recurrence. Methylation changes appear to be the most promising with methylated *RASSF1A* and *SOX17* being independent predictors of overall survival. Despite this, the sensitivity and positive predictive value reported for some of these biomarkers may not be sufficiently high enough to guide therapeutic decisions.

TABLE 3 | Accuracy of circulating tumor biomarkers for gastric cancer recurrence and survival (excluding duplicate studies and those with unclear number or fewer than 20 recurrences or deaths).

Author, year	Biomarkers	Stage	Pre-op, post-op or time of recurrence	No. recurrence/ total	Sensitivity for recurrence	Specificity for recurrence	NPV for recurrence	PPV for recurrence	HR for recurrence (95% CI)	Adjusted HR (95% CI)	Mean/Median recurrence-free survival
RECURRENCE											
Fang; 2016 (8)	DNA copy number (cyclophilin)	All stages	Pre-op	129/244	49.6%	N/A	N/A	N/A	1.19 (0.86–1.63)	N/A	N/A
SURVIVAL											
Author, year	**Biomarkers**	**Stage**	**Pre-op or post-op**	**No. deaths/total**	**Sensitivity for death**	**Specificity for death**	**NPV for death**	**PPV for death**	**HR for poor survival (95% CI)**	**Adjusted HR (95% CI)**	**Mean/Median survival**
Fang; 2016 (8)	DNA copy number (cyclophilin);	All stages	Pre-op	179/277	N/A	N/A	N/A	N/A	N/A	N/A	High DNA levels: 39.2% (vs. 45.8%), $p = 0.039$
	DNA mutations										DNA mutations stage III-IV: 5.6% (vs. 31.5%), $p = 0.028$
Balgkouranidou; 2013 (72)	Methylation: *SOX17*	Operable gastric cancer	Pre-op	38/73	68%	51%	60%	61%	2.0 (1.0–3.9)	3.0 (1.2–7.8)	37.7 months (vs. 66.9 months), $p = 0.049$
Balgkouranidou; 2015 (71)	Methylation: *APC*, *RASSF1A*	Stage I-III	Pre-op	38/73	*APC:* 94.7% *RASSF1A:* 65.8%	N/A	N/A	*APC:* 59.0% *RASSF1A:* 56.5%	N/A	*APC:* 4.6 (1.1-20.3) *RASSF1A:* Not significant	*APC:* 46.0 months (vs. 85.0 months), $p = 0.008$ *RASSF1A:* 56.0 months (vs. 43.0 months), $p = 0.683$

CI, Confidence interval; HR, hazard ratio; N/A, not applicable; NPV, negative predictive value; PPV, positive predictive value; post-op, post-operative; pre-op: pre-operative.

CTDNA BIOMARKERS FOR PROGNOSIS OF GASTROINTESTINAL STROMAL TUMORS

Two studies looked at the role of ctDNA in prognosis of GIST. In 92 patients with recurrent GIST, Rawnaq et al found an association between loss of heterozygosity in microsatellite DNA and recurrence ($p = 0.03$), but no association with overall survival (76). A study by Yoo et al on 30 patients with tyrosine kinase inhibitor-refractory GIST found that a detection of secondary kinase mutations (*KIT* exon 17) prior to treatment was associated with lower overall survival (HR 2.7, 0.047) (77).

Summary

Only the study by Rawnaq et al. (76) had a moderate sample size, and has been summarized in **Table 4**. There have been no investigations into methylation markers of ctDNA, and the existing studies have not found a biomarker that is an independent predictor of either recurrence or survival. More studies are clearly needed for this type of gastrointestinal cancer.

CTDNA BIOMARKERS FOR PROGNOSIS OF COLORECTAL CANCER

DNA Levels, Integrity and Copy Numbers

As demonstrated with other gastrointestinal cancers, level of cfDNA correlates with presence and stage of tumors. Metastatic CRC was found to have highest cfDNA levels, with these decreasing for all patients post resection ($n = 205$) (51). Cassinotti et al and Frattini et al noted that DNA levels increased prior to recurrence in all stages of CRC ($n = 223$, $n = 70$) (78, 79). In two different studies of 38 primary CRC patients, Czeiger and colleagues found that pre-operative DNA level was a better indicator of prognosis than TNM staging for both disease-free survival (HR 6.03) and overall survival (HR 3.53) for all cancer stages. They also showed that DNA levels out-performed pre-operative CEA results, which was not significantly associated with disease-free survival (80, 81). Guadaljara et al found that a high level of pre-operative cfDNA in all CRC stages was correlated with presence of metastases at the time of the surgery or during follow up, but was not associated with overall survival ($n = 73$) (82). Schwarzenbach et al only assessed 55 stage IV CRC patients and found that high DNA levels prior to surgical resection was associated with a shorter survival period (83). Shorter overall survival has also been found to be associated with high pre-operative DNA levels measured as DNA fragments (ALU244 and ALU83, which are thought to represent the amount of the DNA released from non-apoptotic process and the total cfDNA) and DNA copy numbers (measured with DNA binding protein CPP1; $n = 114$, $n = 45$ respectively) (84, 85).

In metastatic CRC patients being treated with chemotherapy, high levels of cfDNA correlated with a worse outcome for the patient. Spindler et al ($n = 100$) found patients with high level of DNA prior to second-line treatment with irinotecan had shorter progression-free survival and overall survival ($p < 0.0001$) (86). In another study Spindler et al assessed 229 patients with

TABLE 4 | Accuracy of circulating tumor biomarkers for gastrointestinal stromal tumor recurrence (excluding duplicate studies and those with unclear number or fewer than 20 recurrences).

Author, year	Biomarkers	Stage	Pre-op, post-op or time of recurrence	No. recurrence/total	Sensitivity for recurrence	Specificity for recurrence	NPV for recurrence	PPV for recurrence	HR for recurrence (95% CI)	Adjusted HR (95% CI)	Mean/Median recurrence-free survival
RECURRENCE											
Rawnaq; 2011 (76)	12 microsatellite markers for loss of heterozygosity	Advanced or recurrent GIST	Post-op	29/83	58%	75%	N/A	N/A	N/A	N/A	N/A

CI, Confidence interval; HR, hazard ratio; N/A, not applicable; NPV, negative predictive value; PPV, positive predictive value; post-op, post-operative; pre-op: pre-operative.

chemorefractory metastatic CRC, and patients with high DNA levels had an impaired overall survival, with each increase in cfDNA quartile having an independent prognostic value ($p = 0.0006$) (87). In 49 patients with therapy resistant metastatic CRC being treated with gemcitabine and capecitabine, it was shown that high DNA levels prior to therapy was associated with lower overall survival (88). Schou et al assessed cfDNA levels longitudinally in 123 patients with locally advanced rectal cancer receiving chemotherapy and found that a high baseline level was associated with a higher risk of local/distant recurrence and a shorter time to recurrence ($p = 0.002$) (89).

DNA Mutations

The relationship between pre-operative ctDNA and survival or recurrence, using mutation markers, in CRC patients of all stages has been explored in a number of studies. Lin et al quantified ctDNA by amplifying mutations in 74 genes and showed that ctDNA, lower than the median value, was associated with a higher 5-year overall survival ($p = 0.001$) ($n = 191$) (90). Möhrmann et al assessed mutations in *BRAF*, *KRAS,* and *EGFR* genes in ctDNA of 20 advanced CRC patients and also found that lower ctDNA corresponded with longer survival (91). These findings were supported by a study of 37 patients that evaluated the presence of *KRAS* mutations and p16 hypermethylation in all stages of CRC and found a strong association between detection of ctDNA, and a shorter survival and higher risk of recurrence (92). Similarly, Wang et al concluded that detection of genetic alterations in *APC*, *p53,* and *KRAS* in a sample of 104 pre-operative CRC patients was linked to increased incidence of recurrence and metastases (93).

Many studies have assessed ctDNA prior to and after CRC resection and determined its clinical utility in detecting recurrence. Ryan et al ($n = 78$) contradicted some of the studies above with their finding that pre-operative KRAS2 mutations in ctDNA was not an independent prognostic factor for disease recurrence. However, they did find that KRAS2 ctDNA was positive in patients after surgery and preceding recurrence, which occurred a median of 4 months before CEA elevations (94). Reinert et al had a similar finding with detection of somatic structure variants in post-operative ctDNA an average of 10 months before recurrence in 6 out 9 CRC patients of all stages (95). Several different studies quantified the level of ctDNA from a panel of commonly mutated genes to assess prognosis. In a small study of 18 patients Diehl et al found that detection of high levels of ctDNA post-operatively was associated with recurrence, and ctDNA was a better biomarker than CEA ($p = 0.03$) (47). Schøler et al compared post-operative ctDNA with radiological evidence of recurrence in 14/45 patients in the study who relapsed and found that ctDNA was detected an average of 9.4 months before CT scans (85). Kidess et al assessed 38 patients undergoing liver metastectomy along with CRC resection and found that post-operative ctDNA levels anticipated recurrence earlier than conventional tools—CEA and radiological imaging (96). Pre- and post-operative ctDNA levels have also been evaluated for clinical utility in determining survival. Shin et al assessed *KRAS* mutations in 62 stage III/IV CRC patients undergoing surgery and found a higher rate of ctDNA mutation detection in patients with metastases, and that detectable ctDNA *KRAS* mutations correlated with a shorter overall survival ($p = 0.03$) (97).

Several studies have assessed recurrence in CRC patients based on selection of ctDNA mutations following primary tumor tissue analysis, including a study by Ng et al ($n = 44$) who found certain patients were positive pre-operatively, negative post-operatively and then positive again prior to recurrence before any clinical or radiological evidence (85). Tie et al found that post-operative ctDNA was predictive of recurrence in both locally advanced rectal cancer patients ($n = 159$) (98) and in stage II CRC ($p = 0.001$) ($n = 178$) (99). These findings were irrespective of adjuvant therapy.

Research has also been conducted on patients undergoing chemotherapy. Studies evaluated pre-therapy ctDNA and longitudinal ctDNA collection during treatment and its prognostic role in predicating survival. In 97 metastatic CRC patients, it was shown that high level of cfDNA and high mutation loads of *KRAS* exon2, *BRAF* V600E in pre-therapy ctDNA was associated with shorter overall survival (100). Similar results were also obtained by Spindler et al, detection of *KRAS* mutation in ctDNA correlated with shorter overall survival and progression free survival ($p = 0.001$; $p = 0.002$) in a sample of 140 patients with chemotherapy resistant metastatic CRC (101). Janku and colleagues longitudinally assessed advanced CRC patients receiving chemotherapy in four different studies. In 62 patients receiving BRAF/MEK inhibitors, detection of a high percentage of *BRAF* V600 ctDNA was associated with shorter overall survival and time to failure ($p = 0.005$; $p = 0.045$) (102). In another study of 71 patients, detection of >6.2% *KRAS* G12/13 ctDNA was correlated with shorter survival ($p = 0.001$) (103). Additionally, in a similar study with a cohort of advanced cancer patients (68 colorectal and 3 gastroesophageal), detection of >1% *KRAS*, *EGFR*, *BRAF*, or *PIK3CA* mutant ctDNA was associated with a shorter median survival (104). They also tested detection of 61 cancer related genes in 14 CRC patients and found that patients with low variant allele frequency survived longer and the time to treatment failure was also longer ($p = 0.018$; $p = 0.03$). Another important finding in this study was that the allele frequency in patients receiving systemic therapy changed in synchronization with radiological response ($p = 0.02$) (105).

A few studies also looked specifically at using ctDNA as a tool for treatment monitoring and assessing prognosis of metastatic CRC. In a study of 211 patients, Spindler et al found that patients with *KRAS* mutations in pre-therapy ctDNA, did not respond to second-line irinotecan treatment and had shorter overall survival and progression free survival ($p = 0.04$; $p < 0.0001$; $p = 0.01$) (106). In another study of 140 patients, Spindler et al found that pre-therapy DNA levels strongly correlated with *KRAS* ctDNA levels and this was associated with poor disease control using third-line treatment with cetuximab and irinotecan ($p = 0.009$) (107). Tie et al assessed mutations in primary tumor present in ctDNA in 53 patients and found that the changes in level of mutant DNA correlated with radiological response to first-line chemotherapy treatment and major reductions in ctDNA seemed to be associated with a trend for increased progression free and overall survival (108).

DNA Methylation

Methylation changes of certain genes has been investigated by many studies to determine prognosis in different patient groups undergoing surgical resection and/or chemotherapy. There is interest in whether methylated ctDNA markers parallel those using mutations, and one study showed a significant correlation between the two, with both being detectable prior to clinical signs of recurrence (109). Liu et al ($n = 165$) found a significant association between pre-operative ctDNA methylation of *SST* and *MAL* and cancer specific deaths. Methylation of *SST* also correlated with tumor recurrence (31).

Several studies have shown prognostic value of methylated DNA markers. Matthaios et al ($n = 155$) found an association between methylation of *APC* and *RASSF1A* in pre-operative ctDNA and poor survival in early and advanced CRC patients (110). A study of 397 CRC patients under surveillance, assessed accuracy of a panel of methylated ctDNA biomarkers (*BCAT1* and *IKZF1*) and found that sensitivity and specificity for recurrence was 68 and 87% respectively, significantly higher than sensitivity of CEA (32%) with no significant difference in specificity (94%) (37). While most studies have assessed hypermethylation, one study ($n = 95$) found that hypomethylation of *CBS* promoter induced by folate deficiency was also linked to recurrence and cancer-related death (111).

Several studies have investigated the prognostic value of DNA methylation for metastatic CRC patients and/or following adjuvant chemotherapy. Prior to therapy, two studies ($n = 467$ and $n = 82$) showed that detection of methylated *HPP1, WIF1,* and *NPY* in blood have been shown to be associated with poor overall survival (112, 113). The second study showed that a decrease in ctDNA during chemotherapy was associated with longer median progression-free survival and overall survival ($p < 0.001$; $p < 0.001$) (113). Methylation of 30 gene promoter regions was assessed by Rasmussen et al in 193 patients prior to receiving chemotherapy, and a higher number of methylated regions was correlated with an increased risk of metastases. *RARB* and *RASSF1A* methylation was associated with more aggressive disease indicating poor survival (114). In two separate studies Philipp et al ($n = 311$ and $n = 259$) showed that methylation of *HLTF* or *HPP1* was associated with larger and more advanced CRC stage, shorter overall survival and metastases (115, 116).

Summary

There have been a larger number of studies performed in CRC patients with survival as the key outcome compared to recurrence (**Table 5**). Recurrence in cases with early stage CRC is a particular challenge for finding prognostic markers that justify individualized therapy aimed at reducing the chance of recurrence. The majority of studies searching for prognostic ctDNA biomarkers for CRC focussed on DNA mutations, with the use of blood biomarkers that have been personalized from primary tumor tissue analysis, showing promising sensitivity. Such biomarkers are most effective though when based on known mutations in surgically resected cancer. Methylated DNA biomarkers are better suited for pre-operative prognostication and hence have been the subject of more studies of this type, with pre-operative detection of methylated *SST* showing promise for independent prediction of recurrence, and methylated *SST*, *RASSF1A,* and *RARB* being independent predictors of overall survival. More studies are warranted in this field.

DISCUSSION

Following cancer diagnosis, clinical decisions regarding treatment and surveillance frequency are largely driven by pathological stage. Despite this there are a considerable proportion of patients who still have cancer recurrence and poor survival. Non-invasive biomarkers that can provide an accurate prognosis assessment independent of stage are therefore warranted. While there have been a large number of studies conducted in gastrointestinal cancers, the majority have assessed prognosis for CRC. Very few studies report diagnostic accuracy for either recurrence or death (sensitivity and specificity), and many are limited by small numbers of patients with endpoints of recurrence or mortality. In addition, out of all of the studies reviewed (when limiting analysis to those studies with at least 20 events of interest), very few ctDNA biomarkers are independent predictors of recurrence or survival. For oesophageal cancer and GIST there were no independent biomarkers for prognosis. For gastric cancer methylated *SOX17* and *APC* were independent predictors of survival, with an adjusted HR of 3.0 (95% CI 1.2–7.8) (72) and 4.6 (95% CI 1.1–20.3) (71) respectively. For CRC there were a number of ctDNA biomarkers that were independent predictors of prognosis including DNA levels and fragments, tumor-specific DNA mutations and DNA methylation. A personalized ctDNA panel based on tumor tissue analysis gave the greatest independent prediction of recurrence with a HR of 28 (95% CI 11–68) (99). Other independent predictors for recurrence included methylated *SST* (HR 2.60, 95% CI 1.37–4.94) (31) and hypomethylated *CBS* (HR 1.54, 95% CI 1.18–3.02) (111). For independent prediction of survival, seven potential biomarkers (all analyzed in pre-operative blood samples) were found: Alu83 (HR 2.71, 95% CI 1.22–6.02) (84), Alu244 (HR 2.70, 95% CI 1.25–5.84) (84), DNA copy number (HR 2.61, 95% CI 1.31–5.19) (90), methylated *SST* (HR 1.96, 95% CI 1.06–3.62) (31), methylated *RARB* (HR 1.99, 95% CI 1.07–3.72) (114), methylated *RASSF1A* (HR 3.35, 95% CI 1.76–6.38) (114), and hypomethylated *CBS* (HR 1.35, 95% CI 1.09–2.41) (111). As can be seen, in most cases the reported hazard ratios for prognosis were not stronger than those found with the clinicopathological variables reported in **Table 1**.

Limitations in Studies of ctDNA

In this review we have not taken into consideration the methodological differences between studies which can affect results, leading to false positives or negatives. Variations in blood collection tubes, storage times, and temperatures, DNA isolation methods, and nature of analysis (automated or manual) are all relevant to assessing benefit. One study that compared different blood collection tubes for analysis of epigenetic alterations in ctDNA found that some could only be stored cold for 24 h, while others could be stored at room temperature for 48 h (119). In addition, the use of plasma or serum can introduce

TABLE 5 | Accuracy of circulating tumor biomarkers for colorectal cancer recurrence and survival (excluding duplicate studies and those with unclear number or fewer than 20 recurrences or deaths).

RECURRENCE											
Author, year	**Biomarkers**	**Stage**	**Pre-op, post-op or time of recurrence**	**No. recurrence/ total**	**Sensitivity for recurrence**	**Specificity for recurrence**	**NPV for recurrence**	**PPV for recurrence**	**HR for recurrence (95% CI)**	**Adjusted HR (95% CI)**	**Mean/ Median recurrence-free survival**
Ng; 2017 (117)	Mutations: based on tumor tissue findings	All stages (with mutations identified in patient tumor tissue prior to plasma testing)	Post-op; at recurrence	26/44	73%; 96%	83%	N/A	N/A	N/A	N/A	N/A
Ryan; 2003 (94)	Mutation: *KRAS2*	All stages	Pre-op; Post-op	Pre: 20/123 Post: 20/94	52.6%	92%	N/A	62.5%	Pre: 2.07 (0.3-14.8) Post (in stage I-III): 6.37 (2.30-18.0)	N/A	N/A
Tie; 2016 (99)	Mutations: based on tumor tissue findings	Stage II, no chemo (with mutations identified in patient tumor tissue prior to plasma testing)	Post-op	27/178	48%	100%	90.2%	78.6%	18 (7.9-40)	28 (11-68)	N/A
Wang; 2004 (93)	Mutations: *APC, p53, KRAS*	All stages	Pre-op	31/104	87%	81%	91%	75%	N/A	N/A	N/A
Liu; 2016 (31)	Methylation: *SST, MAL, TAC1, SEPT9, EYA4, CRABp1, NELL1*	Stage I-III	Pre-op	43/150	N/A	N/A	N/A	N/A	*SST*: 2.40 (1.27-4.55) *MAL*: 1.15 (0.63-2.09) *TAC1*: 0.96 (0.53-1.74) *SEPT9*: 0.77 (0.42-1.40) *EYA4*:1.18 (0.65-2.15) *CRABp1*: 1.15 (0.63-2.10) *NELL1*:0.73 (0.37-1.44)	*SST*: 2.60 (1.37–4.94) All others: not significant	*SST:* 18.7% (vs. 38.7%), $p = 0.005$
Young; 2016 (37)	Methylation: *BCAT1, IKZF1*	All stages	At recurrence	28/122	68%	87%	N/A	N/A	N/A	N/A	N/A
Xue; 2017 (111)	Hypomethylation: *CBS*	All stages	Pre-op	43/95	74.4%	59.6%	74%	62%	1.62 (1.29-3.68)	1.54 (1.18-3.02)	N/A
SURVIVAL											
Author, year	**Biomarkers**	**Stage**	**Pre-op or post-op**	**No. deaths/ total**	**Sensitivity for death**	**Specificity for death**	**NPV for death**	**PPV for death**	**HR for poor survival (95% CI)**	**Adjusted HR (95% CI)**	**Mean/ Median survival**
Schwarzenbach; 2008 (83)	DNA level (spectrophotometric quantification at 260 and 280nm)	Stage IV	Pre-op	33/55	N/A	N/A	N/A	N/A	N/A	N/A	Association with shorter survival ($p = 0.02$)
Li; 2017 (118)	cfDNA copy number variation	Stage III-IV	Post-op	23/35	N/A	N/A	N/A	N/A	5.33 (6.76-94.44)	N/A	15.87 months (vs. 68.53 months)

(Continued)

TABLE 5 | Continued

Author, year	Biomarkers	Stage	Pre-op or post-op	No. deaths/ total	Sensitivity for death	Specificity for death	NPV for death	PPV for death	HR for poor survival (95% CI)	Adjusted HR (95% CI)	Mean/ Median survival
Bedin; 2017 (84)	DNA fragments (Alu83 and Alu244); Methylation: *OSMR, SFRP1*	All stages	Pre-op	28/114	N/A	N/A	N/A	N/A	Alu83: 3.49 (1.58-7.71) Alu244: 2.70 (1.25-5.84)	Alu83: 2.71 (1.22-6.02) Alu244: 2.40 (1.11-5.19)	Methylation: No association with survival
Lin; 2014 (90)	DNA copy number (cyclophilin); Mutations: 74 genes (including *KRAS, APC, TP53, PIK3CA, BRAF*)	All stages	Pre-op	62/191	N/A	N/A	N/A	N/A	DNA copy number: 3.25 (1.66-6.45) Mutations: p>0.05	DNA copy number: 2.61 (1.31-5.19)	DNA copy number: 43% (vs. 78%), $p = 0.001$ Mutations: 48.8% (vs. 77%), $p = 0.008$
Tie; 2015 (108)	Mutations: based on tumor tissue findings (including *KRAS*)	Stage IV (with mutations identified in patient tumor tissue prior to plasma testing)	Post-op	20/53	N/A	N/A	N/A	N/A	N/A	N/A	No association between change in ctDNA and survival
Herbst; 2017 (112)	Methylation: *HPP1*	Stage IV (treated with a combination therapy containing a fluoropyrimidine, oxaliplatin, and bevacizumab)	Pre-op	246/467	N/A	N/A	N/A	N/A	1.86 (1.37-2.35)	N/A	21.9 months (vs. 35.2 months)
Liu; 2016 (31)	Methylation: *SST, MAL, TAC1, SEPT9, EYA4, CRABp1, NELL1*	All stages	Pre-op	58/165	N/A	N/A	N/A	N/A	*SST*: 2.40 (1.35-4.28) *MAL*: 2.26 (1.29-3.96) *TAC1*: 1.15 (0.67-1.97) *SEPT9*: 1.02 (0.59-1.74) *EYA4*:1.24 (0.73-2.12) *CRABp1*: 1.10 (0.64-1.87) *NELL1*:1.19 (0.68-2.07)	*SST*: 1.96 (1.06-3.62) All others: not significant	*SST* in stage II and III 16.1% (vs. 41.9%), $p = 0.003$
Philipp; 2012 (116)	Methylation: *HLTF, HPP1*	All stages	Pre-op	190/311	N/A	N/A	N/A	N/A	N/A	N/A	*HLTF:* 36.3 months (vs. 80.2 months), $p = 0.0001$ *HPP1:* 12.6 months (104.7 months), $p < 0.0001$

(Continued)

TABLE 5 | Continued

Author, year	Biomarkers	Stage	Pre-op or post-op	No. deaths/ total	Sensitivity for death	Specificity for death	NPV for death	PPV for death	HR for poor survival (95% CI)	Adjusted HR (95% CI)	Mean/ Median survival
Rasmussen; 2018 (114)	Methylation: *ALX4, BNC1, HIC1, RARB, RASSF1A, SDC2, SEPT9, SRFP1, SRFP2, SPG20, TFP12, THBD, WIF1, APC, BMP3, BRCA1, CDKN2A, HLTF, MGMT, MLH1, NDRG4, NPTX2, NEUROG1, OSMR, PHACTR3, PPENK, SST, TAC1, VIM, WNT5A*	All stages	Pre-op	74/193	N/A	N/A	N/A	N/A	*ALX4:* 2.18 (1.41-3.38) *BNC1:* (2.93 (1.69-5.07) *HIC1:* 3.04 (1.46-6.32) *RARB:* 2.06 (1.31-3.23) *RASSF1A:* 2.75 (1.57-4.81) *SDC2:* 1.94 (1.23-3.07) *SEPT9:* 1.91 (1.21-3.02) *SRFP1:* 1.91 (1.21-3.02) *SRFP2:* 2.42 (1.53-3.84) *SPG20:* 2.66 (1.67-4.24) *TFP12:* 2.67 (1.38-5.19) *THBD:* 2.95 (1.68-5.18) *WIF1:* 3.26 (1.83-5.83) *HLTF:* 1.87 (1.04-3.38) *MHMT:* 2.27 (1.04-4.93) *PPENK:* 1.94 (1.07-3.50) *TAC1:* 1.56 (1.01-2.42) *VIM:* 1.82 (0.84-3.96) All others: not significant	*RARB:* 1.99 (1.07-3.72) *RASSF1A:* 3.35 (1.76-6.38) All others: not significant	N/A
Xue; 2017 (111)	Hypomethylation: *CBS*	All stages	Pre-op	37/95	75.7%	56.9%	80%	53%	1.49 (1.15-2.64)	1.35 (1.09-2.41)	N/A

CI, Confidence interval; HR, hazard ratio; N/A, not applicable; NPV, negative predictive value; PPV, positive predictive value; post-op, post-operative; pre-op, pre-operative.

differences in results. Serum typically has higher yields of DNA (85, 120), but this may be from contamination of the sample with DNA from white blood cells, which lyse during serum processing (120). A study showed that DNA levels from serum and plasma did not correlate. Serum DNA was associated with the presence of liver metastases, while only DNA from plasma was predictive for recurrences (121). Another study showed that serum samples compared to plasma samples had a decreased *KRAS* allele frequency (122). This suggests that plasma is the optimal specimen type for analysis of ctDNA (123), but despite this, approximately one-third of the studies that we reviewed had used serum (20% of CRC studies, 29% of oesophageal cancer studies, 62% of gastric cancer studies, and 100% of GIST studies).

Other features that need to be considered for ctDNA studies are amplicon lengths and time of collection. As circulating DNA is highly fragmented, targeted regions of the DNA need to account for this. By using a short amplicon assay, KRAS mutated DNA was detected in significantly more blood samples compared to using a long amplicon assays (124). Time of blood collection may also influence levels of ctDNA, as it has been shown that total DNA and levels of methylated Septin 9 (*SEPT9*) have diurnal variations (125). In patients with CRC, highest concentrations were measured at midnight (125).

All studies, whether of mutations or methylation markers, are subject to the chance that detection of biomarkers might not be associated with the tumor of interest. This was supported by a study of *TAC1* hypermethylation in oesophageal cancer which found that ∼13% of their cohort had the biomarker present in plasma but not in the matched tumor tissue (61). They proposed that this could indicate a risk for developing malignant disease in the future; that it could be derived from a pre-cancerous lesion; or it could be derived from a cancer elsewhere in the body. It is possible that ctDNA biomarkers may not be specific to just one cancer. While hypermethylation of the promoter region of *SEPT9* shows promise for screening and monitoring of CRC, methylated *Sept9* was also detected in 44.3% of lung cancer patients (126). In the current review, the lack of specificity for one cancer was seen for methylated *APC* and *RASSF1A* that have prognostic potential in both gastric (70, 71) and colorectal cancers (110, 114). These studies highlight the importance of optimization of ctDNA assays.

Choice of Biomarker

Many of the studies of prognosis have used DNA mutations as ctDNA biomarkers. Due to tumor heterogeneity, assessment of mutations is not easily implemented in practice, with the common genes (*KRAS, BRAF, APC, TP53*) mutated in only 15–40% of CRC (127). This is why several of the studies that we reviewed applied tumor tissue analysis to personalize ctDNA biomarkers. Extensive analysis of tumor prior to blood may reduce cost effectiveness of the liquid biopsy, and in addition, this limits the ctDNA biomarkers to assessment of certain tumor subtypes rather than being a universal marker of prognosis. Measurement of DNA methylation may be an easier test to apply. Methylated DNA is present in a higher proportion of tumors than mutations, for example 82% of primary tumor tissue displays *SEPT9* promoter methylation (128). There is also evidence that aberrant methylation is more common and frequently precedes the mutational changes (53). The consequences of promoter methylation can include transcriptional silencing which might facilitate tumor progression by allowing the accumulation of additional genetic and/or epigenetic changes (129). As the metastatic capacity of a cell is determined at an early stage of tumor progression (130) it seems possible to identify epigenetic biomarkers that point to tumor aggressiveness.

Other Clinical Management Strategies for CtDNA

Besides its use for prognosis, there is a lot of interest in the use of ctDNA in relation to treatment strategies. As ctDNA provides real-time results that reflect the current molecular profile of the tumor tissue which are likely to be more representative of the entire tumor rather than a single biopsy (131), ctDNA results could be used to plan appropriate therapy. Analysis of ctDNA from patients with gastrointestinal malignancies showed that most of the patients tested had one or more alterations potentially actionable by experimental or approved drugs (132). ctDNA can also be used in monitoring treatment efficacy with one such example shown with changes of ctDNA *HER2* copy number with trastuzumab treatment in gastric cancer (133). In relation to monitoring efficacy, ctDNA also shows value in detecting the development of secondary resistance to therapy. Examples have been shown in both CRC and gastric cancer with ctDNA detecting growth of mutated clones. For example, in patients with metastatic CRC, *RAS* mutations emerged during therapy with anti-EGFR mAB which indicated resistance (134). Similarly use of serial ctDNA measurements have shown emerging resistance to crizotinib use in gastric cancer (135). It is also possible that the detection of new mutations with ctDNA, or detection of a number of biomarkers identifies tumor heterogeneity, and indicates prognosis as well as guiding therapy. Clinical use of ctDNA for monitoring of therapies will allow the use of ineffective therapies to be ceased earlier. The use of ctDNA for metastatic CRC is supported by physicians, with 69% of physicians reporting that it was more convenient than tissue testing, 59% believing ctDNA to be the superior method to guide experimental therapy choice, and reporting that 89% of their patients were satisfied with the ability of this method to improve quality of care (136).

CONCLUSION

Application of new strategies for prognostication and personalized management are needed to improve survival from gastrointestinal cancers. This can be achieved with ctDNA. Due to heterogeneity of disease, single biomarkers are less likely to have sufficient sensitivity and specificity and therefore a combination of biomarkers and techniques could maximize diagnostic accuracy. Our review shows that the use of ctDNA shows great promise as prognostic biomarkers for recurrence and survival, however caution should be taken

with interpreting results from studies with limited sample sizes. As well as prognostication, markers might allow early detection of recurrence. This will result in survival benefits from resection when lesions are treatable, as well as permitting earlier commencement of therapy.

AUTHOR CONTRIBUTIONS

ES and GY came up with the manuscript concept. HS and ES reviewed all of the articles and drafted the manuscript. GY and CK contributed clinical advice. SP contributed molecular advice. GY, CK, and SP thoroughly revised and amended the manuscript.

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SUPPLEMENTARY MATERIAL

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Assessing the Impact of Circulating Tumor DNA (ctDNA) in Patients With Colorectal Cancer: Separating Fact From Fiction

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Significant advances and increased awareness have been in made in the field of non-invasive liquid biopsies for cancer, spanning several malignancies from gastrointestinal, pulmonary, and other etiologies. Broadly, the genetic source material for liquid biopsies includes circulating tumor cells, cell-free circulating tumor DNA (ctDNA), or cell-free circulating tumor microRNA (mRNA). In this review, we specifically focus on ctDNA and its current role in colorectal cancer. While there are several commercially available assays that detect ctDNA, the utility of these products is still variable and therefore the clinical applications of ctDNA in the management of patients with cancer has yet to be determined. This is reflected by the recent joint review set forth by the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP), clarifying and somewhat tempering the present role of ctDNA in patients with cancer. This review provides additional detail regarding ctDNA in the limited setting of colorectal cancer. The increasing importance and promise of ctDNA remains an area of active research, and further prospective studies may enhance the clinical utility of ctDNA in the future.

Keywords: circulating tumor DNA, colorectal cancer, biomarker, cancer diagnosis, treatment

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INTRODUCTION

The concept of circulating, cell-free DNA was initially proposed through work by Mandel and Métais in the late 1940s (1, 2). The broad correlation between cell-free DNA and cancer was later observed through work by Leon et al. in the 1970s, where levels of cell-free circulating DNA (though not necessarily tumor specific cell-free DNA) corresponded to the burden of metastatic disease for patients with a variety of tumors, including lymphoma, colorectal, lung, gynecological, breast, and brain tumors (3). The now commonly used term "liquid biopsies" was introduced by Pantel and Alix-Panabières in the late 1980s as a potential means to obtain diagnostic data from the peripheral blood of cancer patients that would have the same function as that derived from tumor specimens (4). The diagnostic source derived from the peripheral blood includes a variety of soluble factors, including cell-free circulating tumor DNA, circulating tumor microRNA (5), proteins and other biomarkers, and intact circulating tumor cells themselves (6). Since then and most significantly with the last decade, research investigating circulating tumor DNA (ctDNA) has expanded rapidly. In fact, a PubMed search using the term "ctDNA" will generate over 3,500 citations on this topic. This number of citations decreases to 403 during a search for "ctDNA and colorectal cancer."

ctDNA refers to the cell-free DNA released by tumor cells through a variety of proposed mechanisms, including secretion of tumor-associated DNA, necrosis from nonviable tumor cells, or through phagocytosis by tumor infiltrating immune cells (7). Interestingly, the ctDNA fragments themselves may not only represent solid tumor DNA, but also may have the ability to induce oncogenic changes in normal cells that ctDNA encounters, potentially serving as a mechanism for cancer metastasis (8). While these observations have been made only in animal models incorporating human ctDNA, this data suggests a functional role for ctDNA in addition to its more highly studied diagnostic and prognostic roles.

Indeed, there has been much attention recently to ctDNA and its diagnostic/prognostic roles in cancer (9–11). Like all applications for liquid biopsies, ctDNA offers potential advantages over traditional solid tumor biopsies. These include a less invasive means of obtaining diagnostic information through a blood test (as opposed to a percutaneous or open approach to sampling tumor tissue), which can be more frequently and easily repeated with minimal risk to the patient. In addition, the use of ctDNA may result in obtaining a more thorough representation of the tumor heterogeneity that is present within the tumors themselves (12, 13). In contrast, percutaneous tumor biopsies are more limited in sampling heterogeneous areas of the tumor. While a general correlation between high levels of cell-free DNA and cancer has been noted by several groups (2, 3), ctDNA that is specific to known mutations associated with a given malignancy has been targeted as a potential clinical biomarker. In this regard, several studies have demonstrated that ctDNA possesses clinical validity, meaning that it correlates to whether the patient actually has cancer. However, as will be discussed, it remains unclear whether ctDNA possesses clinical utility, meaning the ability of ctDNA to positively impact patient outcomes as a biomarker. In this review, we focus on ctDNA and colorectal cancer, evaluating and its current and potential clinical roles as well as its benefits and limitations (**Table 1**).

THE ROLE OF CTDNA IN COLORECTAL CANCER

Targeted Mutations in ctDNA

A number of common and lesser known biomarkers has been the focus of several studies investigating ctDNA in colorectal cancer. Mutated genes encoding KRAS, BRAF, APC, and p53 are among the more common targeted biomarkers, and each of these has been shown to be involved in the carcinogenesis of colorectal tumors. In 2003, Ryan et al. showed that mutant KRAS2 could be detected in the serum of patients with colorectal cancer prior surgery in 41% of cases; the same KRAS2 mutation was confirmed in 53% of resected tumors, supporting the use of ctDNA as a detection method for mutations reflective of the primary tumor (14). Thierry et al. later showed that multiple KRAS mutations and the BRAF V600E mutation could be reliably detected from ctDNA in patients with metastatic colorectal cancer (15). Detection of APC mutations, like KRAS mutations that are thought to be early changes in the development of colorectal cancer, have also been detected with the use of ctDNA, as well as the detection of P53 mutations, which are thought to be involved in later stage development of colorectal cancer (16).

In addition to these better known mutation targets, a number of other potential ctDNA biomarkers has also been studied. The presence of abnormally methylated septin 9 (mSEPT9) DNA, a GTPase involved in a variety of cellular processes related to carcinogenesis, has been shown to correlate with colon cancer at all stages (17, 18). Toth et al. showed that circulating levels of mSEPT9 were detected in patients with colorectal cancer, but not in patients with precancerous adenomas, providing evidence of its ability to discriminate between malignant and benign tumors (19). Other candidate targets for ctDNA detection have been investigated, including PIK3CA, CDH1, BCAT1, IKZF1, and ALX4, among several others (20–27). While the majority of targets have been aimed at detecting colorectal cancer, some of these new targets, such as ALX4, are being investigated to detect precancerous lesions as well (22). Novel targets continue to be investigated, although their contemporary role in the detection of colorectal cancer remains to be seen.

ctDNA in Early and Late Colorectal Cancer

The clinical utility of ctDNA as a diagnostic and prognostic biomarker varies based on its use in early vs. late colorectal cancer. Ideally, detection of ctDNA mutations would be valuable in identifying precancerous lesions or early cancers when intervention would be most beneficial. Historically, the investigation of ctDNA was performed in colorectal cancer patients with metastatic disease or pooled from a cohort having any stage of disease. Lefebure et al. investigated KRAS and RAS-SF2A mutations in 29 patients with metastatic colorectal cancer, showing that 41% of patients had detectable serum mutations that matched the mutations within the primary tumor (28). In patients where ctDNA mutations were detected, the prognosis was significantly worse as compared to patients without detectable ctDNA mutations. A more recent study by Bachet et al. found a much higher correlation between paired plasma and tumor samples with respect to RAS mutations and a cohort of 412 patients (29). Specifically, the correlation between plasma and tumor mutations was ∼70% for patients with colorectal liver metastases. Other studies by Bettegowda et al. and Schmiegel et al. showed that the correlation between plasma ctDNA and tumor mutations was similarly high (∼90%) in patients with metastatic colorectal disease (30, 31). This was further validated by a large study of nearly 1,400 patients by Strickler et al. (32). For patients with metastatic disease and detectable (often high) levels of plasma ctDNA, many studies report worse disease-free or overall survival compared to patients without detectable ctDNA (33–35). However, while these more recent studies showed a correlation between ctDNA detection, colorectal metastases, and prognosis, it is unclear whether this information will provide superior outcomes for these patients.

In contrast to metastatic disease, a number of studies have investigated whether ctDNA can be used in the detection of

TABLE 1 | Summary of studies involving ctDNA and colorectal cancer, presented by topic subheading within the review.

Result	Study(ies)	Design
TARGETED MUTATIONS IN ctDNA		
KRAS detection	Ryan et al. (14) Mouliere et al. (12) Thierry et al. (15)	Prospective study of 94 patients showed mutated KRAS ctDNA detection in all stages of CRC Mouse xenograft model of human CRC cell line showed ctDNA serum production and detection Prospective study of 106 patients showed high detection ability of mutated KRAS ctDNA
BRAF detection	Mouliere et al. (12) Thierry et al. (15)	Mouse xenograft model of human CRC cell line showed ctDNA serum production and detection Prospective study of 106 patients showed high detection ability of mutated BRAF ctDNA
APC detection	Wang et al. (16)	Retrospective analysis of 104 patients with CRC showed detection of APC mutated ctDNA
mSEPT9 detection	deVos et al. (17) Grutzmann et al. (18) Toth et al. (19)	Prospective study of 97 patients showing high specificity of mSEPT9 ctDNA with CRC Prospective study of 354 patients showing high specificity of mSEPT9 ctDNA with CRC Prospective study of 60 patients showing high specificity of mSEPT9 ctDNA with CRC but not in benign adenomas
ctDNA IN EARLY AND LATE COLORECTAL CANCER		
ctDNA detection associated with worse prognosis in metastatic CRC	Lefebure et al. (28) Bachet et al. (29)	Retrospective analysis of 29 patients with metastatic CRC showed association of ctDNA with worse disease-free survival Prospective study of 425 patients showed correlation between plasma ctDNA and tumor mutations of approximately 70% in patients with liver metastases
ctDNA detection was not associated with worse prognosis in metastatic or locally advanced disease	Strickler et al. (32)	Retrospective analysis of 1,397 patients with mutated EGFR ctDNA
ctDNA in the detection of early CRC disease has not been shown	Lecomte et al. (32) Lin et al. (20)	Retrospective study of 191 patients with stage I-III CRC showed low sensitivity of ctDNA to detect early disease Prospective study of 191 patients with stage I-III CRC showed low sensitivity of ctDNA to detect early disease
ctDNA may be used as a screening tool, but has not been definitively shown	Flamini et al. (41) Mead et al. (40)	Prospective study of 75 patients with known CRC showing elevated ctDNA compared to healthy patients Prospective study of 26 patients showed mutated ctDNA was associated with invasive carcinoma among polypectomies when combined with CEA levels
ctDNA TO PREDICT PROGNOSIS FOLLOWING SURGERY AND DURING SYSTEMIC TREATMENT		
ctDNA levels may be associated with recurrent disease following surgery	Tie et al. (42) Pedersen et al. (21)	Retrospective analysis of 230 patients with stage II disease showed ctDNA levels were associated with recurrence-free survival after surgery Prospective study including 12 patients with paired pre- and post-surgery assays showing reduction in mutated ctDNA (BCAT1 and IKZF1) following surgery
ctDNA level changes may be associated with response during systemic treatment for metastatic CRC	Tie et al. (43)	Prospective study of 53 patients with metastatic disease showed association of changes in ctDNA levels with radiographic responses
ctDNA TO DETECT RESISTANCE TO SYSTEMIC THERAPIES AND GUIDE TREATMENT SELECTION		
ctDNA levels may be associated with anti-EGFR resistance	Misale et al. (44) Mohan et al. (45) Sclafani et al. (46)	Retrospective analysis of 21 patients showed correlation of ctDNA levels with anti-EGFR response Prospective study of 10 patients with metastatic disease showed correlation of ctDNA levels with anti-EGFR response Retrospective analysis of 97 patients with locally advanced rectal cancer did not show survival benefit with ctDNA detection

early colorectal disease. As concluded by the recent joint review by the American Society of Clinical Oncology (ASCO) and the College American Pathologists (CAP), there is little evidence of the clinical validity of ctDNA in early stage disease (36). Part of this tempering message regarding ctDNA stems from the fact that many of the studies investigating ctDNA in the detection of early stage colorectal disease are comprised by a heterogeneous patient population or do not show correlation of ctDNA mutations with those found within the tumor specimens (37). For example, Lecomte et al. investigated mutated KRAS2 in a small cohort of patients where only 29 of 58 patients had either stage I or II colorectal cancer with the inability to detect mutations in 5/29 of these early stage patients (38). In addition, unlike contemporary studies in metastatic patients,

there was a relatively low correlation between detection of KRAS2 mutation in plasma with detection of the same mutation with in the tumor of 45% (29). In a similar study by Lin et al., the sensitivity of ctDNA mutations was relatively low in early stage disease, specifically 24% in stage I colorectal cancer and 45% in stage II (20). In addition, it is known that the proportion of circulating mutated genes is quite small compared to the number of normal circulating DNA fragments, making it challenging to detect ctDNA in patients with low disease burdens (39). In a study by Diehl and colleagues, multiple ctDNA assays were tested for a small cohort of patients with metastatic colorectal cancer, and the median percentage of mutant DNA fragments was only 0.18% (range, 0.005–11.7% for the 10th and 90th percentiles) (38).

Lastly, while there is much enthusiasm to develop new screening techniques based on ctDNA for the detection of precursors to colorectal cancer in asymptomatic patients, there have been no studies showing the benefit of ctDNA in this role (36). Small studies have investigated the value of ctDNA when combined with other biomarkers, such as CEA levels. Mead et al. developed a predictive model incorporating ctDNA mutations for multiple targets and the serum CEA level, resulting in a positive predictive value of ∼80% for cancer (40). A similar study by Flamini et al. also developed a prognostic algorithm incorporating ctDNA and CEA levels, showing high diagnostic predictive value for patients with early stage cancers (41). These predictive stools, however, have yet to be validated or be used as the standard of care for early detection of colorectal cancer.

ctDNA to Predict Prognosis Following Surgery and During Systemic Treatment

In addition to the detection of early or late stage colorectal cancer, applications of ctDNA have been investigated in predicting recurrent disease following surgery as well as response to disease during systemic treatment. Tie et al. performed a study of 230 patients with resected stage II colon cancer treated (42). In this study, the authors detected ctDNA in 8% of patients who did not receive adjuvant chemotherapy, in whom the majority (79%) had recurred. This is in contrast to a 10% recurrence rate in patients in whom no ctDNA was detected. Other studies have suggested similar conclusions, albeit in smaller cohorts and in more heterogeneous populations, including patients with stage I to stage III disease (47, 48). However, whether the use of ctDNA following surgery results in a benefit to patients with detectable levels of ctDNA, or whether this detection represents a lead-time bias of recurrent disease, has not been addressed by these studies.

A greater body of literature has described the use of ctDNA in monitoring the response metastatic disease to systemic therapies. Current monitoring techniques include serum CEA measurements and the use of different imaging modalities, the interpretation of which is typically based on the Response Evaluation Criteria In Solid Tumors (RECIST) criteria (49, 50). These methods, however, are hindered by several limitations. CEA levels may be falsely low in patients whose tumors do not secrete release CEA, and there may be several reasons for falsely elevated CEA levels (51). Use of the RECIST criteria can often prove challenging, as there can be inter-observer variation and heterogeneous responses among different sites of metastases within an individual patient. Thus, ctDNA holds some promise as a more effective means to monitor response to systemic treatments, thus functioning as a predictive biomarker (52, 53). However, limited data exist to support this proposed use for ctDNA. A study conducted by Tie et al. prospectively followed 53 patients with metastatic colorectal cancer receiving first-line chemotherapy, the majority of whom had oxaliplatin-based followed by irinotecan-based chemotherapy with or without bevacizumab (42). Although this cohort was relatively small, significant reductions in ctDNA levels were observed after the first cycle of chemotherapy. These reductions, defined by a >10-fold decrease in the baseline ctDNA, were not statistically significantly associated with progression-free survival (14.7 vs. 8.1 months, $p = 0.27$, for patients with significant ctDNA reductions vs. not) (43). Thus, the clinical utility of ctDNA in monitoring response to systemic treatment remains to be established and warrants further study.

ctDNA to Detect Resistance to Systemic Therapies and Guide Treatment Selection

Multiple tumors have developed mechanisms to evade the effects of systemic treatments. This has been shown for colorectal cancers, with much of the research performed in anti-EGFR resistance. Anti-EGFR monoclonal antibodies, including cetuximab and panitumumab, are used to treat patients with metastatic colorectal cancer, but are ineffective in tumors that have mutations in the RAS pathway (EGFR-RAS-RAF-MEK signaling cascade) (54). Thus, measurement of ctDNA for RAS mutations may help identify patients who would be non-responders to this targeted therapy or monitor patients who later develop resistance to anti-EGFR antibodies (55). In fact, many patients will initially respond to anti-EGFR therapy, but later develop resistance, and measurement of related mutations has been shown to correlate with this change (56, 57). Newer agents have also been investigated with ctDNA levels, such as Sym004, which is a combination of two anti-EGFR monoclonal antibodies, futuximab, and modotuximab (58). In a phase II randomized trial comparing Sym004 with standard second and third line chemotherapy in ∼250 patients with acquired resistance to anti-EGFR monoclonal antibodies (cetuximab and panitumumab), an up to 30-fold decrease in EGFR ctDNA was detected in some patients. However, this response did not translate into a survival benefit (58).

One study by Mohan et al. detected mutations in KRAS and MET ctDNA in a small cohort of patients who developed resistance to anti-EGFR therapy (45). In a study by Misale et al., analysis of metastases from a small cohort of patients who developed resistance to anti-EGFR therapy showed the emergence of KRAS amplification or other KRAS mutations in 60% of cases. Interestingly, the KRAS mutant ctDNA were detectable in the blood of anti-EGFR treated patients as early as 10 months before radiographic disease progression (44). Additional studies by this group also reported the detection

of ctDNA mutations to NRAS prior to disease progression while on anti-EGFR therapy, and showed using a mouse xenotransplant model that tumor from a colorectal cancer patient who had initially responded and then relapsed while on anti-EGFR therapy significantly responded to combination treatment with both anti-EGFR and anti-MEK inhibition (59). On the contrary, a recent retrospective study by Sclafani et al. analyzed KRAS and BRAF ctDNA in a cohort of about 100 patients with locally advanced CRC, and showed that there was no difference in outcome between patients with or without detectable ctDNA (46).

Taken together, it is still unclear whether ctDNA may lead to improved individualized care for patients who develop resistance to current targeted treatments, such as anti-EGFR therapy, and guide management for the selection new therapies or combination therapies. However, it is important to stress that these findings are based on very small cohorts of patients and should be further evaluated in prospective studies in order to better establish the benefits of using ctDNA to guide these patient treatment decisions.

Practical Aspects of ctDNA in Colorectal Cancer

There are several commercially available assays used to obtain, process, and analyze ctDNA from patients. As discussed in the joint review by ASCO and CAP, while there is some consensus on the methodology of ctDNA collection, there are still many questions regarding the optimal processing methods for ctDNA analysis (36, 60). Nuances in ctDNA collection and storage can affect the validity of ctDNA quantification, and there is no current consensus or regulation on the standards for these methods (61). Differences in collection and purification methods have been reported with regard to the quality of extracted ctDNA. For example, Kloten et al. recently reported that silica-based membrane methods improved extraction of long cell free DNA fragments, whereas a magnetic bead system improved extraction of short cell free DNA fragments in serum of cancer patients (62). In general, there are a variety assays for ctDNA, and several specific assays for ctDNA derived from patients with colorectal cancer have been used. Many of these incorporate next generation sequencing in order to detect specific mutations in colorectal cancer as described above (63), while others take a broader approach to ctDNA analysis.

Recognizing that abnormally methylated DNA correlated with the presence of colorectal cancer, He and colleagues developed a polymerase chain reaction (PCR) assay to detect the mutation status of several colorectal cancer selected genes (mSEPT9, ALX, TMEFF2) called the MethyLight assay (64). The sensitivities using these three genes as biomarkers for the detection of colorectal cancer were high both in primary tumor tissue and peripheral blood samples (84 and 81%, respectively, with specificities of 87 and 90%). Later studies have expanded on the number of gene mutations analyzed. Some groups, such as Lin et al., have targeted the identification of hypermethylated genes associated with microsatellite instability (MSI), including D5S345, D2S123, BAT25, BAT26, and D17S250 (65). Detection of three or more of these hypermethylated markers was correlated with metastatic disease and worse disease-free survival. Interestingly, Mouliere et al. developed a multi-gene assay detecting seven KRAS mutations and one BRAF mutation (V600E) that was combined with a type of high resolution scanning microscopy. This imaging technique, known as Atomic Force Microscopy, detects low levels of fragmented DNA in the serum (66). This combined approach yielded a positive predictive value ∼90% in the detection of colorectal cancer using a cohort of 124 patients. Recognizing the limitations of ctDNA collection due to the low levels of circulating cell free DNA, other groups have developed enrichment methods in order to increase the detection mutated ctDNA (67–69). Indeed, with the wide breath of techniques available for collecting and analyzing ctDNA, further study is warranted to efficiently compare the reliability, reproducibility, and utility of these methods, in addition to gauging the cost effectiveness among these different assays.

CONCLUSION

Over 50 years has passed since the credited discovery circulating cell free DNA, and in the last 15 years much attention and enthusiasm has been given to ctDNA as liquid biopsies to obtain diagnostic and prognostic information for patients with cancer, particularly for patients with colorectal cancer. Advances have been made in the application of ctDNA for (1) detecting early or late stage colorectal cancer, (2) generating predictions of response to systemic therapy, (3) using changes in mutated ctDNA to modify systemic treatments, and (4) utilizing ctDNA to for surveillance of disease recurrence following surgery. Yet many of these studies are limited by their retrospective design and small sample size, and therefore the clinical utility of measuring ctDNA for colorectal cancer has yet to be established. In addition, the high number of different assays available to collect, purify, and analyze ctDNA introduces practical challenges to establishing standard clinical use and utility of these methods. Thus, the fervor over ctDNA has recently been tempered (36, 70, 71). Nonetheless, the advantages of ctDNA are promising, and with novel prospective, collaborative studies, the true benefits of ctDNA in colorectal cancer as well as other malignancies may soon become realized.

AUTHOR CONTRIBUTIONS

EG drafted the manuscript and performed critical review. SB performed critical review.

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CASE REPORT
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Mutation Detection in Tumor-Derived Cell Free DNA Anticipates Progression in a Patient With Metastatic Colorectal Cancer

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Background: The observation of tumor-derived cell-free DNA (ctDNA) in plasma brought new expectations to monitor treatment response in cancer patients.

Case presentation: In an exploratory case of a 57-year-old man diagnosed with metastatic sigmoid adenocarcinoma, we used a hotspot panel of cancer-associated gene mutations to identify tumor-specific mutations in the primary tumor and metastasis. Results: Five mutations were detected (*KRAS,* p.Gly12Val; *TP53*, p.Arg175His; *RB1*, p.Ile680Thr; *ALK,* p.Gly1184Glu; and *ERBB2*, p.Lys860Lys), of which three were detected in both tissue types (primary tumor and metastasis). All five mutations were monitored in the ctDNA of six serial plasma samples. Only *KRAS* and *TP53* mutations were detected at a high frequency in the first plasma sample. After 1 month of chemotherapy the allele frequencies of both mutations fell below the detection limit. From the third month of systemic treatment onward, the allele frequencies of both mutations were detectable in plasma, displaying a continual increase thereafter. The remaining three mutations were not detected in plasma samples. Signs of disease progression in ctDNA during the treatment period were evident while computed tomography (CT) measurements suggested stable metastatic lesions throughout the treatment.

Conclusions: Liquid biopsies revealed tumor heterogeneity and predicted tumor progression, demonstrating the potential of ctDNA analysis to be a sensitive and specific tool for monitoring treatment responsivity and for early identification of treatment resistance.

Keywords: liquid biopsy, ctDNA, colorectal cancer, NGS, gene panel

BACKGROUND

Recently, there have been substantial advances in the development of minimally invasive techniques for cancer diagnostics and the monitoring of treatment response and disease progression. In this context, liquid biopsies are emerging as a valuable tool in several clinical scenarios.

Liquid biopsies can be use to detect tumor biomarkers, such as circulating tumor cells, tumor microvesicles and circulating tumor DNA (ctDNA) in plasma and other body fluids. One of the most validated liquid biopsies applications is the detection of tumor-specific mutations in cell-free DNA (cfDNA) that has been released into circulation. Owing to the non-invasive nature of liquid biopsies making them amenable to repeat procedures, ctDNA detection in liquid biopsies has been demonstrated to be useful for several oncological applications, including monitoring treatment response, disease progression, and tumor relapse information (1–6). Additionally, this method enables assessment of tumor variability and heterogeneity, once tumor DNA are expelled from cells from different regions of the tumor and metastases (3, 7, 8).

Until recently, detection of ctDNA was hindered by the insufficient sensitivity of routine techniques for assessing the tumor-specific mutations in total cfDNA. Because cfDNA contains DNA from both normal and cancer cells, with the latter being found at lower levels and in much more degraded fragments, highly sensitive techniques are needed to detect tumor-specific mutations (3, 6, 9, 10).

A great number of variables can also limit cfDNA concentrations and hinder ctDNA detection. For example, trauma, infection, autoimmune disease, and intensive exercise, can alter cfDNA concentrations in plasma (11, 12). Furthermore, because ctDNA has a half-life is under 2.5 h, the time elapsed between sample collection and processing can reduce the ctDNA detection potential (13, 14). Moreover, ctDNA levels appear to be associated with tumor burden, such that more advanced tumors are more likely to produce higher amounts of ctDNA in plasma (6), whereas a favorable treatment response can decrease tumor DNA quantities in circulation (13, 15). Another important issue is the clonal hematopoiesis of indeterminate potential, commonly referred to as CHIP. Several studies have demonstrated that aging can increase somatic mutations in cfDNA of healthy individuals, especially in genes associated with hematopoietic elements, such as *DNMT3A* and *TET2* (16–18). Thus, it is important to take precautions with respect to these variables to avoid erroneous diagnostic conclusions.

To obtain the sensitivity and specificity necessary to incorporate ctDNA analysis into clinical practice, existing techniques may be modified and/or new techniques may be developed. To date, the most used technologies have been digital PCR, real-time PCR, BEAMing (beads, emulsions, amplification, and magnetics) (19, 20), and, most recently, next generation sequencing (NGS).

NGS has been shown to be a reliable tool for detecting tumor-specific mutations in ctDNA with great sensitivity and specificity (>94%, and >98%, respectively) (5, 6). Changes in ctDNA levels corresponding to tumor dynamics in response to treatment have been demonstrated across several tumor types, including lung, breast, colorectal, and melanoma cancers among others (2, 4, 6, 14, 21–23). Increases in ctDNA mutation allele frequencies were shown to occur prior to clinical or imaging evidences of tumor progression (24). Because treatment resistance can result from the acquisition of new somatic mutations in cancer cells, genomic ctDNA profiling may enable detection of emerging subclonal actionable mutations for which targeted therapies can be applied.

Here, we examined the relevance of ctDNA analysis in a tumor kinetics study involving a patient diagnosed with metastatic colorectal cancer using a NGS hotspot panel of 50 genes frequently mutated in cancer. We evaluated both primary and metastatic tumor tissues and monitored ctDNA extracted from plasma samples collected over the course of patient's treatment.

CASE PRESENTATION

A 57-year-old man presented with a complaint of increasing abdominal pain in June of 2014. A colonoscopy performed in July of the same year showed a stenotic and ulcerated lesion with an infiltrative aspect in the sigmoid region; the stenosis prevented advancement of the colonoscopy beyond the lesion. Computed tomography (CT) revealed hepatic nodules with peripheral contrast enhancement in segments II, IV, I, VIII, V, and VI. The largest hepatic nodule measuring 2.5 cm, was found in segment II. Additionally, a hypodense nodular formation, measuring 2.8 cm, was found in the right adrenal gland and a focal wall thickening, with an area of 6.0 × 3.2 cm, was found in the descending colon measuring. A subsequent magnetic resonance imaging (MRI) examination conducted in August of 2014 revealed hepatic nodules larger than 4.4 cm in segment I. A thoracic CT performed on the same date showed pulmonary micronodules suggestive of secondary implants.

Due to the obstructive sigmoid lesion, a laparoscopic sigmoidectomy with primary colorectal anastomosis was considered the first treatment option, followed by palliative chemotherapy. The patient received FOLFOX (10 cycles) as a first-line treatment and FOLFIRI (3 cycles) as second-line regimen. Further evaluations of the hepatic lesions were made every 2–3 months by CT imaging. Carcinoembryonic antigen (CEA), a serum marker used to monitor carcinoma progression, was evaluated at the time of diagnosis (145 mg/dl) and before commencement of the second-line treatment (1,678 mg/dl).

Microscopic evaluation of the surgical specimen revealed a moderately differentiated sigmoid adenocarcinoma (5.6 × 3.4 cm) with mucinous pattern areas and a pathology stage of pT4apN2apM1. The lesion had an invasive front compromising the serous layer. Lymph-node metastases with capsular extravasation were detected in four of fourteen lymph-nodes dissected from adjacent adipose. Surgical margins were

Abbreviations: CEA, carcinoembryonic antigen; ctDNA, circulating tumor DNA; CT, Computed tomography; cfDNA, cell free DNA; NGS, Next Generation Sequencing; CHIP, Clonal hematopoiesis of indeterminate potential; COSMIC, Catalog of Somatic Mutations in Cancer; MRI, magnetic resonance imaging; PS, Plasma sampling; RECIST, Response Evaluation Criteria In Solid Tumors.

tumor-free. Biopsies of hepatic growths at the moment of the primary tumor surgery confirmed a diagnosis of metastatic colorectal adenocarcinoma. Immunohistochemistry showed positive labeling for the mismatch repair proteins MLH1, MSH2, MSH6, and PMS2. An activating *KRAS* mutation was identified by routine molecular testing for metastatic colorectal cancer at our institution.

Targeted resequencing was performed in the Ion Proton platform with the Ion AmpliSeq^{TM} Cancer Hotspot Panel v2 (Thermo Fisher Scientific, Waltham, MA), which covers approximately 2,800 COSMIC mutations from 50 oncogenes and tumor suppressor genes. A detailed description of the sequencing methods are provided in a **Supplementary File**. Tumor-specific genomic DNA mutations were assessed for the primary tumor, and metastasis. cfDNA from six plasma samples (PS1–6) were also assessed: one before treatment and five after surgery and during palliative chemotherapy.

Sequencing analysis of the primary tumor identified five tumor-specific mutations, including an activating *KRAS* mutation (p.Gly12Val), confirming previous analysis, a loss-of-function mutation in *TP53* (p.Arg175His), two somatic mutations of unknown clinical impact in *RB1* (p.Ile680Thr) and *ALK* (p.Gly1184Glu), and a synonymous *ERBB2* (p.Lys860Lys) alteration (**Table 1**). Evaluation of hepatic metastatic lesions detected three of these somatic mutations (*KRAS, TP53,* and *RB1*) with a 1% threshold criterion. In the plasma sample collected before surgery (PS1), only the two mutations (*KRAS* and *TP53*) detected with high allele frequency in both primary and metastatic samples were detected in ctDNA.

The remaining five additional plasma samples, PS2–6, were collected monthly, starting 1 month after the beginning of chemotherapy (**Figure 1**). Interestingly, the allele frequency of the two aforementioned tumor-specific mutations in *KRAS* and *TP53* decreased significantly, dropping below the 1% detection cut-off of by PS2 (**Figure 1**). ctDNA mutations remained undetectable by NGS in PS3. The noticeable decrease in allele frequency mutations after primary tumor resection and during palliative chemotherapy can be related to a response to treatment. By PS4, in February of 2015, 3 months of FOLFOX treatment, the allele frequencies of both mutations started to rise, *TP53* 6% and *KRAS* 4%, approaching PS1 frequencies in PS5 and PS6. Although the initial decrease in mutation frequencies was in accord with an initial treatment response; subsequent increases in mutation frequencies anticipated tumor progression, albeit CT imaging showed maintenance of the number and size of the patient's liver lesions throughout palliative chemotherapy treatment. The timeline of the patient's peripheral blood collection, palliative chemotherapy, and follow-up scheme, as well as the ctDNA identification through NGS, along with their frequencies, are shown in **Figure 1**. In April of 2015, the patient presented signs of disease progression and FOLFIRI was started. After 3 cycles of FOLFIRI, the patient's clinical condition deteriorated, and he died due to liver failure in May of 2015. It is important to highlight that ctDNA analysis was not performed concurrently with plasma collection, and all ctDNA analysis were performed at a later time, such that ctDNA results did not alter the clinical treatment protocol.

DISCUSSION

In this case study we were able to detect five tumor-specific mutations in primary or metastatic tumor tissues and use them to monitor tumor dynamics in plasma samples. Two mutations that are strongly associated with colorectal cancer (*KRAS*, and *TP53*) exhibited robustly elevated allele frequencies in the preoperative plasma sample. The allele frequencies of both mutations decreased sharply after resection of primary tumor and continued decreasing in the first month of chemotherapy, suggesting that the metastatic lesions were responding to the treatment. The subsequent increasing trend in these mutated allele frequencies were suggestive of tumor progression. Interestingly, all CT evaluations performed in this timeframe suggested disease stability, with no evidence of increase in the number or size of the major lesions. Disease progression was detected in an MRI exam (data not shown) performed 24 days after the last CT; showing innumerous diffuse and confluent liver metastases interpreted as unequivocal disease progression. Although MRI has a higher sensitivity than CT, the tumor burden change observed was significant and would probably have been detected by CT before patient's death. Nevertheless, we cannot infer neither that these further lesions were not present earlier nor that MRI could have detected tumor progression if used for continuous monitoring of the patient. Notwithstanding, the early change in the dynamics of the ctDNA was predictive of a poor outcome, as shown in our ctDNA analysis timeline.

Morphological imaging-based criteria are still the main parameter utilized to monitor solid tumor evolution, but have limitations. A critical drawback is the delay between tumor progression/regression and a perceptible change in tumor size. Detecting tumor enlargement by imaging can take weeks or months, which can delay critical decisions in patient management. The present data support this conclusion.

Several evidences have indicated that ctDNA frequencies are associated with cancer patient prognosis and can anticipate disease progression, in a manner that is more precise than the methods currently used for monitoring chemotherapy response. In the present case, CEA, a tumor marker use to monitor treatment response in patients with colorectal cancer patients, did also show concordance with clinical progression, however, several limitations have been recurrently reported regarding this issue. About 30% of patients diagnostic with colorectal cancer do not show alterations in CEA levels (25); not every patient has abnormal CEA elevation in the presence of disease and during relapse; CEA flare post and during chemotherapy are not always related to cancer progression; among others.

Similar to our results, Bettegowda et al. observed a correlation between elevations in ctDNA frequency and poor prognosis and overall survival in various malignancies. Moreover, Dawson et al. (15) observed increased ctDNA frequencies in a group of women with metastatic breast cancer, earlier to imaging exams. Thus, ctDNA seems to be a trustworthy biomarker when compared to the current ones used in diagnosis including imaging diagnostic methods.

NGS provides a window into tumor dynamics and resistance mechanisms by providing data for a larger number of mutations.

TABLE 1 | Somatic mutations identified in the primary tumor, metastasis and plasma.

Gene	Chr: position	Ref allele	Mut allele	Codon change	Protein change	Variant type	VAF%		
							Tumor	Metastasis	PS1
KRAS	chr12: 25398284	C	A	c.35G>T	p.Gly12Val	Missense	43.28	35.86	23.23
TP53	chr17: 7578406	C	T	c.524G>A	p.Arg175His	Missense	80.23	61.35	28.27
RB1	chr13: 49033902	T	C	c.2039T>C	p.Ile680Thr	Missense	11.11	1.08	ND
ALK	chr2: 29443666	C	T	c.3551G>A	p.Gly1184Glu	Missense	25.89	ND	ND
ERBB2	chr17: 37881388	A	G	c.2580A>G	p.Lys860Lys	Synonymous	19.66	ND	ND

Chr, chromosome; Ref, reference allele; Mut, mutated allele; VAF, variant allele frequency; PS1, plasma sample #1 (pretreatment) ND, not detected.

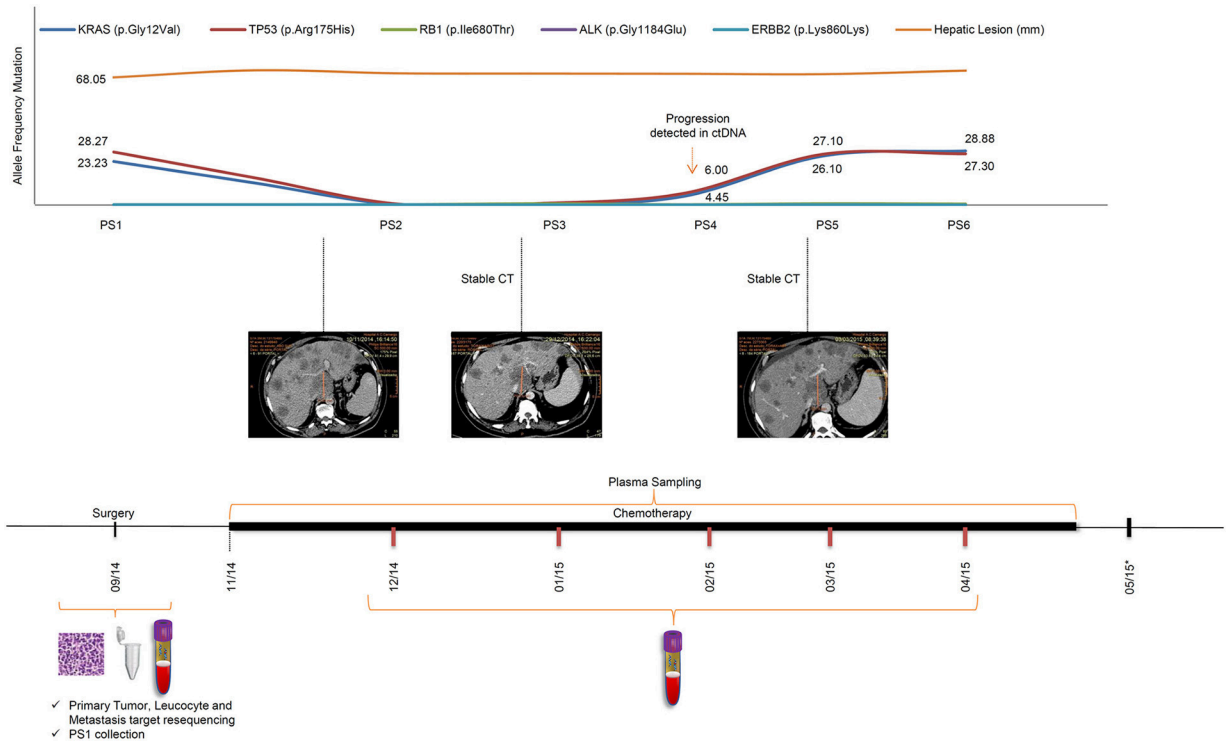

FIGURE 1 | Timeline of patient's treatment, follow-up and ctDNA mutation levels during disease progression. The upper panel shows the frequencies of tumor mutations detected in each plasma sample, and hepatic lesion size throughout the course of palliative chemotherapy. An increase in *KRAS* and *TP53* mutation frequencies observed in PS4 preceded significant increases in tumor marker frequencies in PS5 and PS6. Sequential CT measurements were suggestive of disease stability throughout the treatment period. The lower panel shows the patient's course of treatment (FOLFOX followed by FOLFIRI) with surgery date and serial PS collection. PS, plasma sample; CT, computed tomography; *, patient death.

The information obtained is of an inestimable value because it may enable early detection of disease progression, which may affect the planning for combination treatments and the use of alternate therapies, thereby providing the potential to hasten and improve disease management decisions (9, 10, 15, 26). Using a 50-gene panel for screening the primary tumor, metastatic and plasma samples, we were able to assess the heterogeneity among them and the clonal dynamics of tumor cells in response to treatment.

It is noteworthy that we identified only *KRAS* and *TP53* mutations in ctDNA. Activating mutation in *KRAS* and loss-of-function mutation in *TP53* seem to be essential for tumor maintenance and progression (27). The *KRAS* activating mutation p.Gly12Val results in constitutive activation of RAS GTPase and is considered to be a driver mutation in colorectal cancer (28). Meanwhile, *TP53* p.Arg175His, a known pathogenic mutation, has been observed in 6% of colorectal cancers and 3% of head and neck cancers [(29); COSMIC database]. Our data reinforce the notion that investigating a group of tumor mutations enables a broad assessment of tumor mutation burden dynamism during treatment and disease progression.

Another interesting finding of our study was the fact that not all mutations could be tracked by ctDNA analysis. Initially, we found five point mutations in the primary tumor, some

with potential clinical relevance (*ALK* and *ERBB2*). However, metastasis sequencing revealed only three mutations *KRAS* and *TP53,* at high-level, and *RB1,* at low-level. None of the additional mutations (*ALK, ERBB2,* and *RB1*) were detected in the plasma samples.

The RECIST imaging criteria is used universally to evaluate tumor response during and after systemic treatment for solid tumors. This criteria, which is related to tumor size changes, strongly correlates with clinical outcome (progression, symptoms, and death). However, it is becoming increasingly clear that other methods not based solely on tumor size should be developed and validated to improve our ability to evaluate treatment efficacy in oncology. The liquid biopsy technique represents one promising option. In the present case, despite no tumor alterations was detected on CT after treatment, we observed early decreases and subsequent increases in ctDNA. In a scenario where multiple and personalized drugs are available, this strategy may inform rapid changes in an ongoing treatment plan.

CONCLUSIONS

Our results reinforce the potential applicability of plasma ctDNA for anticipating disease progression efficiently in patients with colorectal cancer and highlight the value of NGS in revealing clonal dynamics of tumors in response to therapy.

ETHICS STATEMENT

Patient has signed an informed consent form to participate in the study and for case report publication. This study was performed in compliance with the Helsinki Declaration and was approved by the ethics committee of the A. C. Camargo Cancer Center under number 1819/13.

AUTHOR CONTRIBUTIONS

BDFB carried out the sequencing experiments. BK was responsible for the patient's follow up. RC evaluated CT imaging results. CALM, SAJ, SJS, and DMC designed the study. BDFB, GTT, CALM, RC and DMC analyzed the data and wrote the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2018.00306/full#supplementary-material

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CASE REPORT
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Cell-Free DNA Profiling to Discover Mechanisms of Exceptional Response to Cabozantinib Plus Panitumumab in a Patient With Treatment Refractory Metastatic Colorectal Cancer

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MET amplification is rare in treatment-naïve metastatic colorectal cancer (CRC) tumors, but can emerge as a mechanism of resistance to anti-EGFR therapies. Preclinical and clinical data suggest that patients with *MET* amplified tumors benefit from MET-targeted therapy. Cabozantinib is an inhibitor of multiple tyrosine kinases, included c-MET. Panitumumab is an inhibitor of EGFR. This report describes a patient with *KRAS*, *NRAS,* and *BRAF* wild-type metastatic CRC who experienced disease progression on all standard chemotherapy and anti-EGFR antibody therapy. The patient was enrolled in a clinical trial evaluating the combination of cabozantinib plus panitumumab. After only 6 weeks of treatment, the patient experienced a significant anti-tumor response. Although tumor tissue was negative for *MET* amplification, molecular profiling of cell-free DNA (cfDNA) revealed *MET* amplification. This case represents the first report showing the activity of cabozantinib in combination with panitumumab in a patient with metastatic CRC, and suggests that *MET* amplification in cfDNA may be a biomarker of response. A clinical trial targeting *MET* amplified metastatic CRC is currently underway.

Keywords: *MET* amplification, metastatic colorectal cancer, cabozantinib, cell-free DNA, ctDNA

BACKGROUND

The receptor tyrosine kinase c-MET (mesenchymal-epithelial transition factor), is implicated in tumorigenesis, proliferation, invasiveness, metastasis, and resistance to cancer treatment (1). Encoded by the *MET* proto-oncogene, c-MET is a disulfide-linked glycoprotein consisting of an extracellular α-subunit and a membrane spanning β-subunit (1). Hepatocyte growth factor (HGF) is the only known ligand for c-MET, and is predominantly secreted in a paracrine fashion by stromal cells. HGF binding induces c-MET receptor dimerization which in turn activates various downstream signaling pathways (2). HGF/c-MET signaling plays an essential role in diverse physiological processes such as embryonic development, epithelial branching morphogenesis and postnatal organ regeneration (3). Aberrant MET activation can occur via multiple mechanisms, including *MET* gene amplification (4).

MET gene amplification has been observed in multiple tumor types, including colorectal cancer (CRC) (5, 6), gastric cancer (7, 8), genitourinary cancers (9), head and neck cancer (10), non-small cell lung cancer (NSCLC) (11, 12), neuroblastoma (13), and ovarian cancer (14, 15). *MET* amplification is one of the key mechanisms mediating both primary (16) and acquired resistance (17) to epidermal growth factor receptor (EGFR) inhibition in patients with NSCLC. It has been shown that *MET* amplification leads to acquired resistance to EGFR tyrosine kinase inhibitors (TKI)s by persistent activation of ERBB3 signaling (18) and *MET* amplification can be detected with or without the presence of the *EGFR* T790M "gatekeeper" mutation (19). The prevalence of *MET* amplification is low (∼3 %) in patients with untreated NSCLC, but increases to 5–22% in patients who develop acquired resistance to EGFR TKI therapy (17, 19, 20). The emergence of *MET* amplification under the selective pressure of anti-EGFR therapy supports the notion that *MET* amplification is a driver of acquired treatment resistance (21).

In patients with metastatic CRC, *MET* amplification is associated with resistance to anti-EGFR antibodies, including cetuximab and panitumumab. In mice engrafted with *MET* amplified CRC tumors, treatment with cetuximab is ineffective, suggesting that *MET* amplification may be responsible for intrinsic resistance to anti-EGFR antibodies (22). Functional crosstalk between c-MET and EGFR provides compensatory signal transduction leading to constitutive activation of downstream MAPK and PI3K pathways, thereby circumventing upstream EGFR blockade (23). *MET* amplification is found in less than 3% of patients with metastatic CRC who have not been exposed to anti-EGFR antibodies. Given the fitness advantage of *MET* amplification under the selective pressure of anti-EGFR therapies, *MET* amplification is much more common after exposure to anti-EGFR antibodies. Bardelli et al. (22) found that *MET* amplification emerged in post-treatment tumor biopsies of 3 out of 7 patients with metastatic CRC who developed acquired resistance to cetuximab or panitumumab (22). In a separate cohort of 22 patients with *RAS* and *BRAF* wild-type, HER2/MET negative metastatic CRC who developed resistance to anti-EGFR therapy, *in situ* hybridization (ISH) of the tumor tissue biopsies identified *MET* amplification as one of the most common genomic alterations (24).

Molecular profiling of blood-based circulating cell-free DNA (cfDNA) also supports *MET* amplification as a driver of EGFR antibody resistance. In a study by Siravegna et al. *MET* amplification was detected in 3 out of 16 patients who developed acquired resistance to anti-EGFR therapy (25). In another cohort of 53 patients with metastatic CRC, *MET* amplification was detected in in 22.6% (12/53) of patients with RAS wild-type tumors after exposure to anti-EGFR antibody therapy, but not found at an elevated frequency in anti-EGFR antibody-naïve patients (26). In addition, *MET* amplification was uncommon in *RAS* mutated patients (26). These findings have two major implications. First, it supports the utility of *MET* amplification as a biomarker of treatment resistance in patients with *RAS* wild-type EGFR antibody refractory metastatic CRC. Second, it demonstrates that *MET* amplification can be detected in cfDNA, thus supporting the clinical validity of cfDNA profiling to select patients for MET-targeted therapy.

The efficacy of MET inhibition in anti-EGFR antibody refractory metastatic CRC has been demonstrated in many preclinical studies. For example, in *MET* amplified patient-derived colorectal cancer xenograft models, MET tyrosine kinase inhibitors (TKIs) reversed resistance to EGFR blockade (22). Synergistic inhibitory effects between MET TKI and EGFR blockade was shown in a CRC xenograft mouse model expressing human HGF, where more pronounced tumor regression with concomitant MET TKI and cetuximab was observed *in vivo* in comparison to MET inhibition or cetuximab alone (27).

Cabozantinib is an orally bioavailable TKI that targets c-MET and VEGFR2, as well as RET, ROS1, AXL, KIT, and TIE-2. Cabozantinib is approved by the United States Food and Drug Administration (FDA) for use as monotherapy for metastatic medullary thyroid cancer[1] and advanced renal cell carcinoma[2]. Panitumumab is an anti-EGFR monoclonal antibody FDA-approved for use in patients with *KRAS* and *NRAS* wild-type metastatic CRC[3]. Here we present a case report of a dramatic response to cabozantinib and panitumumab in a patient with *MET* amplified, EGFR antibody refractory metastatic CRC.

CASE REPORT

A 57-year-old male was initially diagnosed with locally advanced rectal cancer (T3N1M0) and treated with neoadjuvant chemoradiation followed by surgical resection (**Figure 1**). He subsequently received adjuvant modified (m) FOLFOX6 followed by colostomy reversal.

Two years later, CT imaging demonstrated new retroperitoneal lymphadenopathy suspicious for metastatic disease, and retroperitoneal lymph node (LN) biopsy revealed metastatic adenocarcinoma consistent with CRC primary. He received first-line treatment with FOLFIRI plus bevacizumab, but eventually experienced disease progression. He then progressed on a clinical trial combining capecitabine with an investigational therapy, followed by progression on regorafenib.

As his tumor was *KRAS* and *NRAS* wild-type, he was then treated with anti-EGFR antibody therapy (panitumumab). After 7 months of disease control, imaging revealed a new hypermetabolic LN at the right common iliac chain, and irinotecan was added to panitumumab. This treatment was eventually discontinued due to disease progression. A new biopsy of a mediastinal LN was performed and next generation sequencing (NGS) revealed that the tumor was still *KRAS*, *NRAS*, and *BRAF* wild-type, and there was no evidence of *MET* amplification (*see* **Table 1**). After progression on another phase I clinical trial with an investigational therapy, he was then enrolled in a phase Ib clinical trial combining cabozantinib and panitumumab (NCT02008383). At the time that he started

[1]COMETRIQ (cabozantinib) full prescribing information, revised 1/2018.
[2]CABOMETYX (cabozantinib) full prescribing information, revised: 12/2017.
[3]VECTIBIX (panitumumab) full prescribing information, revised 6/2017.

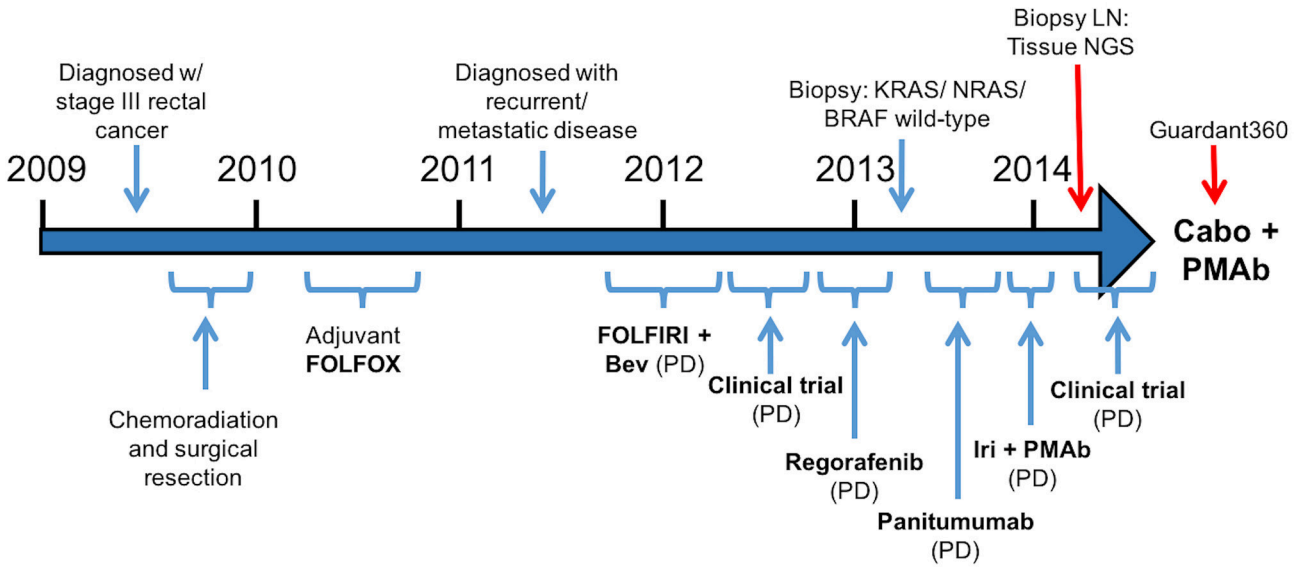

FIGURE 1 | Treatment course (Bev, bevacizumab; Iri, irinotecan; PMAb, panitumumab; LN, lymph node; Tissue NGS, tissue next generation sequencing; Guardant360, cell free DNA profiling; Cabo, cabozantinib).

TABLE 1 | Tissue-based next-generation sequencing (NGS) and blood-based cfDNA NGS.

Gene	LN biopsy (NGS) (2/27/2014)	Blood cfDNA (5/28/2014)
APC	Y935fs*1	Y935N†
BRAF	Not detected	G466E†
EGFR	G465R - subclonal	G465R†, G465E†, S464L†
		Amplified (pCN 2.2)
FAM123B	G348fs*29	Not tested
FGFR1	Amplified	Not tested
KRAS	Not detected	G13D†, G12S†, Q61H†
MET	Not detected	Amplified (pCN 2.3)
NF1	Rearrangement int30	Not tested
TP53	R213*	R213*

†*Minor alterations: Defined as alterations with relative variant allele frequency (rVAF) less than 10% of the alteration with the highest VAF. In this case TP53 R213* is the alteration with the highest VAF.*

treatment, he was increasingly symptomatic due to extensive pulmonary metastases, with worsening cough and shortness of breath. After ∼6 weeks of treatment, CT demonstrated dramatic improvement in his pulmonary tumor burden (see **Figure 2**), as well as resolution of dyspnea and cough. As part of the trial protocol, plasma-EDTA was collected before the start of treatment to explore potential drivers of treatment response and/or resistance. CfDNA profiling utilizing a 54-gene targeted NGS panel (Guardant 360™) was performed on this sample. Blood-based profiling revealed subclonal *EGFR*, *KRAS* and *BRAF* resistance mutations. Additionally, *EGFR* amplification and *MET* amplification were observed in cfDNA, but not in tissue obtained 3 months prior (**Table 1**). Unfortunately his treatment course was complicated by anastomotic dehiscence and leak with abscess evolution. Because the dehiscence was apparently related to marked treatment response and tumor involution, treatment was discontinued. CfDNA profiling performed after 28 days of treatment revealed loss of *MET* and *EGFR* amplification (**Figure 3A**), while the mutant allele frequency (MAF) of *KRAS* G13D increased from 0.3 to 0.6%. There was also a nearly 10-fold decrease in the MAF of *TP53* R213* post treatment, likely correlating with the dramatic reduction of tumor burden (**Figure 3B**).

After 2 months off therapy, his CEA increased and his dyspnea and cough returned. Capecitabine was initiated and panitumumab was added 2 months later for additional control. He experienced brief stabilization of disease on capecitabine and panitumumab, with subjective improvement of his pulmonary symptoms. He then experienced disease progression and was transitioned to hospice. He died ∼10 months after discontinuing cabozantinib and panitumumab.

DISCUSSION

Despite advances in the treatment of CRC, it remains the second leading cause of cancer-related death in the United States (28). Patients with *RAS* wild-type metastatic CRC are eligible for treatment with the anti-EGFR antibodies panitumumab or cetuximab (29, 30). The clinical benefit of anti-EGFR antibodies is modest, with a single agent response rate of ∼20% and a median progression free survival of 4 months (31). Even among patients who experience benefit from EGFR antibodies, acquired resistance is nearly universal (32, 33).

Multiple mechanisms of acquired resistance to anti-EGFR therapy have been identified in metastatic CRC, including *BRAF* mutations, acquired mutations in the *EGFR* extracellular

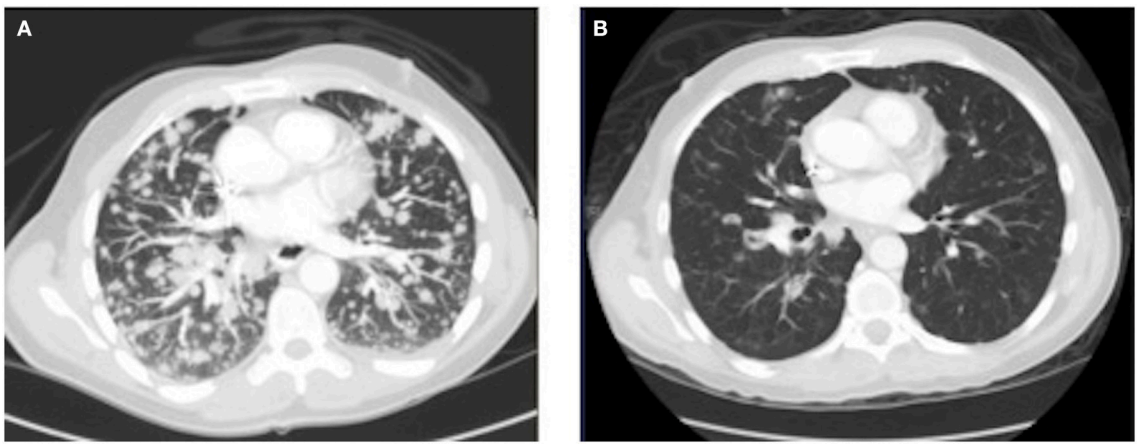

FIGURE 2 | Chest CT image **(A)** before the start of cabozantinib plus panitumumab and **(B)** after 42 days of cabozantinib plus panitumumab.

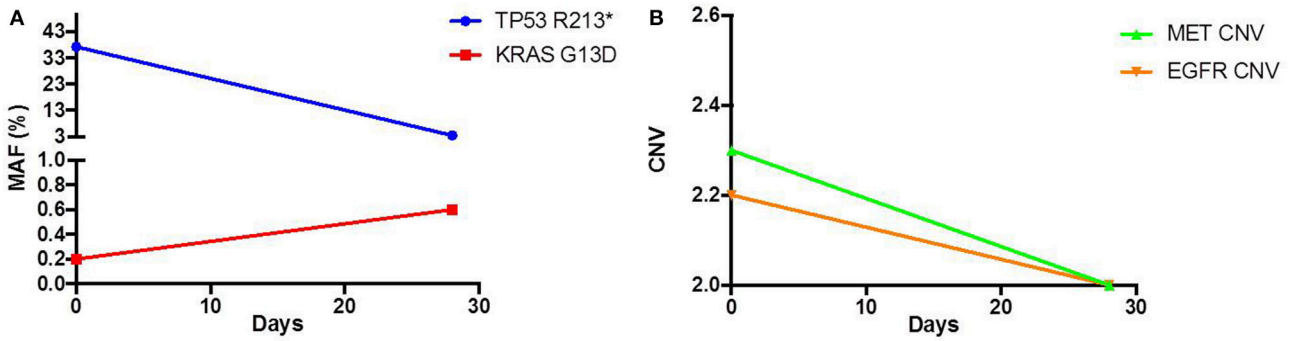

FIGURE 3 | Pre and Post treatment cfDNA profile of **(A)** mutant allele frequency (MAF) and **(B)** copy number variation (CNV).

domain (34, 35), *KRAS* and *NRAS* mutations (36, 37), and *MET* amplification (22) and these mutations often co-occur (38). Of these, *MET* amplification is potentially treatable with tyrosine kinase inhibitors and antibodies in development. Previous preclinical studies have demonstrated the potential activity of MET inhibitors in treating cetuximab or panitumumab refractory metastatic CRC. For example, treatment with a selective MET TKI successfully restored sensitivity to cetuximab in two cetuximab-resistant human colon cancer cell lines *in vitro*. The two cell lines displayed MET signaling pathway activation but *MET* amplification was not examined (39). Using a CRC cell-line harboring *MET* amplification in a murine xenograft model derived from a patient who developed acquired resistance to anti-EGFR therapy, tumor growth *in vivo* was effectively inhibited by crizotinib, a MET/ALK inhibitor (22).

To date, several MET TKIs have been developed with variable kinase selectivity against c-MET. Many of these are under different stages of clinical evaluation, either alone or in combination with other targeted therapy in patients with advanced solid tumors (40, 41). The MErCuRIC phase I/II clinical trial aims to assess the safety and efficacy of the combination of crizotinib and a MEK1/2 inhibitor, binemetinib, in patients with MET over-expressing, RAS-mutant or RAS wild-type metastatic CRC (42). Subgroup analysis from this study suggested potential benefit in patients with high c-MET expression (43).

Although the mechanisms of treatment response in this case are not fully known, the response to cabozantinib and panitumumab may be explained by the restoration of sensitivity to panitumumab or potentially synergy from dual MET and EGFR inhibition. Of note, other objective responses to small molecule MET inhibitors have been reported in patients with metastatic NSCLC and gastric cancer who had *MET* amplification detected by cfDNA profiling (44, 45). Alternatively, the anti-angiogenic properties of cabozantinib may have contributed to the overall response. To better understand whether treatment with cabozantinib alone drives response for patients with *MET* amplified metastatic CRC, this trial has been expanded to treat patients with *MET* amplified metastatic CRC with cabozantinib monotherapy.

MET amplification is not routinely tested in clinical practice due to its low prevalence and unproven actionability. Additionally, access to treatment-refractory tumor tissue and molecular heterogeneity complicates testing efforts (24, 46).

Given these limitations, cfDNA profiling may be the optimal approach for detection of *MET* amplification in the treatment refractory setting (47). In our patient, *MET* amplification was not detected in a tissue biopsy sample but was detected in plasma cfDNA ∼3 months later (**Table 1**). One explanation for the discrepancy between tissue and blood profiling results is that *MET* amplification represented a subclonal alteration that was not consistently present throughout the same lesion (intratumoral heterogeneity) or between different lesions throughout the body (intertumoral heterogeneity), as described previously (48, 49). This possibility is supported by the notion that mutations known to mediate acquired anti-EGFR resistance, e.g., *KRAS* and *BRAF* mutations, were seen in blood, but not the LN biopsy, suggesting temporal evolution from a common clonal origin. Alternatively, tumor cells harboring *MET* amplification may not have been present at a sufficiently high allele frequency to be detected by the tissue-based NGS assay.

This is the first case, to our knowledge, showing the activity of cabozantinib in combination with panitumumab in a patient with metastatic CRC. *MET* amplification, which is an established driver of EGFR antibody resistance, may have played a critical role in sensitizing this refractory tumor to the combination of an anti-MET TKI and anti-EGFR therapy. To further understand the drivers of sensitivity and resistance, studies are ongoing to evaluate the activity of cabozantinib treatment, either alone or in combination with panitumumab, in *MET* amplified metastatic CRC.

CONCLUSIONS

MET amplification is an important driver of EGFR antibody resistance. Anti-MET therapy is active in patients with *MET* amplified tumors, and may be a clinically actionable target in patients with *MET* amplified metastatic CRC. Clinical investigations are underway to determine how best to target *MET* amplified metastatic CRC, and to determine whether targeting *MET* amplification has meaningful anti-tumor activity. Furthermore, cfDNA profiling is a promising diagnostic technology to detect genomic alterations in the treatment refractory setting. Prospective clinical trials utilizing cfDNA to identify and treat *MET* amplified metastatic CRC are ongoing.

Written informed consent has been obtained from the next of kin for the publication of this case report.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations and approval of the Duke University Cancer Protocol Committee and the Duke University Institutional Review Board. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

JJ contributed to conception and design, analysis and interpretation of data, writing, review, and revision of the manuscript, and technical, material support. MM, RN, RL, and JS contributed to conception and design, analysis and interpretation of data, writing, review, and revision of the manuscript, and technical/material support.

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BRIEF RESEARCH REPORT
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Cell-Free DNA From Metastatic Pancreatic Neuroendocrine Tumor Patients Contains Tumor-Specific Mutations and Copy Number Variations

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Background: Detection of tumor-specific alterations in cell-free DNA (cfDNA) has proven valuable as a liquid biopsy for several types of cancer. So far, use of cfDNA remains unexplored for pancreatic neuroendocrine tumor (PNET) patients.

Methods: From 10 PNET patients, fresh frozen tumor tissue, buffy coat and plasma samples were collected. Whole-exome sequencing of primary tumor and germline DNA was performed to identify tumor-specific variants and copy number variations (CNVs). Subsequently, tumor-specific variants were quantified in plasma cfDNA with droplet digital PCR. In addition, CNV analysis of cfDNA was performed using shallow whole-genome sequencing.

Results: Tumor-specific variants were detected in perioperative plasma samples of two PNET patients, at variant allele fractions (VAFs) of respectively 19 and 21%. Both patients had metastatic disease at time of surgery, while the other patients presented with localized disease. In the metastatic patients, CNV profiles of tumor tissue and cfDNA were significantly correlated. A follow-up plasma sample of a metastatic patient demonstrated an increased VAF (57%) and an increased chromosomal instability, in parallel with an increase in tumor burden.

Conclusions: We are the first to report the presence of tumor-specific genetic alterations in cfDNA of metastatic PNET patients and their evolution during disease progression. Additionally, CNV analysis in cfDNA shows potential as a liquid biopsy.

Keywords: pancreatic neuroendocrine tumors, circulating tumor DNA, cell-free DNA, biomarkers, droplet digital PCR, whole-exome sequencing, shallow whole-genome sequencing

INTRODUCTION

Pancreatic neuroendocrine tumors (PNETs) are rare tumors with an incidence rate of 0.48 per 100,000 according to the Surveillance, Epidemiology, and End Results (SEER) program (1). Surgical resection of a PNET is often curative in early-stage disease, but 50% of cases present with unresectable disease at time of diagnosis (2). Patient diagnosis, follow-up and treatment are based on imaging, tumor (re)biopsies and biomarker assessment. Taking a biopsy is associated with potential complications and is therefore not feasible in some cases. Currently, Chromogranin A is the most widely used circulating biomarker in PNETs, but its diagnostic sensitivity and specificity are low. In addition, recent reports show limited value for Chromogranin A as a follow-up marker (3). Hence, new biomarkers are needed (4). Circulating tumor DNA (ctDNA) is the proportion of cell-free DNA (cfDNA) in the blood plasma that is released by a tumor as a result of apoptosis, necrosis and active secretion (5). The ctDNA can be detected and quantified in cfDNA through tumor-specific genetic alterations. ctDNA has been extensively studied in cancer patients as an alternative for tissue biopsies and for its biomarker potential in different stages of disease, as summarized by Wan et al. (6). In PNETs, however, ctDNA remains largely unexplored. This study aimed to demonstrate the presence of ctDNA in PNET patients through the detection of both tumor-specific point mutations and copy number variations (CNVs) using droplet digital PCR (ddPCR) and shallow whole-genome sequencing (sWGS), respectively.

MATERIALS AND METHODS

Patients

Ten patients diagnosed with a sporadic PNET and undergoing surgery for their primary tumor at the Antwerp University Hospital (UZA) were prospectively included in this study. Eight patients presented with limited, localized disease, while two patients had metastatic disease at time of surgery. All patients underwent surgery with curative intent. Since patient no. 3 presented with metastatic WHO2010 grade 3 disease (**Supplementary Figure 1**), he first started cisplatin-etoposide treatment. Only after showing a sustained partial response after 6 cycles, the decision was made to perform debulking surgery with curative intent. The other metastatic patient (no. 7; **Supplementary Figure 2**) was planned to undergo a two-stage-procedure, first a pancreatectomy with lymph node clearance and in a later moment, a liver transplantation to clear liver metastases. However, disease recurred before transplantation could be performed. In all patients, fresh frozen tumor tissue from pancreatic resection, perioperative blood samples in EDTA tubes and clinicopathological data were collected with informed consent. From case 7, an additional blood sample was taken during follow-up, 23 months after surgery and 12 days before succumbing to his disease. After a median follow-up time of 20 months (range: 11–31 months), seven patients were alive and disease-free, while patients 3 and 7 died due to their disease. One patient was lost to follow-up. The human biological material was provided by Biobank@UZA (Antwerp, Belgium; ID:BE71030031000)[1] and the study was approved by the local ethics committee (Antwerp University Hospital/University of Antwerp).

DNA Extraction

DNA was isolated from primary tumor tissue (tumor DNA), buffy coat (germline DNA) and plasma (cfDNA) using the AllPrep DNA/RNA Micro kit (Qiagen, Hilden, Germany), QIAamp DNA Blood Mini kit (Qiagen) and the Maxwell RSC ccfDNA Plasma Kit for large volumes (Promega, Madison, WI, USA), respectively. DNA concentrations were assessed using Qubit 2.0 fluorometer (Thermo Fisher Scientific, Eugene, OR, USA).

Whole-Exome Sequencing to Detect Tumor-Specific Alterations

Tumor and germline DNA were subjected to whole-exome sequencing (WES), using hybridization-based target enrichment with NimbleGen SeqCap EZ Human Exome v3.0 (Roche, Basel, Switzerland), on an Illumina NextSeq500 platform (Illumina, San Diego, CA, USA). Further analysis was performed using in-house analysis pipelines and paired variant callers VarScan2 (v2.4.2) (7) and MuTect2 (v1.1.5) (8) were used to call tumor-specific variants. Because WES data analysis provides multiple tumor-specific variants per patient, variant filtering in VariantDB (9) and prioritization were performed to select one target per patient for ddPCR. First, only rare non-synonymous single nucleotide variants (minor allele frequency$<$0.01 in dbSNP v142 (10), ExAC v03 (11) and 1000Genomes april2012 (12)) were identified. Then, alterations with a variant allele fraction (VAF) lower than 20% were excluded to allow validation of tumor-specific state using Sanger sequencing. Next, variants were prioritized that lie in previously described neuroendocrine tumor-associated genes, in COSMIC v82 cancer census genes (13) or variants with a high predicted pathogenicity by CADD PHRED (14) and SIFT effect (15). Selected variants, one per patient, were validated using Sanger sequencing on the 3130xl Genetic Analyser (Applied Biosystems Inc., Foster City, CA, USA) platform.

For CNV analysis, we developed an in-house pipeline that employs an algorithm to divide the genome into non-overlapping 50 kb-bins and subsequently counts all mapped sequencing reads for each sample within each bin. Next, logR-ratios were calculated for every tumor/normal pair.

Droplet Digital PCR for Single Nucleotide Variants

Custom-made, variant-specific primer/probe assays were ordered from Bio-Rad to perform genotyping of cfDNA on the QX200 Droplet Digital PCR System (Bio-Rad, CA, USA). Specific sequences of primers and probes are not disclosed by Bio-Rad. However, sequences containing the 60–100 bp-sized amplicons are given in **Supplementary Table 1**. In short, 20 μL reaction mixtures consisting of 10 μL Supermix for Probes (no dUTP; Bio-Rad), 1 μL ddPCR assay mix (Bio-Rad) and 9 μL

[1] *Biobank@UZA, BBMR-ERIC, Belgian Virtual Tumourbank funded by the National Cancer Plan; No. Access: (2), Last: April, 12, 2017. [BIORESOURCE].*

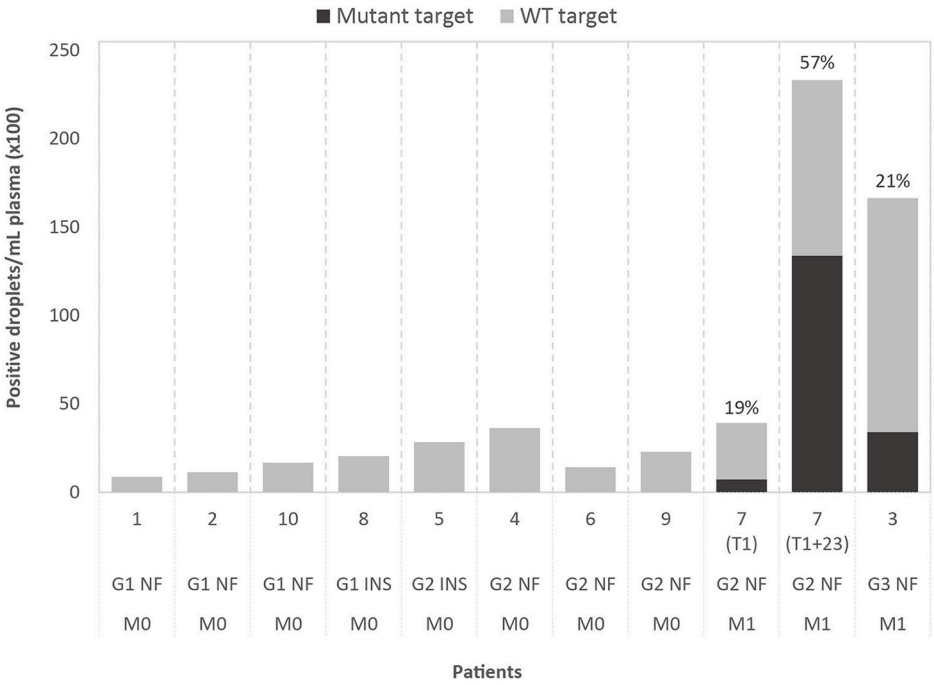

FIGURE 1 | Results of droplet digital PCR (ddPCR) on cell-free DNA of ten patients, grouped by WHO2010 grade (G), functionality of the tumor (NF, non-functional; INS, insulinoma) and presence of metastasis (M0, no metastasis; M1, metastasis present). The graph shows the number of positive droplets per 1 mL of plasma, for both mutant (black) and wild-type (WT; gray) target. For mutant-positive patients, the variant allele fractions (VAFs) are indicated. The selected ddPCR targets are, from left to right, chr3:g.98251584T>A (*GPR15*), chr2:g.46603856C>T (*EPAS1*), chr21:g.39754856C>A (*ERG*), chr20:g.46288182C>T (*SULF2*), chr5:169477296C>T (*DOCK2*), chr2:g.111416130T>G (*BUB1*), chr2:g.204073466C>G (*NBEAL1*), chr16:g.15854467G>A (*MYH11*), chr11:g.64575561C>A (*MEN1*), chr6:g.33288573G>A (*DAXX*) (in GRCh37/hg19). For case 7, results obtained with the same ddPCR assay are shown for the perioperative plasma sample (T1) and the follow-up plasma sample (T1+23 months).

DNA and nuclease-free water, were partitioned in approximately 20,000 nanoliter-droplets with the QX200 Droplet Generator. Droplets were transferred to a PCR plate and subjected to PCR amplification (95°C × 10 min, (94°C × 30 s, 55°C × 1 min) × 40, 98°C × 10 min, 4°C hold; ramp rate 2.5°C/s) followed by read-out. Tumor and germline DNA were used, respectively, as positive and negative control for the mutation. Additionally, template-negative reactions were run. Droplets were manually called as mutant-only, wild-type (WT)-only, double-positive or template-negative using the QuantaSoft software package v1.7.4 (Bio-Rad).

Shallow Whole-Genome Sequencing of Cell-Free DNA

10–20 ng of cfDNA was used as input for sWGS aiming for a coverage of 0.3-fold. Library preparation was performed using the Truseq Nano DNA HT library prep kit (Illumina) with dual-indexing and sequencing was performed on the NextSeq500 platform (Illumina). CNVs were detected by applying the R-package QDNAseq (16).

RESULTS

Whole-Exome Sequencing and Variant Selection

WES was performed on primary tumor tissue and corresponding germline samples with an average target base coverage of 108 ± 8-fold and 35 ± 7-fold, respectively. The goal of the WES analysis was to identify a tumor-specific variant for every patient which could then be detected in cfDNA of the corresponding patient. By applying the filters described in the methods section, we were able to select a single variant for every patient, which was validated with Sanger sequencing to confirm tumor-specificity (legend **Figure 1**). Our analysis revealed several interesting mutations in known PNET-associated genes, including missense mutations in *MEN1* and *EPAS1* and a stopgain mutation in *DAXX* (17, 18).

Tumor-Specific Variants Can be Detected in Cell-Free DNA of Metastatic Patients

Custom ddPCR assays were designed for detection of the selected tumor-specific variants (mutant targets) with normal, WT targets as control. Analysis was performed on DNA extracted from tumor tissue, buffy coat and plasma. In tumor DNA, both mutant and WT targets could be detected by ddPCR, with VAFs showing a significant correlation with VAFs detected by WES (Pearson's $r = 0.8786$; $p < 0.001$). WT targets could be detected in cfDNA of all patients and two of the cases also tested positive for the tumor-specific mutation, with VAFs of respectively 19 and 21%. Droplet counts per mL plasma are shown in **Figure 1**. Assuming a limit for ctDNA-positivity of two mutant-positive droplets, our median detection limit based on the total amount of positive droplets is 0.27% (range: 0.06–0.63%). Remarkably, both patients that tested positive presented with metastatic disease before surgery, while the others presented with localized disease.

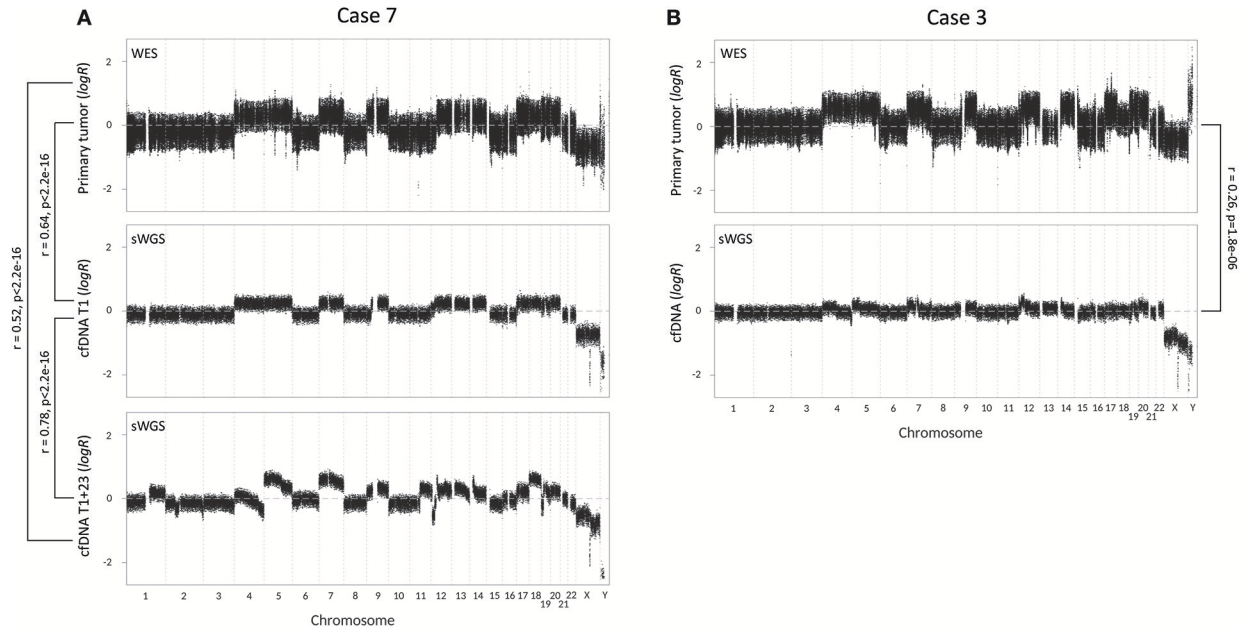

FIGURE 2 | Copy number variation (CNV) profiles with correlations (Pearson's r) of tumor tissue and cell-free DNA (cfDNA) samples of the two metastatic cases. CNV profiles of tumor tissue and cfDNA were created respectively by whole-exome sequencing (WES) and shallow whole-genome sequencing (sWGS). **(A)** CNV profiles of case 7, with from top to bottom the CNV profile of tumor tissue, perioperative cfDNA sample (T1) and follow-up cfDNA sample, 23 months later (T1+23). **(B)** CNV profiles of case 3 (top: tumor tissue; bottom: cfDNA).

The median plasma cfDNA concentration, estimated by Qubit, was 16 ng/mL (range: 4–30 ng/mL) for patients with localized disease, which is considerably lower than cfDNA concentrations in patients with metastatic disease (50 ng/mL and 81 ng/mL).

For case 7, two plasma samples were available, one perioperative (T1) and one follow-up sample, taken 23 months after surgery (T1+23 months). Plasma of both timepoints tested positive for the mutation, with an increase in VAF from 19 to 57% and in cfDNA concentration from 50 to 423 ng/mL, in line with the diffuse liver and bone invasion on T1+23 (**Supplementary Figure 3**).

Reclassification of WHO Grade 3 Patient Based on a Liquid Biopsy

Case no. 3 was diagnosed with metastatic WHO2010 grade 3 disease. In 2017, however, a new WHO grading system was implemented that distinguishes between well-differentiated grade 3 neuroendocrine tumors and poorly differentiated grade 3 neuroendocrine carcinomas. Tang et al. (19) described the most common molecular alterations associated with both types. In DNA extracted from both tumor tissue and plasma of our grade 3 case, we were able to detect a *DAXX* loss-of-function mutation, suggestive for classification as a well-differentiated grade 3 neuroendocrine tumor (19). To confirm our hypothesis based on molecular analysis, review by a dedicated pathologist was performed (**Supplementary Figure 1**). This showed indeed a morphologically well-differentiated PNET with a high Ki-67 (>20%). Remarkably, expression of the Ki-67 marker varied strongly across the tumor with hotspot regions reaching Ki-67 values as high as 66%, indicating tumor heterogeneity.

CNVs Detected in cfDNA and Tumor Tissue Show a Good Correlation

To further assess the biomarker potential of cfDNA, we constructed CNV profiles of cfDNA and primary tumor samples of our two metastatic cases (**Figure 2**). CNV profiles of primary tumor tissue and cfDNA(T1) of case 7 show a significant correlation (Pearson's $r = 0.64$, $p < 2.2e^{-16}$). The CNV profile of the follow-up sample, cfDNA(T1+23), shows increased chromosomal instability, which is reflected by a lower Pearson's r-value than cfDNA(T1), when compared to the primary tumor ($r = 0.52$, $p < 2.2e^{-16}$). The higher correlation between the two cfDNA samples ($r = 0.78$, $p < 2.2e^{-16}$) can be explained by uniformity of the technique and the fact that sWGS creates more data points and, hence, a more stable CNV profile than WES. CNV profiles of primary tumor and perioperative cfDNA sample for case 3 are also significantly correlated ($r = 0.26$, $p = 1.8e^{-06}$), but the correlation is less strong. In general, however, the same chromosomal regions seem to be affected in the tumor and the cfDNA sample, but logR ratios are closer to zero in cfDNA.

DISCUSSION

This study provides the first evidence for the presence of ctDNA in plasma of metastatic PNET patients, through ddPCR genotyping of cfDNA for tumor-specific variants. Tumor-specific variants were obtained for all patients through WES analysis of primary tumor tissue and germline DNA, but when genotyping variants in cfDNA of cases with localized disease, the variants could not be detected. This suggests that there is no ctDNA

present or that a lower detection limit is required to detect it. Absence or presence of lower levels of ctDNA during early stage cancer have been described previously and the fact that PNETs are often indolent tumors, could also explain the absence of ctDNA in plasma (20). ctDNA-negative patients did not relapse during follow-up, while the two ctDNA-positive cases succumbed within 2 years after surgery to their disease, despite histology-confirmed R0 resection in case 3. Therefore, ctDNA analysis might help to differentiate between localized and metastatic disease, which has important prognostic and therapeutic implications, or help to detect relapse. This should be evaluated in further studies. Interestingly, we were able to detect a *DAXX* mutation in tumor tissue and plasma of a WHO2010 grade 3 patient (no. 3), which is suggestive for classification as a WHO2017 grade 3 well-differentiated neuroendocrine tumor, as opposed to a poorly differentiated neuroendocrine carcinoma (19). Pathology revision confirmed this diagnosis, showing potential for ctDNA to differentiate between the two types of WHO2017 grade 3 tumors, and possibly in the future also between other WHO grades as molecular research is ongoing (19, 21).

In both metastatic cases, a significant correlation was found between CNV profiles of tumor tissue and corresponding cfDNA, but there was a marked difference in the strength of the correlation. This might be explained by a difference in ctDNA fraction, if wrongly estimated by mutation analysis with ddPCR, or by tumor heterogeneity. Since central pathological review has demonstrated the presence of tumor heterogeneity in case 3, in which a weaker correlation was identified, tumor heterogeneity is the most likely explanation. CNV profiles are often characteristic for a certain tumor type (22). As many neuroendocrine tumors present with an unknown primary, CNV analysis of cfDNA to identify the primary tumor site might be a potential application.

Mutation and CNV analysis of a follow-up blood sample at progression showed an increase in cfDNA concentration, VAF and chromosomal instability. The increase in cfDNA concentration and VAF indicates an association with tumor burden, hinting toward a potential role for ctDNA as a follow-up marker for detection of treatment response or progression. The detection of an altered CNV profile, caused by disease progression and treatment pressure, means that cfDNA provides a real-time representation of cancer dynamics.

Our approach, where we first sequence the resected tumor followed by detection of tumor-specific variants in plasma, can only be applied for monitoring tumors in postoperative survey. However, other approaches might be explored in further studies, such as sequencing of tissue biopsies to identify tumor-specific variants, or detection of ctDNA by CNV analysis (as shown), methylation markers or sequencing of cfDNA (23, 24). In metastatic PNET cases, our results suggest that cfDNA might be a novel alternative to tissue biopsies for molecular profiling. Research on PNET tissue is being performed to identify prognostic and predictive genetic alterations, but few alterations have been validated so far (25). The possibility to detect genetic alterations in the blood instead of tissue would facilitate this research and future applicability due to easier access to tumor material in different stages of disease or treatment, evading the need for repeated tissue biopsies. Patients without treatment options could also benefit from ctDNA profiling through identification of actionable molecular alterations to allow inclusion in "molecular trials" with targeted therapies. Additionally, it is believed that all tumor cells release DNA, hence, molecular profiling of ctDNA creates a representation of alterations in the whole tumor, thereby evading the typical tumor heterogeneity problem of tissue biopsies.

To conclude, we report the first evidence for the presence of ctDNA in plasma of metastatic PNET patients and demonstrate its potential as a novel biomarker for PNETs. However, additional research on larger sample sizes and with multiple sampling timepoints per patient is required to further explore the possibilities of ctDNA in PNET patient management.

AVAILABILITY OF DATA AND MATERIALS

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the ethics committee of the Antwerp University Hospital/University of Antwerp with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the ethics committee of the Antwerp University Hospital/University of Antwerp (approval number 16/46/490).

AUTHOR CONTRIBUTIONS

GB, TV, MP, GVC, and KOdB contributed to conception and design of the study and wrote the manuscript. GB, TV, and MB performed experiments and data analysis. GR and TV collected the samples. AD performed pathology review. All authors contributed to data interpretation and manuscript revision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2018.00467/full#supplementary-material

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REVIEW
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The Potential Clinical Utility of Circulating Tumor DNA in Esophageal Adenocarcinoma: From Early Detection to Therapy

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Esophageal adenocarcinoma (EAC) is a lethal cancer requiring improved screening strategies and treatment options due to poor detection methods, aggressive progression, and therapeutic resistance. Emerging circulating tumor DNA (ctDNA) technologies may offer a unique non-invasive strategy to better characterize the highly heterogeneous cancer and more clearly establish the genetic modulations leading to disease progression. The presented review describes the potential advantages of ctDNA methodologies as compared to current clinical strategies to improve clinical detection, enhance disease surveillance, evaluate prognosis, and personalize treatment. Specifically, we describe the ctDNA-targetable genetic markers of prognostic significance to stratify patients into risk of progression from benign to malignant disease and potentially offer cost-effective screening of established cancer. We also describe the application of ctDNA to more effectively characterize the heterogeneity and particular mutagenic resistance mechanisms in real-time to improve prognosis and therapeutic monitoring strategies. Lastly, we discuss the inconsistent clinical responses to currently approved therapies for EAC and the role of ctDNA to explore the dynamic regulation of novel targeted and immunotherapies to personalize therapy and improve patient outcomes. Although there are clear limitations of ctDNA technologies for immediate clinical deployment, this review presents the prospective role of such applications to potentially overcome many of the notable hurdles to treating EAC patients. A deeper understanding of complex EAC tumor biology may result in the progress toward improved clinical outcomes.

Keywords: esophageal cancer (EC), circulating tumor DNA (ctDNA), targeted therapy (TT), immunotherapy, personalized medicine

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INTRODUCTION

Esophageal cancer (EC) is currently the 7th deadliest cancer in the United States with an estimated 17,290 newly diagnosed cases and 15,850 deaths in 2018, with an overall 5-years survival rate under 20% (1). For locally advanced disease, standard of care treatment currently includes neoadjuvant chemoradiotherapy plus surgery (2–4). However, ∼40% of patients present with unresectable late-stage disease at diagnosis, reducing their 5-years survival to <5%, and further underscoring the urgent need for earlier detection and improved treatment strategies (5, 6).

Circulating tumor DNA (ctDNA) is fragmented tumor-derived DNA in the blood stream that serves as a non-invasive diagnostic and prognostic tool for a number of cancer types (7, 8). Current

standard cancer liquid protein biomarkers, such as PSA, CA 125, CEA, CA 19-9, AFP, etc., are limited by poor specificity and are known to be elevated in a variety of benign conditions. In contrast, ctDNA offers increased specificity of dysregulated genetic tumor markers, with levels of detection correlating well with premalignant to malignant progression (9). Moreover, ctDNA has a shorter half-life than protein biomarkers, and the real-time information that may be gathered from such "liquid biopsies" may provide unique insight to improve screening methodologies, therapeutic monitoring, and personalized therapy to improve outcomes in a cost-effective widely accessible manner (7, 10). Recently, Park et al. demonstrated that deep sequencing of ctDNA KRAS mutations sensitively detects pancreatic ductal adenocarcinoma and correlates with therapeutic response and disease progression (11). Additionally, ctDNA has shown promise for the detection of post-surgical recurrence of colon, breast, and lung cancers (12–14). The Cancer Genome Atlas (TCGA) recently characterized numerous deregulated genes in esophagogastric adenocarcinoma such as TP53, CDKN2A, SMAD4, ARID1A, ERBB2, VEGFA, CCNE1, GATA4, and GATA6 (15). Many of the identified markers overlapped with significant genes in gastric cancer pathogenesis; however, EAC DNA was consistently more hyper-methylated (11). ctDNA technologies may differentiate these important variances through the detection of not only point mutations, but also copy number variations, chromosomal rearrangements, epigenetic alterations, insertions, and deletions (10). The purpose of this review is to describe the potential utility of ctDNA to improve the detection, monitoring, treatment strategies, and prognosis for EAC patients.

LITERATURE REVIEW METHODS

A thorough literature review was performed by searching the PubMed database for all relevant articles through September 1st, 2018. The following search criteria were applied: ("circulating tumor DNA" OR "ctDNA" OR "cell free DNA" OR "tumor DNA") AND ("esophageal" OR "esophageal" OR "gastrointestinal" or "gastroesophageal") AND ("cancer" OR "tumor" OR "malignancy"). The search produced 165 items, and two independent reviewers (JK and TP) screened the results. References from relevant articles were screened as an additional source of literature. Exclusions included articles that evaluated GI malignancies other than esophageal cancer and articles that were not in English. Additional specific searches of current EAC epidemiology statistics, TCGA data, ctDNA methodologies, and EAC therapeutics were performed to supplement background information.

CTDNA METHODOLOGY

ctDNA detection was first established though Sanger sequencing, however limitations such as high cost and complicated protocols have lead to the development of various methodologies to improve efficiency, cost-effectiveness, sensitivity, and specificity (16). Currently, the two main methods of detection include Digital PCR (dPCR) and Next-Generation Sequencing (NGS) (17). dPCR methods, such as Droplet Digital PCR and BEAMing, carry the advantage of being relatively easy, inexpensive methods with short turn around times, which can be a critical advantage when treating aggressive malignancies (18, 19). Additionally, these PCR methods offer superior precision, sensitivity, and reproducibility (19). Specifically, BEAMing has reported up to 100% sensitivity of multiple markers in a variety of cancers (20–22). Although dPCR methods carry higher sensitivity than NGS, it can only detect mutations within a limited number of loci, usually within a single gene, a disadvantage that can be overcome with NGS (23, 24). ctDNA NGS protocols include tagged-amplicon deep sequencing (Tam-Seq), Safe-Sequencing System (Safe-SeqS), and capture based sequencing (CAPP-seq) (23). CAPP-seq targets only areas of interest and is therefore more cost effective and focused than whole exome or whole genome sequencing (16). It also confers the lowest detection limit and background error rate of any NGS-based method and is therefore considered a superior NGS method for practical implementation (16).

Application of ctDNA technologies offers potential unique advantages to clinical care over tissue biopsy, and the concordance of ctDNA with tissue biopsy has been validated across a variety of cancers (25–29). Primarily, tissue DNA collection requires invasive procedures that many frail and elderly patients may not tolerate well (30). Less than 15% of esophageal cancers are diagnosed before the age of 55, so non-invasive detection and monitoring methods may confer particular benefit to the EAC patient population (1). Additionally, it has been well-established that many cancers, including EAC, are very heterogenic in nature and gain additional mutations throughout progression, treatment, and metastasis (31). Tissue biopsy samples only a small section of primary tumor, so obtained samples may not truly reflect all relevant and targetable mutations (32). Comparatively, ctDNA offers a more global perspective of the entire tumor, including metastases, that may offer improved detection of spatial and temporal tumor heterogeneity, which can carry great value by providing up-to-date information on tumor evolution and mutational status throughout disease course (10, 33). Moreover, unlike tissue DNA, analyzing ctDNA does not require fixation of the sample, which can fragment the DNA and cause sequence artifacts that may be misinterpreted as cancer-associated mutations (34).

Despite the improving technologies, there are current limitations to the clinical applications of ctDNA. First, the use of ctDNA as a reliable mechanism to inform clinical decisions lacks standardization due to limited early data and complex bioinformatics processing (35). To date, the only FDA approved tests include methylation-based test of *SEPT9* for colorectal cancer and qPCR-based test for EGFR in non-small cell lung cancer (36, 37). Second, the yield of ctDNA material from plasma is usually quite low, especially in early stage cancers and precursor lesions. In such cases, whole genome amplification has been utilized to improve sample yield, however, future studies are needed to determine if this may compromise the clinical characterization of the tumor (35). Third, the mechanisms and rate of elimination of ctDNA from the bloodstream has not

yet been fully explored. There is evidence to suggest ctDNA is cleared through the kidney, liver, and spleen, as well as nuclease degradation and phagocytosis; however, the rate of elimination may be very relevant to elucidate to in the setting of therapeutic monitoring or disease progression (38, 39).

CTDNA DETECTION OF EAC

In an at-risk patient population of over 20 million GERD sufferers, it is estimated that only 10–15% develop Barrett's esophagus (BE), 0.5% of patients with BE progress to EAC per year, and 25% of high-grade dysplasia cases progress to EAC (40). The minimal risk of disease progression based on GERD symptoms alone dilutes the practicality of universal esophageal cancer screening protocols. Current testing for EAC and precursor lesions is primarily triggered by persistent GERD symptoms in a goal to detect early disease before development to malignancy. Despite this strategy, ∼93% of diagnosed EAC patients received no prior surveillance (41). Non-invasive screening with ctDNA liquid biopsy may offer a cost-effective non-invasive alternative to identify patients at increased risk for early-stage disease or development of EAC.

Numerous genetic alterations have been identified in association with the development and prognosis of EAC that may serve as ideal ctDNA targets for improved detection. Particularly, the tumor suppressors TP53 and CDKN2A are mutated in ∼72 and 12% of EAC cases, respectively (42, 43). Bettegowda et al. utilized ctDNA technology to detect multiple early and late stage malignancies, including gastroesophageal cancer (GEC), and were able to reliably detect ∼58% of localized GEC (no evidence of metastasis) and 100% of metastatic disease (44). As expected, sensitivity improved from Stage 1 through IV progression across all malignancies (44). Additionally, a meta-analysis by Creemers et al. suggested ctDNA detection of HER2 and MYC may be useful for diagnosis and therapeutic monitoring of GEC (45). In order to improve early detection of EAC, non-invasive ctDNA testing may be strategically applied to patients with clinical risk factors, such as recurrent GERD symptoms, Caucasian ethnicity, obesity, smoking history, age, and/or male sex.

Many of the described mutations that characterize EAC, such as TP53 and CDKN2A, also occur in precursor lesions such as BE and HGD, lowering the specificity of such testing for established cancer (46). Still, alternative ctDNA markers may be useful in identifying patients with BE or HGD who are at increased risk for progression to EAC. Currently, the American College of Gastroenterology recommends patients with established non-dysplastic BE should receive endoscopic biopsy surveillance to assess possible disease progression every 3–5 years; however, 90–95% of these patients have completely stable disease and will never progress to EAC (47–50). In a recent study by Li et al. patients with BE were stratified into 79 progressors and 169 non-progressors to EAC, and biopsies were classified based on genetic expression. The study demonstrated that non-progressing BE lesions had small localized deletions at fragile sites, such as FHIT, WWOX, CDKN2A, and 9p arm loss/copy neutral loss of heterozygosity (LOH) (51). These samples revealed a low level of genetic heterogeneity and remained stable over years of surveillance. Alternatively, lesions that progressed to EAC developed increasing chromosome instability as early as 48 months before progression to EAC with gains and losses of whole chromosomes or chromosome arms, such as loss of 18q (51). Progressors showed significant mutation of SMAD4 and were universally more heterogenetic with progressive diversity and genome doublings (51). Similarly, a high-powered meta-analysis by Gharahkahni et al. suggests HTR3C and ABCC5 may be specific for progression of BE to EAC (52). Rumiato et al. recently utilized circulating cell-free DNA (cfDNA) to evaluate the neoplastic progression of BE and were able to successfully detect notable LOH predicting progression to dysplasia and/or EAC prior to visualization (9, 53). Moreover, post-intervention cfDNA sampling demonstrated a return to baseline levels of expression, further validating the potential of the technology to aid in the evaluation of treatment efficacy (9). Therefore, ctDNA screening for SMAD4, HTR3C, ABCC5, and increasing genetic variability over time may stratify patients with precancerous lesions for more appropriate endoscopic screening or intervention recommendations.

PROGNOSTIC SIGNIFICANCE AND THERAPEUTIC MONITORING

Various previous studies have established ctDNA as a useful tool for the prediction of patient prognosis before and after therapy in a variety of cancer types (54–58). In a recent study evaluating the role of ctDNA for tumor prognosis in breast cancer patients with multiline resistance, Hu et al. revealed unique mutation frequency patterns in those with progression free survival (PFS) $<$3 months vs. $>$3 months (59). Additionally, a TP53+PIK3CA mutation pattern successfully predicted progression within 6 months (59). Similarly, in a study investigating the utility of ctDNA to monitor non-small cell lung cancer (NSCLC) patients, tumor and blood samples before and after surgical resection demonstrated that the presence of ctDNA had a higher positive predictive value than six currently utilized clinical tumor biomarkers (60). As EAC is a notably heterozygous malignancy, a variety of diverse studies have reported unique genomic signatures associated with survival and prognostic response to chemoradiotherapy (**Table 1**) (71). Although, the true clinical utility of these specific genomic profiles has yet to be reliably established, ctDNA may offer an ideal setting for future studies to validate these prognostic indicators, as has been done for other cancers.

Furthermore, 44–61% of patients treated according to the current guidelines of neoadjuvant chemoradiotherapy plus surgery will experience recurrent disease (72). Despite complete resection with pathologically-confirmed clear margins and negative post-therapeutic CT imaging, it is hypothesized that many EAC recurrences are due to minimal residual disease (MRD) or systemic micrometastases (73). Previous studies have demonstrated that ctDNA screening may more sensitively identify small areas of remaining or recurrent disease as

TABLE 1 | Esophageal adenocarcinoma genetic signatures associated with prognosis.

Study	Signature	Results
Peters et al. (61)	Downregulated: DCK, PAPSS2, SIRT2 Upregulated: TRIM44	Reduction in survival from 58 to 14%
Kim et al. (62)	SPARC, SPP1	Significant association with poor survival
Goh et al. (63)	EGFR, MTMR9, NEIL2, WT1	Stratification of patients in 5 survival clusters
Pennathur et al. (64)	165-gene signature	Stratification of patients into good vs. poor survival cluster
Rao et al. (65)	59-gene signature	Stratification of patients into good vs. poor survival cluster
Rao et al. (65)	Upregulated: Ephrin B3	Increased response to chemoradiotherapy
Motoori et al. (66)	Downregulated: PERP Upregulated: DAD1, PRDX6, SELPINB6, and SRF	Decreased response to chemoradiotherapy
Maher et al. (67)	Downregulated: ERB41L3, NMES1, RPNC1, STAT5B Upregulated: RTKN	Increased response to chemoradiotherapy in EAC and ESCC
Tamoto et al. (68)	Upregulated: PERP, S100A2, SPRR3	Characterized complete responders to chemoradiotherapy in EAC and ESCC
Murugasu et al. (69)	Increased tumor heterogeneity	Decreased response to neoadjuvant chemotherapy
Rumiato et al. (70)	SNPs of ABCC2, ABCC3, CYP2A6, PPARG, SLC7A8	Decreased response to platinum-based chemotherapy

compared to standard imaging technology (74, 75). Such sensitive screening methodology may be ideally suited for such an aggressively deadly disease to trigger early intervention and reduce associated mortality. Recently, Chan et al. was able to improve detection of nasopharyngeal carcinoma recurrence by 10 months as compared to standard screening protocols (76). Similarly, ctDNA has also been used to detect MRD and predict recurrence in breast cancer with a mean lead-time of 7.9 months over clinical relapse (12). In a third study for lung cancer, ctDNA mutations predicted recurrence with 94% sensitivity with a median clinical lead-time of 5.2 months (14).

Unfortunately, 50–60% of EAC patients are resistant to standard chemotherapeutic treatment options due to inherent heterogeneity and development of escape mechanisms (69, 77). ctDNA technology provides an additional tool to monitor real-time therapeutic efficacy for more efficient modification of dosing and regimen. Murugasu et al. demonstrated that patients with EAC who had promising response to platinum agents developed decreased C>T mutations and increased C>A mutations through the course of treatment (69). Findlay et al. also confirmed this finding in addition to TT>CT changes, and acquired mutations in SF3B1, TAF1, and CCND2 (78). As neoadjuvant chemotherapy can dramatically and rapidly change the EAC genome profile, real-time monitoring to quickly identify resistance and opportunities for new actionable mutations may lead to clinical benefit. Moreover, ctDNA studies may provide additional insight into disease progression when used in conjunction with clinical imaging. de Figueriredo Barros et al. described an increasing mutation burden correlating with progression in metastatic colorectal cancer, while CT imaging showed stable disease (79).

PERSONALIZED THERAPEUTIC APPLICATIONS

Various targeted therapies have been explored for the treatment of gastroesophageal cancers but have only demonstrated limited efficacy (80, 81). Only trastuzumab has been established as a potential first-line treatment option for advanced GEC in HER2+ patients; however, benefits are minimal with a median overall survival (OS) of 13.8 months vs. 11.1 months (82). Ramucirumab (VEGFR-2) single agent or in combination with paclitaxel are recommended options for second-line treatment demonstrating an OS of 5.2 and 9.6 months, compared to 3.8 and 7.4 months, respectively (83, 84).

Over the last decade, success of immunotherapy across multiple cancer types has prompted exploration of novel immunologic targets for GECs (85). Recently, the late-line KEYNOTE-059 study of pembrolizumab in PD-L1 positive GEC and gastric tumors showed an objective response rate of 13.3% with 58% of the responses lasting 6 months or longer, leading to a third-line FDA approval (86, 87). Similarly, nivolumab has been approved for third-line GEC treatment in Japan after demonstrating an improved median survival of 5.26 months vs. 4.14 months, independent of PD-L1 expression (88, 89). Disappointingly, the most recent KEYNOTE-061 second-line pembrolizumab trial did not demonstrate any significant survival benefit in PD-L1 positive GEC patients (90). Still, pembrolizumab has secured a tumor agnostic approval in microsatellite instability-high or mismatch repair deficient previously treated unresectable or metastatic solid tumors; yet, these criteria only apply to ∼3% of GECs (15, 91).

First generation immuno-oncology agents have demonstrated modest activity and potential application for the treatment of EAC; however, better stratification biomarkers and newer immunotherapeutic combination strategies may be required for enhanced durable responses. Only ∼40% of EAC patients present with baseline PD-1 positivity, and this expression occurs primarily at the invasive margin (92, 93). Therefore, PD-L1 may not be the ideal predictive marker in EAC due to the inconsistent clinical response and relatively low rate of upregulation. There is evidence that the immune microenvironment as a whole is highly reactive to chemoradiotherapy and significantly increases expression of PD-L1 and other developing targets, such as LAG3, TIM3, and OX40 (94). Therefore, ctDNA technology may be a useful tool to explore the dynamic immunoregulation throughout the course of treatment and to better characterize the immunologic profile of EAC beyond the PD-1/PD-L1 pathway. A number of novel targeted molecular and immunotherapeutic agents are currently under investigation for EAC to explore additional potential treatment alternatives

(95). Due to the extreme genomic variability and instability that classically characterize EAC, in addition to the multiple resistance mechanisms that may emerge during treatment, EAC may be an optimal candidate for the application of personalized therapeutics through the use of real-time ctDNA monitoring (96).

Moreover, in patients treated with immunotherapy, radiological evaluation of early disease responsiveness is especially challenging due to immune cell infiltration resulting in pseudo-progression on imaging (97, 98). Cabel et al. showed ctDNA is useful for early monitoring of responsiveness to anti-PD1 immunotherapy and correlated well with PFS and OS (99). Similarly, Xi et al. showed early changes in BRAFV600E ctDNA as early indicator to identify responding from non-responding patients with metastatic melanoma treated with immunotherapy (100). In another study, Raja et al. demonstrated early reduction in ctDNA correlation with survival in lung and bladder cancer after treatment with durvalumab (PD-L1) (101). Lastly, Khagi et al. demonstrated increased ctDNA detection of variants of unknown significance correlated with statistically significant improved PFS and OS in patients with diverse malignancies receiving immunotherapy (102). Exploration into the use of ctDNA technologies for non-invasive therapeutic monitoring of immunotherapy in EAC may be warranted due to the supporting evidence that ctDNA may be a good indicator of immunoresponse and prognosis.

CONCLUSIONS

Esophageal adenocarcinoma is a very deadly disease due to the high percentage of patients presenting with late-stage diagnosis and minimally effective treatment strategies. The emergence of non-invasive and cost-effective ctDNA technologies may offer a unique opportunity to improve screening protocols to more effectively monitor benign disease and detect malignancy earlier. Moreover, many patients with EAC are resistant to first line chemoradiotherapy, and therapeutic monitoring of ctDNA mutations throughout the course of treatment may allow for more efficient adjustments of personalized therapy. Despite only minor successes with targeted therapies due to the highly heterogenic nature of EAC, real-time information regarding response and prognosis may allow for more informed clinical decision-making strategies. Additionally, the integration of ctDNA with developing immunotherapeutic options may open the door for improved prognostic outcomes.

Limitations of ctDNA technologies are rooted in the still early development of this new emerging technology and the lack of strongly validated studies characterizing the precise clinical role it may play to truly improve patient care (30). Rigorous future clinical studies will be required to reliably describe specific discoverable changes in ctDNA throughout disease progression before broad implementation. Still, EAC remains an extremely lethal disease, and further investigation into the potential benefits of ctDNA characterization may offer significant benefits to improve early detection, monitor progression, enhance real-time personalization of therapeutics, and evaluate prognosis to improve clinical care.

AUTHOR CONTRIBUTIONS

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Clinical Utilization Pattern of Liquid Biopsies (LB) to Detect Actionable Driver Mutations, Guide Treatment Decisions and Monitor Disease Burden During Treatment of 33 Metastatic Colorectal Cancer (mCRC) Patients (pts) at a Fox Chase Cancer Center GI Oncology Subspecialty Clinic

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Background: Liquid biopsy (LB) captures dynamic genomic alterations (alts) across metastatic colorectal cancer (mCRC) therapy and may complement tissue biopsy (TB). We sought to describe the utility of LB and better understand mCRC biology during therapy.

Methods: Thirty-three patients (pts) with mCRC underwent LB. We used permutation-based *t*-tests to assess associations between alts, and clinical variables and used Kendall's tau to measure correlations.

Results: Of 33 pts, 15 were women; 22 had colon, and the rest rectal cancer. Pts received a median of two lines of therapy before LB. Nineteen pts had limited testing on TB (*RAS/RAF/TP53/APC*), 11 extended NGS, and 3 no TB. Maxpct and alts correlated with CEA ($p < 0.001$, respectively). In 3/5 pts with serial LB, CEA correlated with maxpct trend, and CT tumor burden. In 6 pts, mutant *RAS* was seen in LB and not TB; 5/6 had received anti-EGFR therapy prior to LB, suggesting *RAS* alts developed post-therapy. In two pts *RAS*-mutated by TB, no *RAS* alts were detected on LB; these pts had low disease burden on CT at time of LB that also did not reveal *APC* or *TP53* alts. In six patients who were KRAS wt based on TB, post anti-EGFR LB revealed subclonal KRAS mutations, likely a treatment effect. The median number of alts was higher post anti-EGFR LB ($n = 12$) vs. anti-EGFR naïve LB ($n = 22$) (9.5 vs. 5.5, $p = 0.059$) but not statistically significant. More alts were also noted in post anti-EGFR therapy LB vs. *KRAS* wt anti-EGFR-naïve LB ($n = 6$) (9.5 vs. 5) among patients with *KRAS* wild-type tumors, although the difference was not significant ($p = 0.182$).

Conclusions: LB across mCRC therapy detects driver mutations, monitors disease burden, and identifies sub-clonal alts that reflect drug resistance, tumor evolution, and heterogeneity. Interpretation of LB results is impacted by clinical context.

Keywords: liquid biopsy, precision oncology, molecular target, tumor heterogeneity, drug resistance, tumor burden, cfDNA

INTRODUCTION

A key factor contributing to the lethal outcome of cancer, therapeutic failure, and drug resistance is intra-tumoral heterogeneity and clonal evolution of tumors caused by accumulation of somatic mutations (1–3). The advent of next-generation sequencing has enabled more powerful analysis of tumor evolution and has improved our understanding of tumor initiation and development (1, 2, 4, 5). In patients with advanced colorectal cancer who receive multiple lines of therapy during the course of treatment, understanding the evolution of genetic alts during treatment can inform clinical management, and clinical trial design (6–9). This is becoming important in the era of precision oncology where acquired mutations may suggest novel options for therapy or resistance to targeted agents (10–12).

Guardant360 is an assay that utilizes next generation sequencing of cell-free DNA (cfDNA) to comprehensively profile 73 cancer-related genes in peripheral blood to establish circulating tumor (ct)-DNA presence, mutation patterns, and quantity (13). Multiple validation studies have been published utilizing this assay, including analytical studies and clinical validations in patients with advanced non-small cell lung cancer, colorectal cancer, and other solid tumors; such studies demonstrate high concordance between clinical plasma- and tissue-based genotyping methods which supports the clinical accuracy of the Guardant360 LB assay (3, 14, 15). An analysis of the landscape of cfDNA alts detected in a large cohort of colorectal cancer patient samples analyzed with this assay showed high similarity with genomic alts from tissue studies (7).

Here we present a case series of patients with advanced colorectal cancers who underwent LB testing at various time points during the course of their treatment at a Fox Chase Cancer Center GI Oncology Clinic. We describe our experience and delineate the utility of LB in practice and try to better understand the biology of metastatic colorectal cancer. Our findings point to the utility of LB in clinical practice during the care of patients with advanced colorectal cancer at an academic NCI-designated comprehensive cancer center. They suggest the importance of clinical context with regard to interpretation of LB test results, and illustrate uses, and information gained beyond which specific mutations are detectable. LB can reveal changes in tumor burden with ongoing therapy, a range of sub-clonal mutations likely due to acquired drug resistance, and clinical insight into tumor heterogeneity. Our retrospective case study was not designed to allow for clinical practice recommendations but rather to demonstrate preliminary clinical use patterns at an academic GI cancer clinic such that in the future specific uses or outcomes of interest can be further investigated.

METHODS

Patient Selection

Patients who underwent LB from Jan 2016 to April 2018 in a single colon cancer specialty clinic (W.S.E-D.) at Fox Chase Cancer Center were identified and studied. This was an institutional review board (IRB)-approved retrospective study. Clinical information including date of diagnosis, age at diagnosis, gender, type of cancer: colon vs. rectal, stage at diagnosis, lines of therapy, date of LB, reason for LB, date of tissue biopsy, CEA at time of LB and tissue biopsy, tumor burden on CT scan at time of LB and tissue biopsy, and last date of follow-up were recorded.

Genomic Testing

All patient samples were collected and processed in accordance with the Guardant360 clinical blood collection kit instructions (Guardant Health, Inc.). Guardant360 interrogates cfDNA for single nucleotide variants (SNVs) in 73 cancer-related genes, indels in 23 genes, copy number amplifications (CNAs) in 18 genes, and fusions in six genes. A routine blood draw (two, 10-mL Streck tubes) was obtained in the clinic and sent to Guardant Heath, a Clinical Laboratory Improvement Amendments (CLIA)-licensed, College of American Pathologists-accredited, New York State Department of Health-approved clinical laboratory. No refrigeration or local centrifugation was needed. For each sample, cfDNA was extracted from stabilized whole blood and between 5 and 30 ng of cfDNA input per sample was analyzed as described previously (3, 13). While the input was 5–30 ng of extracted ctDNA, ∼2/3 of the samples used 30 ng, but the minimum required was 5 ng of extracted ctDNA. In brief, DNA fragments were labeled at high efficiency with non-random oligonucleotide adapters ("molecular barcodes"), and used to prepare sequencing libraries, which were then enriched using hybrid capture and sequenced. Sequencing reads were then used to reconstruct individual cfDNA molecules present in the original patient sample with high fidelity using proprietary double-stranded consensus sequence representation. From the LB report, genomic alts, type of alts, and somatic alteration burden (maxpct—defined as the percentage frequency of the alteration with highest mutant allele frequency reported in the sample) were recorded.

Tissue Biopsy

For patients who had undergone tissue biopsy during their clinical course, the genes tested, and mutations identified in the tissue biopsy were recorded. Some patients with tissue biopsy had a restricted panel of next-generation sequencing (NGS) testing for alts in *RAS, RAF, TP53,* and/or *APC,* while others had more

extensive testing using various commercially available NGS test panels (FoundationOne, Caris, Nanthealth, Tempus, Omniseq).

Statistical Analysis

We used Kendall rank correlation tau to measure associations between continuous variables. For comparing numbers of alts between groups, we applied permutation-based two-sample *t*-tests. When laboratory measurements were available from multiple time points for the same patient we selected data from the blood sample collected at time of disease progression for this comparison. All tests were two-sided with a 5% type I error. Data were analyzed in statistical software R (version 3.5.0) and SAS (version 9.4).

RESULTS

Clinical Characteristics/Patient Demographics

Of 33 patients, 11 patients had rectal tumors and 22 had colon cancer. There were 18 women and 15 men. Median age at diagnosis was 52 years (range 20–76). Four patients initially presented with stage 2 disease, 9 patients with stage 3 disease, and 20 patients presented with metastatic disease at diagnosis. All patients were metastatic at the time of LB. Patients received a median of two (range 0–7) lines of therapy before LB.

Tissue and Liquid Biopsy Characteristics

Nineteen patients had limited tumor tissue NGS, 11 patients had extensive tumor NGS testing, and 3 patients had no NGS on tumor tissue. Eighteen patients were *KRAS* wild-type and 12 patients were *KRAS* mutant (**Figure 1**). One patient's tumor was MSI-high, 13 patients had microsatellite stable tumors, and 9 patients had not had MSI testing on the tumor. All detected mutations and their allele frequencies are listed in **Supplementary Table 1**.

In 5 patients LB was obtained due to inability to obtain tissue biopsy and in 28 patients to assess mutation load/identify targetable alts. Five patients had serial LBs. Of these, 1 patient had LB four times and the rest had LB twice during their treatment course. When including all LB results, including serial LB, median maxpct was 11.6% (range 0–83.9%). The median number of alts detected was 6 (range 0–43). Median CEA at the time of LB was 56 (range 1–4090). The median time from blood draw to obtaining results for LB was 14 days.

Correlation Between Tumor Burden and LB Alts

We hypothesized that LB results obtained at a given time might provide representative information on tumor burden, and may correlate with other measures appropriate to the clinical context. CEA is a good surrogate blood-based marker for tumor burden in most patients with colorectal cancer. In three patients (one of whom had four serial biopsies) CEA was always normal (<3 ng/ml) despite a high tumor burden indicating that their tumor did not produce CEA (**Figure 2**). We found that CEA correlated positively with maxpct (Kendall's Tau = 0.436, $p = 0.001$) and number of alts (Kendall's Tau = 0.451, $p < 0.001$) present in the LB consistent with our hypothesis (**Figure 2**). One of three pts with 0 LB alts detected had no measurable disease on CT scan, and the others had several lesions noted on CT scan. In the other 2 patients, LB did not have *APC*, *p53, KRAS,* or *PIK3CA* mutations, which may suggest that the LB specimen may not have captured any ctDNA. In 3/5 pts with serial LBs, CEA correlated with maxpct trend and CT tumor burden (**Figure 3**). In one of the patients in whom the CEA, number of alts and maxpct did not correlate with CT tumor burden, the allele frequency was very low (2–4%) and is probably due to low disease burden on CT. In another patient with an unexpected pattern of the LB results and CT scan tumor burden, necrosis demonstrated in the CT scan may have led to unreliable ctDNA results. Relationships between tumor burden and findings of LB results, including the presence or absence of accompanying tumor gene mutations involved in CRC, provided an indication that the clinical context in which an LB was performed would ultimately impact the results and their interpretation.

Liquid Biopsy to Guide Treatment Decision

We hypothesized that the presence of *RAS* mutations in LB may not only guide treatment decision for mCRC but might also reveal developing subclonal mutations in mCRC patients receiving anti-EGFR therapy. In two patients, due to insufficient tissue available for testing, *RAS* status could not be determined. LB obtained at the time of disease progression ultimately revealed clear evidence for *KRAS* mutation and anti-EGFR therapy was avoided in this patient. In another patient referred from an outside institution, *RAS* testing was not sent on tissue biopsy. LB in this patient revealed KRAS mutation and thus anti-EGFR therapy was not recommended as a first-line treatment option. In another patient, serial LBs revealed *KRAS* G12D mutation in both instances with allele frequency 0.49 and 6.46%, respectively (patient three in **Figure 1**). However, a tissue biopsy obtained at a time point between the two LB did not reveal *RAS* mutations, likely an example of tumor heterogeneity. In this patient, anti-EGFR therapy was avoided. LB can overcome some shortcomings of tissue biopsy, as a complementary test.

Association Between Number of Alts and Anti-EGFR Therapy

We hypothesized that anti-EGFR therapy might increase the number of genomic alts in the tumor. We compared the median number of alts in LBs of patient's pre- and post-anti-EGFR therapy. Overall, the median number of alts was higher post anti-EGFR ($n = 12$; median = 9.5) vs. anti-EGFR naïve ($n = 22$; median = 5.5) LB, however the difference did not quite reach the pre-defined level for statistical significance ($p = 0.059$) (**Figure 4**). Likewise, among patients with *KRAS* wild-type tumors more alts were noted in post anti-EGFR therapy LB ($n = 12$; median = 9.5) vs. anti-EGFR naïve ($n = 6$; median = 5) LB. This difference was not statistically significant ($p = 0.182$). Of note, in six patients who were KRAS wt based on tissue biopsy, post anti-EGFR subclonal KRAS mutations developed, and is likely an effect of treatment. One patient post-cetuximab anti-EGFR therapy had five KRAS gene mutations (A146T*, G12V, G13D, G60R, K117N), 3 EGFR alterations (N493D, P373S, Y1172Y), 7 p53 mutations (A161T, C176R, Q354R*, R282W,

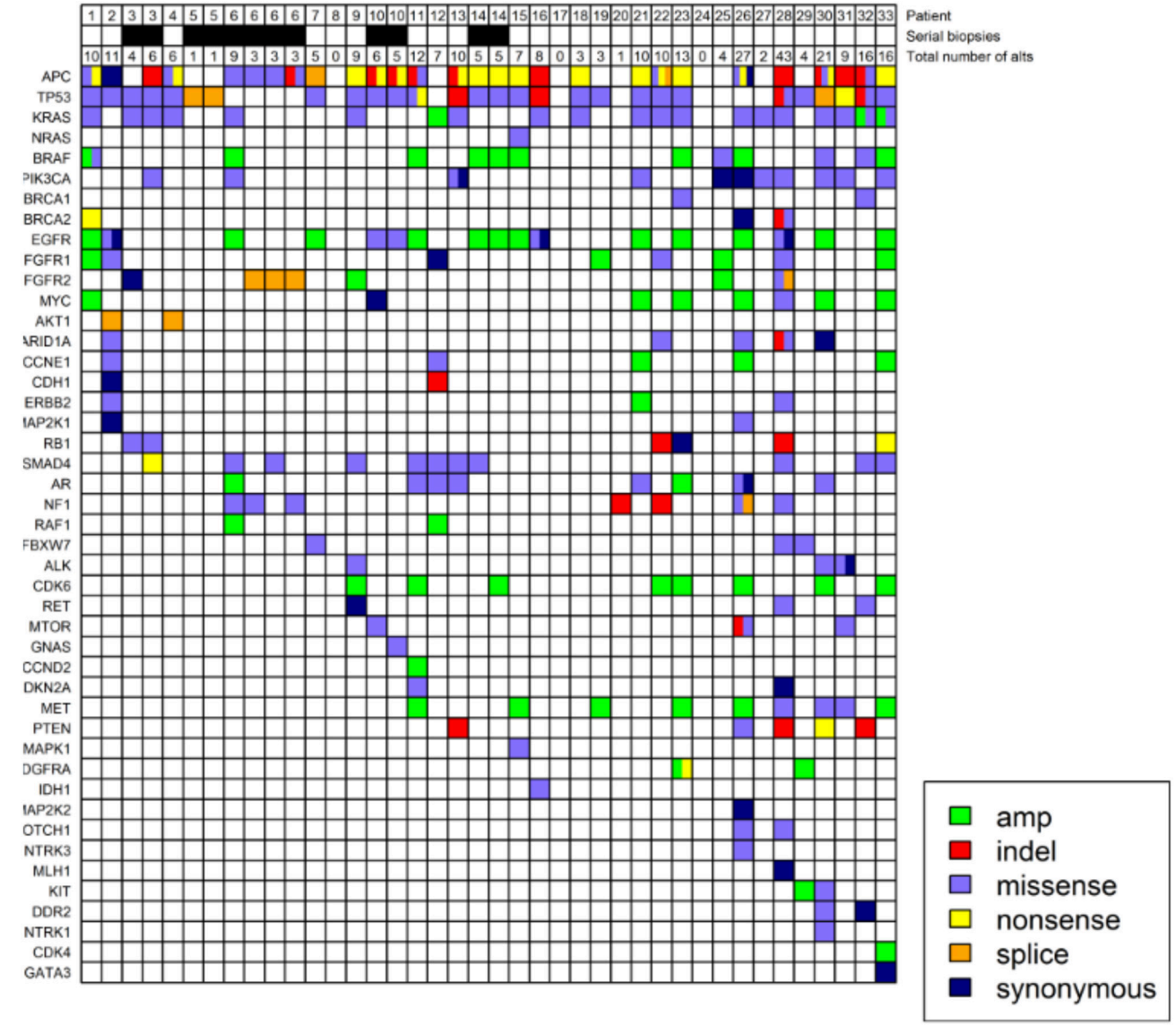

FIGURE 1 | Genomic profiling of circulating free tumor DNA in 33 patients with metastatic colorectal cancer. Four patients underwent serial LB as indicated in the second row. The number of alts detected on LB are listed in the third row. Colors denote different types of alts. Allele frequencies associated with the alts can be found in **Supplementary Table 1**.

p.Lys382fs, p.Ser2fs*, p.Val73fs), among others. Some of the mutations were present at fairly high allele frequencies, with an "*" indicating allele frequency >4%. The value of 4% allele frequency was set empirically for one case to highlight specific enriched alleles in that case where there were multiple mutations in a number of driver and drug-resistance genes found post-cetuximab therapy. The value is on the high end to show that for example in the case of the KRAS gene there was a dominant allele (A146T) and multiple other less frequent alleles likely reflecting the tumor's heterogeneity and the subclonal nature of the mutations. In a recently published large clinical sample set using the guardant technology (~21,000 patient samples) the median mutant allele fraction for alterations was 0.4% and the mean was 3.67% (16), and so 4% would be on the higher end of the spectrum. Thus, patients receiving anti-EGFR therapy can develop a high number alterations found in LB that likely represent acquired resistance mechanisms, e.g., multiple sub-clonal KRAS mutations.

DISCUSSION

Our results provide a clinically and genomically annotated case series in mCRC detailing the clinical experience with LB results in a cohort receiving systemic chemotherapy combinations, and includes several patients who underwent serial LB. In CRC, the most common mutations are *APC* (incidence 80%), *TP53* (50%),

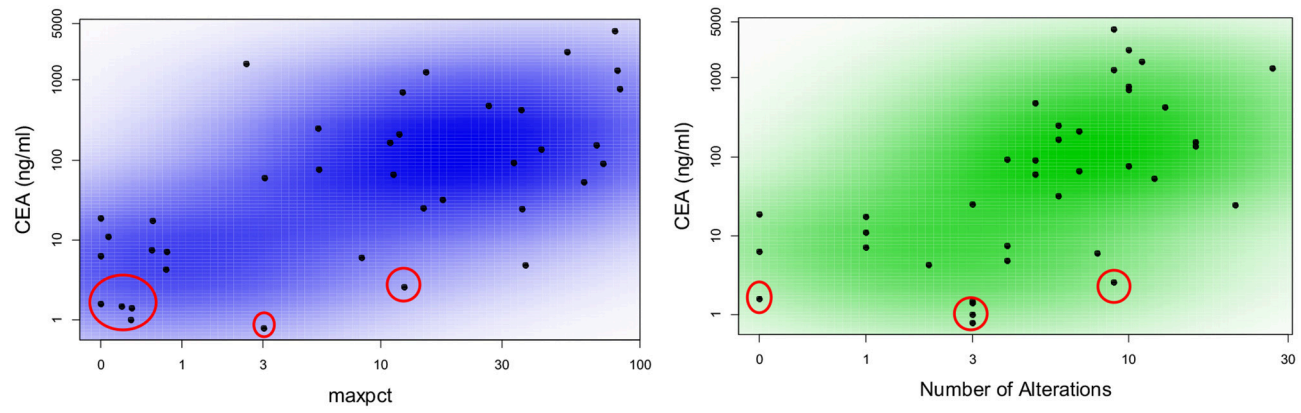

FIGURE 2 | Correlation between CEA as a marker of tumor burden and number of alterations or maximal allele frequency found in liquid biopsy from patients with metastatic colorectal cancer**.** Scatter plot colored by smoothed density demonstrating correlation between maxpct and number of alts with CEA. Plots include data from all available blood samples. There is a direct correlation between maxpct with CEA (Kendall's Tau = 0.436; $p = 0.001$) and number of alts with CEA (Kendall's Tau = 0.451; $p < 0.001$). The values circled in red represent three patients who never had elevated CEA despite high burden of metastatic disease. One of these patients had four serial liquid biopsies.

all *RAS* (40%), *BRAF* (8–10%), and *PIK3CA* (12%) (17–20). These were also the most common mutations identified on LB in this cohort (**Figure 1**). For LB results with complete absence of these or any other mutations, there is a need for caution in interpretation as the results may indicate lack of sufficient ctDNA. For example, LB in patients 8, 17, 20, 24 in **Figure 1** may not have had sufficient ctDNA, and the lack of alts on LB may not be representative of *RAS* status. With very low or absent disease burden, it would be expected that cfDNA may not be measurable. The expected profile of genomic alts (which genes are mutated) will vary among patients with different tumor types based on the observed common drivers. Our results provide insight into what is observed in a typical cohort of mCRC patients.

In 10 patients, there was discordance between the results of *KRAS* mutation in LB and tumor tissue. Importantly, the tissue biopsy and LB were obtained at different time points in all of these cases, and thus tumor evolution or therapy effects could have impacted the results and may explain the discordance. Moreover, in 6 of these 10 cases, LB obtained after anti-EGFR therapy revealed subclonal *KRAS* mutations that were not identified in the tissue biopsy obtained earlier. In two patients, *KRAS* mutation present in TB was absent in LB obtained later. One of these patients had minimal disease on CT scan at the time of LB indicating possible lack of ctDNA. LB also did not reveal *APC* or *TP53* alts in this patient, suggesting undetectable tumor-derived cfDNA overall. One patient with *KRAS* mutation on TB had serial LBs. The first LB showed *KRAS* mutation and the subsequent 3 LBs showed no *KRAS* mutations. This is likely due to the patient's significant tumor response observed on CT scan between the first and subsequent LBs. Of note, this patient did not receive any anti-EGFR therapy. Our results point to the importance and relevance of clinical context with regard to interpretation of LB test results, and further illustrate uses and information gained beyond just which specific gene mutations are detected in cfDNA analysis. LB clearly reveals changes in tumor burden with ongoing therapy, a range of subclonal mutations most likely due to acquired drug resistance, and clinical insight into tumor heterogeneity. This includes heterogeneity post-therapy exposure.

One patient with an MSI-H tumor demonstrated on TB testing had 11 alts on LB (patient two in **Figure 1**), while a patient with 27 alts was MSS (patient 26 in **Figure 1**), and MSI status was not determined in a patient with 43 alts on LB (patient 28 in **Figure 1**). We suspect that patients who were heavily pretreated acquired a significant number of gene mutations that may promote cell survival and resistance to chemotherapy or targeted therapy.

Clonal hematopoeisis (CH) is the somatic acquisition of genomic alts in hematopoietic stem and/or progenitor cells, leading to clonal expansion (21). In patients with cancer, CH is a common occurrence, associated with aging, smoking, and radiation therapy (22). CH is associated with increased risk of therapy-related hematologic malignant neoplasms, and genes frequently mutated in CH such as *DNMT3*, *TET2*, *PPM1* are also commonly altered in hematologic malignant neoplasms (23). While CH mutations in both tissue and LB may be misattributed as somatic tumor variants in patients, the Guardant360 panel did not test for mutations in these frequently mutated CH genes. While *TP53* or *KRAS* can be associated with CH, we believe that clonal hematopoiesis likely has a minimal role in this study as the objective of obtaining LBs in this cohort was to identify therapy-related resistance mechanisms during treatment course and to monitor tumor burden as a measure of therapy response by serial LBs.

In our case series, we show that LBs can help identify actionable driver mutations, guide treatment decisions, and monitor disease burden. LBs offer several advantages over TB. In addition to being non-invasive, LBs can be much more easily performed serially during treatment course, can help monitor disease burden, treatment effect, and developing resistance

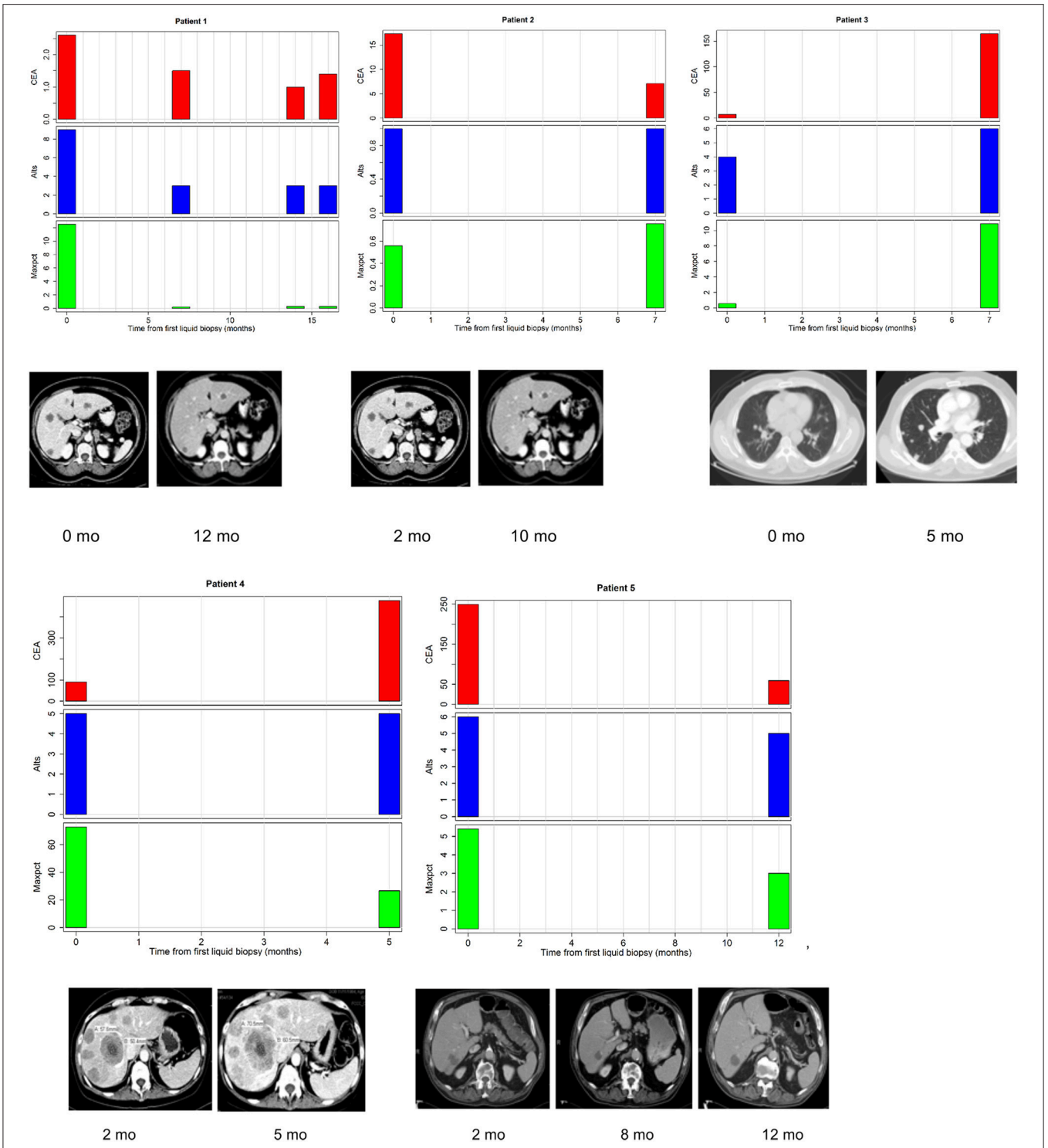

FIGURE 3 | Correlations between tumor burden as assessed by radiographic imaging (CT scans) or CEA (tumor marker) and liquid biopsy mutation parameters (alts or number of alterations/number of mutated genes and maxpct or maximal allele frequency of mutated allele). **(Top Left)** Maxpct, CEA, and alts follow a downward trend as disease on CT scan improves. Center: With growth of mediastinal mass on CT, note rise in maxpct, CEA, and alts. **(Top Right)** As lung disease worsens on CT, maxpct, CEA, and alts increase. **(Bottom Left)** Despite increasing tumor on CT scan and rising CEA, maxpct did not rise. Liquid biopsy did contain APC and TP53 mutations, indicating presence of ctDNA. **(Bottom Right)** Liver metastases decreased between 1/2017 and 7/2017 and then increased in 11/2017. Allele freq. low (2–4%) probably due to low disease burden on CT.

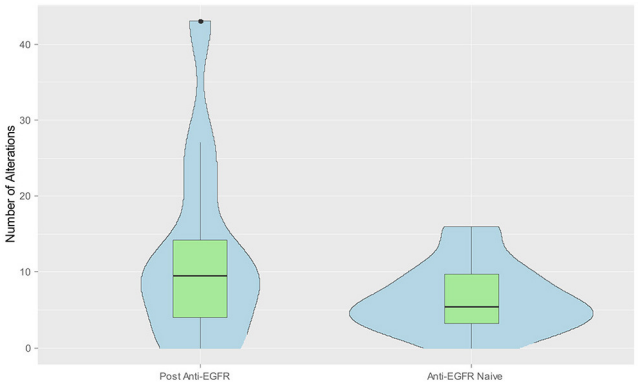

FIGURE 4 | Combined violin and box plot graph demonstrating increased number of alts in liquid biopsy post anti-EGFR therapy. Data from the blood sample collected at time of disease progression was selected when multiple measurements were available from the same patient. Median number of alts were higher post anti-EGFR ($n = 12$) vs. anti-EGFR naïve liquid biopsy ($n = 22$) (9.5 vs. 5.5, $p = 0.058$).

mutations, and can detect tumor heterogeneity that is a limitation with use of tissue biopsy. Our results provide clinical experience with use of this technology in a limited mCRC cohort at an academic center and illustrate how information may be used to impact clinical decision-making. However, larger studies are needed to address any recommendations that may impact on clinical practice. In addition, insights were gained regarding the biology of treatment response and resistance. LB appears to have some clinical utility in the ongoing care of patients with mCRC including the timely identification of *RAS* family gene mutations, and understanding the basis for emerging resistance to anti-EGFR therapy. An important insight gained from our experience is that clinical context matters and can have an impact on the interpretation of LB results. While we believe CH had minimal impact on the uses we describe for LB in our cohort, this is clearly an important consideration that could have impact on the interpretation of LB results especially in certain contexts with rare sub-clonal alts whose origin may not be the patient's tumor. For mCRC patients, the presence or absence of tumor Ras mutations directly impacts on use of anti-EGFR therapy. Thus, it is critically important to have valid information for the Ras gene family status with appropriate interpretation taking into account the possible confounder of effects of clonal hematopoiesis. This suggests that the input of experts in cancer genetics and molecular tumor boards (as well as potentially testing of normal WBC DNA in specific situations) may ultimately enhance the clinical utility of LB in patient care.

In conclusion, based on our experience we suggest considering LB for patients who have not had TB or have insufficient tissue to determine the presence of *KRAS/NRAS/BRAF* mutations, especially in the context of high tumor burden prior to therapy. LB could also be considered in patients who do not have an elevated CEA as serial LB may help monitor disease response during treatment. In patients who appear to have no evidence of disease after therapy, periodic LB during surveillance period may help detect disease relapse if alts are detected. LB may also help monitor the evolution of resistance mechanisms in the tumor and recent data indicate that LB results may allow re-challenge of previously received anti-EGFR therapy (24). In this study the authors note that in *RAS/RAF/EGFR* wild-type patients progressing on anti-EGFR therapy, the clones of *RAS and EGFR*, as detected on LB exponentially decay and knowing the half-life of these clones can help predict the efficacy of re-challenging these patients with anti-EGFR therapy (24). Larger studies in the future need to more definitively establish the ability of liquid biopsies to safely substitute for tissue biopsies in certain clinical settings for mCRC patients and to determine the optimal frequency of obtaining LB in different clinical settings. Our study was a retrospective case series that was neither designed nor intended to make clinical practice recommendations but to motivate larger studies that are statistically powered to allow for specific recommendations in different clinical situations where use of liquid biopsy may be advantageous in the clinical care of patients. However, LB as an adjunct, complementary technology appears to have some utility in the monitoring and treatment decisions for patients with advanced mCRC especially in settings where TB results are unavailable, not possible or impractical.

ETHICS STATEMENT

Upon review, the IRB at Fox Chase Cancer Center determined the submission (IRB# 17-9057: Pilot Analysis of Utility of Liquid Biopsy Results in Advanced Colorectal Cancer) meets the criteria for the approval of research outlined in 45 CFR 46.111. Upon review, the IRB determined the submission meets the criteria for Waiver of Informed Consent outlined in 45 CFR 46.116(d). Upon review, the IRB determined the submission meets the criteria for Waiver of Authorization for Use or Disclosure outlined in 45 CFR 164.512(i)(2). The IRB approved the protocol from 1/12/2018 to 1/11/2019 inclusive.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2018.00652/full#supplementary-material

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CASE REPORT
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Adjunctive Use of Circulating Tumor DNA Testing in Detecting Pancreas Cancer Recurrence

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Liquid biopsies (circulating tumor DNA—ctDNA testing) are increasingly being utilized in clinical trials as well as practice for the detection of cancer, monitoring of tumor genomic abnormalities, response to treatment and early detection of relapse/recurrence. Here, we present a challenging case where liquid biopsy was used to confirm an early recurrence of pancreatic cancer where acquisition of tissue was not safe or feasible on more than one occasion.

Keywords: pancreas cancer, circulating tumor DNA (ctDNA), circulating tumor cells (CTC), liquid biopsy, relapse/recurrence

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BACKGROUND

In cancer, surgical or interventional biopsies are obtained traditionally to characterize the site of origin of the cancer cells as well as to potentially characterize the genetic profile of the tumor. These approaches only represent a limited snap shot of the tumor (1). Furthermore, it is known that cancers can evolve on treatment and are known to have intratumoral and intertumoral heterogeneity. Single tumor biopsies can limit the extent of personalized medicine as they can underestimate the tumor genomic landscape and evolution throughout treatment (2).

Liquid biopsies have been developed recently and improved over time as a potential surrogates for tumor biopsies in cancer screening, detection of genomic alterations, determination of response to treatment, and detection of early recurrence (3). A lot of research is still underway. At present their use is primarily limited to advanced/metastatic cases in practice.

We present a case of a woman with pancreatic cancer were a liquid biopsy was used twice for confirmation of recurrence and prompt initiation of treatment in lieu of a surgical biopsy due to the difficulty of obtaining tissue to confirm recurrence. Serial evaluations by liquid biopsy confirmed response to treatment and then later again recurrence. In all these instances, it was not safe or feasible to obtain tissue.

INTRODUCTION (CASE)

A 70 years old Caucasian female with a history of laparoscopic Roux-en-Y gastric bypass in 2013 complicated by the development of a large ventral hernia in May 2014 was evaluated. As part of a pre-surgical evaluation a CT of the abdomen on December 8, 2015 identified a 3.5 × 2.8 cm mass in the head of the pancreas. MRI of the abdomen on December 29, 2015 showed the mass to be 3 × 2.9 × 3.4 cm. CT chest showed no pulmonary metastases. On January 11, 2016, she underwent open pylorus-preserving pancreaticoduodenectomy, cholecystectomy, and repair of the ventral hernia. Pathology showed a 3.5 cm, invasive well differentiated pancreatic ductal adenocarcinoma, arising in a background of intraductal papillary mucinous neoplasm (IPMN). The tumor invaded the duodenal wall, peripancreatic soft tissues, and extrapancreatic common bile duct. Margins were negative for tumor. IPMN was present at the pancreatic surgical margin without evidence of high-grade dysplasia/carcinoma.

Fourteen of twenty-seven regional lymph nodes were positive for metastatic carcinoma (14/27). Lymphovascular invasion was indeterminate. Perineural invasion was present. Final pathologic staging per TNM classification was IIB (pT3pN1M0). Comprehensive tumor based genetic testing showed mutations in *KRAS G12V, CDKN2A p16INK4a A17fs*21, TP53 S149fs*32,* and *U2AF1 S34F*.

She initiated adjuvant therapy with gemcitabine on February 2016 and completed 2 cycles through April. Course was complicated by abdominal pain and rash. CT abdomen/pelvis with contrast on April 11, 2016 showed interval appearance of a solid mass in the tail of the pancreas worrisome for a new primary cancer. PET scan showed the mass to be hypermetabolic with an SUV 6.4. MRI showed postop Whipple procedure with new hypoenhancing mass in the tail of the pancreatic remnant measuring 1.8 × 2.1 cm, correlating with the hypermetabolic lesion seen on PET scan. There was no evidence of liver metastases. CA 19–9 tumor marker was 12 U/mL (normal <55 U/mL).

On May 10, 2016, she underwent splenectomy, remnant gastrectomy, and total pancreatectomy. Operative note did not report any visible abdominal malignancy. Pathology showed IPMN with focal high-grade dysplasia but no overt cancer. Seven peripancreatic and 4 peri hilar (splenic) nodes were negative for malignancy. Immunohistochemistry showed no expression for PD-L1 and normal expression of *MLH1*, *PMS2*, *MSH2*, and *MSH6*. Postoperative course had a slow recovery. She declined resumption of adjuvant therapy (either radiation or chemotherapy) and she was surveilled with imaging.

On May 12, 2017 a CT of the chest, abdomen and pelvis showed a newly enlarged 1.2 cm low para-aortic lymph node, suspicious for metastatic disease. A single 1.1 cm periportal lymph node was also mildly increased in size from prior. Her case was discussed in the multidisciplinary tumor board. The para-aortic node was in a challenging location for successful biopsy. CA 19-9 was 34 U/mL (normal <55 U/mL). A circulating tumor DNA test (ctDNA, Guardant360®) was sent on May 22, 2017 that showed mutations in *CDKN2A*—that was present at baseline tumor based genetic profile and a new mutation in *ARID1A* (T2138del).

After a thorough discussion with the patient the decision was made to start systemic chemotherapy treatment with Gemcitabine/nab-paclitaxel for recurrent adenocarcinoma of the pancreas. She completed 4 cycles of Gemcitabine/nab-paclitaxel. This was followed by chemoradiation with capecitabine as a radiosensitizer, which she completed in January 2018. A repeat ctDNA in February 2018 showed disappearance of the previous *CDKN2A* and *ARID1A* mutations and no new mutations were detected.

Unfortunately, in March 2018 imaging again showed recurrence in the lungs and liver. The locations still were not amenable to a tissue biopsy and liquid biopsy was utilized that picked up again mutations that were concordant with the patient's tumor (**Table 1**). She is currently on chemotherapy with liposomal irinotecan/5-fluorouracil.

Written informed consent was obtained from the patient for the publication of this case report.

GUARDANT 360

As per the manufacturer, the "Guardant 360 is a whole blood based cell free DNA detection assay. A Guardant sample collection kit is used to obtain two 10 mL of whole blood from the patient. The sample is sent directly to the laboratory at Guardant Health. The test detects single nucleotide variants in a targeted panel of 73 genes, and selected copy number amplifications, fusions/rearrangements, and indels for a specific set of genes. All four types of genomic alterations are reported in a single test. Turnaround time for testing is approximately ≤14 days.

The genes sequenced include: *AKT1; ALK; APC; AR; ARAF; ARID1A; ATM; BRAF; BRCA1; BRCA2; CCND1; CCND2; CCNE1; CDH1; CDK4; CDK6; CDKN2A; CTNNB1; DDR2; EGFR; ERBB2; ESR1; EZH2; FBXW7; FGFR1; FGFR2; FGFR3; GATA3; GNA11; GNAQ; GNAS; HNF1A; HRAS; IDH1; IDH2; JAK2; JAK3; KIT; KRAS; MAP2K1; MAP2K2; MAPK1; MAPK3; MET; MLH1; MPL; MTOR; MYC; NF1; NFE2L2; NOTCH1; NPM1; NRAS; NTRK1; NTRK3; PDGFRA; PIK3CA; PTEN; PTPN11; RAF1; RB1; RET; RHEB; RHOA; RIT1; ROS1; SMAD4; SMO; STK11; TERT; TP53; TSC1;* and *VHL*. Covered exons are completely sequenced to maximize detection of known somatic variants. Sensitivity for genes sequenced is >99.9% if the allelic fraction/copy number is >0.25% with a positive predictive value (PPV) of 99.6. If the allelic fraction is 0.05–0.25% the sensitivity of the test is 63.8% with a PPV of 92.1%.

The following genes are also analyzed for copy number amplifications (CAN): *AR; BRAF; CCND1; CCND2; CCNE1; CDK4; CDK6; EGFR; ERBB2; FGFR1; FGFR2; KIT; KRAS; MET; MYC; PDGFRA; PIK3CA*; and *RAF1*. Sensitivity for CAN is 95% and PPV is 100%.

Genes analyzed for fusions/rearrangements are: *ALK; FGFR2; FGFR3; NTRK1; RET*; and *ROS1*. Sensitivity and PPV for fusions is 100% if allelic fraction is ≥0.3%.

The following genes are also analyzed for indels: *APC; ARID1A; ATM; BRCA1; BRCA2; CDH1; CDKN2A; EGFR; ERBB2; GATA3; KIT; MET; MLH1; MTOR; NF1; PDGFRA; PTEN; RB1; SMAD4; STK11; TP53; TSC1;* and *VHL*. Sensitivity for indels is >99.9% and PPV is 98% if allelic fraction >0.25%."

DISCUSSION

Pancreas cancer represents the 4th leading cause of cancer deaths in both men and women in the United States. In contrast to the improved survival seen in multiple cancer types the progress in improvement in overall survival has been slow for pancreatic cancer with an overall 5 year survival rates of approximately 8% (4). This could be in part secondary to the majority of patients presenting with advanced disease at diagnosis. New biomarkers

Abbreviations: BEAMing, beads, emulsion, amplification, and magnetics; CT, computed tomography; CTC, circulating tumor cells; cfDNA, circulating free DNA; ctDNA, circulating tumor DNA; DNA, deoxyribonucleic acid; IPMN, intraductal papillary mucinous neoplasm; PCR, polymerase chain reaction; PET, positron emission tomography; RNA, ribonucleic acid.

TABLE 1 | Comparison of aberrations detected on baseline tumor tissue based comprehensive genetic testing and later in circulating tumor DNA (ctDNA) at 3 distinct timepoints.

Baseline comprehensive tumor based testing	ctDNA timepoint 1 (recurrence #1)	ctDNA timepoint 2 (post-chemoradiation)	ctDNA timepoint 3 (recurrence #2)
	0.09% Highest variant allele fraction	ND	0.2% highest variant allele fraction
KRAS G12V	ND	ND	***KRAS G12V***
CDKN2A p16INK4a A17fs*21	***CDKN2A p16INK4a A17fs*21***	ND	ND
TP53 S149fs*32	ND	ND	***TP53 S149fs*32***
U2AF1 S34F	ND	ND	ND
VUS			
ARID1A R1869Q	ND	ND	ND
*FOXP1 *115Lext*?*	ND	ND	ND
PIK3R2 P261_S262insP	ND	ND	ND
PLCG2 Q387P	ND	ND	ND
ND	*ARID1A T2138del*	ND	ND
ND	ND	ND	*IDH2 G145G*

Chronological results of circulating tumor DNA (Guardant 360) showing mutations at baseline, at the time of recurrences and post treatment. ND, not detected; ctDNA, circulating tumor DNA; VUS, variant of unknown significance. In bold are the deleterious mutations noted as opposed to variants of unknown significance.

for diagnosis and monitoring treatment of this disease are required to help improve outcomes.

Cancer cells can release cell fragments and dead cells into the circulation. Liquid biopsies rely on analysis of tumor material such as DNA (known as circulating tumor DNA or ctDNA), RNA, proteins, exosomes and/or whole cells (known as circulating tumor cells or CTCs) that can be found in blood, cerebrospinal fluid, saliva, or urine. They have been developed with the goal of detecting the material in a sample that originates from cancer cells (5). They can be detected by several techniques including quantitative real time polymerase chain reaction (PCR), methylation specific or digital PCR, next generation sequencing and/or BEAMing (beads, emulsion, amplification, and magnetics) (6).

A potential application of liquid biopsies is detecting cancer at an early stage when treatment may be most successful; however a concern is a false positive results and/or overtreatment of tumors that may be more harmful than the tumor itself. Another potential application of liquid biopsies is the paradigm of precision medicine by identification of unique molecular characteristics of a tumor that could be used to determine the optimal treatment. Most importantly, it also allows for simultaneous testing of multiple genes depending on the platform used that can be specific for certain types of cancer.

Liquid biopsies can also be used as prognostic or predictive markers. For example, a prospective study by Toledo et al. of 25 patients with newly diagnosed wild type RAS metastatic colorectal cancer treated with FOLFIRI-cetuximab used liquid biopsies by BEAMing for validation and monitoring of ctDNA mutations in *KRAS, NRAS, BRAF,* and *PIK3CA*. They found that patients with prolonged responses to treatment with anti EGFR therapy maintained a wild type *RAS* status. In contrast, patients who showed upsurges in circulating *KRAS* mutations had rapid disease progression with clinical deterioration and spread of metastasis (7).

A widely studied application for liquid biopsies is monitoring treatment response and predicting early relapse/recurrence. Namlos et al. reported a case of a patient with high grade soft tissue sarcoma were they prospectively collected primary tumor sample at diagnosis and several plasma (ctDNA) samples during the disease course. Targeted resequencing of the levels of ctDNA allowed them to detect progression of the disease 6 weeks after surgery and this was corroborated by detection of multiple new metastatic sites on imaging (8). Nakamura et al. retrospectively reviewed ctDNA in 17 patients with several hematological malignancies who achieved remission after first line chemotherapy. Eight patients in the relapsed group had more than doubled the levels of ctDNA at several time points and there was a median 30-days lead-time over clinical relapse. In contrast, in the 9 patients from the remission group, ctDNA remained undetectable (9). Another example from a prospective study by Birkemkamp-Demtröder et al. in 60 advanced bladder cancer patients used ctDNA in plasma and urine to detect metastatic relapse after cystectomy and measure treatment efficacy. Patients with metastatic relapse had higher ctDNA levels compared to disease free patients ($P < 0.001$) and the median positive lead-time between ctDNA detection in plasma and diagnosis of relapse was 101 days (range 0–932). A meta-analysis of the use of liquid biopsy (both CTCs and ctDNA) as a predictor of recurrence after surgery of non-small cell lung cancer showed that positive blood CTCs or ctDNA after surgery was significantly associated with worse progression free survival [Hazard ratio (HR) 3.37, 95% CI 2.28–4.96, $p < 0.001$ and HR 8.15, CI 2.11–31.50, $p = 0.002$, respectively]. One and two year's recurrence rate were higher in both the CTCs and ctDNA groups compared to the negative groups (10).

In pancreatic cancer specifically, few studies have been reported in this area. Sausen et al. demonstrated that the presence of CTCs after resection of the primary tumor did predict relapse and worse outcomes with recurrence detected at a median of 6 months earlier than CT imaging (11). Furthermore, Ren et al. showed a presence of 80.5% of CTCs in 41 advanced pancreatic cancer patients at baseline prior to initiation of 5-fluorouracil based chemotherapy. After 1 week of treatment the presence of CTCs decreased to 29.3% suggesting a potential role for using CTCs as a biomarker for treatment response in this malignancy (12).

Future approaches would include using ctDNA alone or in combination with other imaging or laboratory tests as a marker for early detection of recurrence in pancreas cancer. Currently, an ongoing prospective clinical trial in Korea is evaluating the use of ctDNA for early screening of recurrence of pancreas cancer and its correlation with clinical outcomes (NCT 02934984), which will also help discuss optimal timing of liquid biopsies in monitoring for recurrence. Furthermore, Cohen et al. described that the combination of ctDNA with protein biomarkers (i.e., tumor markers) increased the sensitivity of detection of resectable pancreatic cancer with a sustained high specificity (13).

In the case of our patient the rationale behind using a liquid biopsy to help detection or confirmation of pancreas cancer recurrence was the inability to obtain tissue twice due to the difficult and inaccessible location of the suspected recurrence. Even though ctDNA is not necessarily meant for that purpose, the results which were taken into considerations with patient's underlying prior comprehensive tissue based tumor testing were considered sufficient alongside the clinical and radiographic picture. The positive result of the ctDNA, the inability to obtain a tissue biopsy and a slight worsening of the overall clinical condition represented red flags that recurrence/relapse was underway and treatment needed to be initiated soon to help achieve the best clinical outcomes.

It is interesting to note that in our case the *KRAS* and *TP53* mutations were not detected after the initial chemotherapy regimen. While clones/subclones can evolve, given this is pancreas cancer and the mutations that were not detected were *KRAS/TP53*, it is likely that in those instances cell free DNA was below 0.25% allelic fraction/copy number and was not detected by the test. So it was falsely negative since tumor burden can impact the sensitivity of the assay whereby a positive test is helpful but a negative test could be negative as a consequence of limited tumor burden. It is also important to realize that clonal hematopoiesis of indeterminant potential (CHIP) is an entity that can be misinterpreted as ctDNA. Comparison of test results with baseline tissue based tumor testing and interpreting results in context of the particular tumor can help.

To our knowledge this is the first case reported of realtime clinical use of liquid biopsy to confirm recurrence twice in a patient with pancreas cancer when tissue biopsy was not considered safe or feasible. Liquid biopsies are safe, tolerable, and sensitive tools that can be incorporated into routine clinical care of cancer patients to help with detection of early recurrence/relapse. It is important to have baseline comprehensive tumor based genetic testing to avoid erroneous diagnoses from clonal hematopoiesis of indeterminate significance. As noted in **Table 1**, given different coverage, levels of ctDNA, and testing platforms, results of these assays may not always be concordant. This further argues to not to interpret such results in the absence of baseline tumor based genetic testing.

CONCLUDING REMARKS

Our article highlights a real-time example of using a commercially available assay to help with confirmation of a clinically suspicious recurrence event in a patient with inaccessible lesions that were not deemed safe for a biopsy. We cannot make any conclusions about if we made an impact on the patient's overall survival by acting early on recurrence. A larger study would be needed to formally study this. However, in the right patient's context with baseline tumor based genetic testing results available, ctDNA testing can be of considerable value.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Disease Control With FOLFIRI Plus Ziv-aflibercept (zFOLFIRI) Beyond FOLFIRI Plus Bevacizumab: Case Series in Metastatic Colorectal Cancer (mCRC)

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Background: The prognosis of patients with metastatic colorectal cancer (mCRC) is poor, especially after failure of initial systemic therapy. The VELOUR study showed modestly prolonged overall survival (OS) with ziv-aflibercept plus 5-fluorouracil, leucovorin, and irinotecan (zFOLFIRI) vs. placebo+FOLFIRI after progression on 5-fluoruracil, leucovorin, and oxaliplatin (FOLFOX) ± bevacizumab. The utility of zFOLFIRI after bevacizumab+FOLFIRI is unknown and not recommended in NCCN guidelines. We explored whether zFOLFIRI may be active beyond progression on bevacizumab+FOLFIRI.

Methods: We undertook a retrospective analysis of patients treated in routine clinical practice. A chart review was conducted for a cohort ($N = 19$) of advanced cancer patients (18 mCRC) who received zFOLFIRI from 2014 to 2018 at Fox Chase Cancer Center (FCCC). Analysis included time on zFOLFIRI, PFS, OS, CEA trends and adverse events. A second mCRC cohort ($N = 26$) from the Flatiron Health EHR-derived database treated with zFOLFIRI after prior bevacizumab+FOLFOX and bevacizumab+FOLFIRI was analyzed for time-on-treatment and overall survival.

Results: Median age of mCRC cohort at zFOLFIRI treatment was 54 (FCCC; $N = 18$) and 62 (Flatiron Health-cohort; $N = 26$). Of 18 FCCC mCRC patients, 1 patient had prior bevacizumab+FOLFOX and ramucirumab+irinotecan prior to zFOLFIRI for 8.5 months. Of 17 FCCC mCRC patients with prior bevacizumab+FOLFIRI who received zFOLFIRI, 13 had mutant-KRAS, 3 WT-KRAS, and one BRAF-V600E. The patient with BRAF-V600E mutation achieved stable disease on zFOLFIRI after multiple BRAF-targeted therapies. One patient (WT-KRAS mCRC) remained on zFOLFIRI for 14 months. Of 14 patients with mutated-KRAS, 8 remained on zFOLFIRI for >5 months including 3 for >15 months. The rate-of-change in CEA measures on zFOLFIRI was significantly different ($p = 0.004$) between rapid progressors and those with PFS>4 months. For mCRC patients treated with zFOLFIRI in the 3rd line or greater ($N = 18$), median PFS was 7.1 months

(214 days) and median OS was 13.8 months (416 days). Median time-on-treatment with zFOLFIRI in the Flatiron Health cohort was 4.4 months, median OS was 7.8 months, and longest time-on-treatment with zFOLFIRI was 266 days.

Conclusions: In these small real-world cohorts, clinical meaningful stable disease and overall survival on zFOLFIRI beyond progression on bevacizumab+FOLFIRI was observed in patients with mCRC. Further exploration of this approach is warranted.

Keywords: ziv-aflibercept, FOLFIRI, mCRC, colorectal cancer, metastasis, disease control, zFOLFIRI

INTRODUCTION

Colorectal cancer is the third most common cancer diagnosed in the USA with an estimated 97,220 new cases of colon cancer and 43,030 cases of rectal cancer in 2018. It is also the third leading cause of cancer-related death among both men and women killing an estimated 50,630 people this year alone (1).

5-fluoruracil, leucovorin, and oxaliplatin (FOLFOX) or 5-fluorouracil, leucovorin, and irinotecan (FOLFIRI) remain standard front-line therapy backbones for patients with metastatic colorectal cancer (mCRC); (NCCN guidelines Version 2.2018). Hurwitz and colleagues first showed in 2004 that the addition of bevacizumab to fluorouracil-based combination chemotherapy significantly prolonged overall survival in patients with mCRC (2). The addition of anti-angiogenic therapy with bevacizumab thus became an acceptable component of standard of care in the frontline setting for patients with metastatic disease (3, 4).

After progressing on FOLFOX or FOLFIRI plus bevacizumab in the first line, the randomized phase III TML study (ML18147) demonstrated an overall survival benefit in the second line with the use of bevacizumab plus FOLFIRI vs. either FOLFIRI or FOLFOX alone, and patients frequently switched from FOLFOX to FOLFIRI or vice versa while continuing on bevacizumab (5). More recently, other antiangiogenic agents have been developed including large molecules such as ziv-aflibercept (Zaltrap or VEGF-TRAP) and ramucirumab (6). The RAISE study was a randomized double-blind multicenter phase III study of FOLFIRI plus ramucirumab or placebo in mCRC patients who progressed during or following first-line combination therapy with oxaliplatin, a fluoropyrimidine and bevacizumab and showed a 1.6 month increase in overall survival (11.7 months vs. 13.3 months) in the group treated with ramucirumab. Ramucirumab is approved in gastric, lung cancer and mCRC (7).

Aflibercept is a fusion protein containing key domains that recognize human VEGF receptors 1 and 2 and human IgG Fc (8). Ziv-aflibercept blocks all human VEGF-A isoforms, VEGF-B, and placental growth factor (PIGF). Known as "VEGF Trap," ziv-aflibercept binds VEGF-A more tightly than native receptors. The randomized phase III VELOUR study (NCT00561470) investigated the use of ziv-aflibercept in combination with FOLFIRI (zFOLFIRI) in second line therapy for mCRC patients who previously failed prior oxaliplatin-containing therapy (9). VELOUR randomized ∼600 metastatic colorectal cancer patients to each arm for treatment with aflibercept 4 mg/kg IV day 1 plus FOLFIRI every 2 weeks vs. placebo IV day 1 plus FOLFIRI every 2 weeks. Patients who received prior bevacizumab were eligible to enroll in the VELOUR study (9). VELOUR showed that overall survival (OS) was modestly prolonged in the study arm of zFOLFIRI vs. the placebo plus FOLFIRI arm. For the subgroup of patients who had received prior bevacizumab, OS was longer by 2.14 months for the zFOLFIRI vs. the placebo plus FOLFIRI arm (9).

zFOLFIRI was approved by the FDA in 2012 as a second line therapy for patients with mCRC. Current NCCN guidelines include the option to use zFOLFIRI in the second line for mCRC. However, the current NCCN guidelines (Version 2.2018) state that there are no data to suggest activity of zFOLFIRI in a patient who has progressed on FOLFIRI-bevacizumab, and that ziv-aflibercept has only shown activity when given in conjunction with FOLFIRI in FOLFIRI-naïve patients. Current 3rd line options for mCRC include regorafenib, based on the results of the CORRECT study in which a 1.4 month OS benefit was seen vs. best supportive care (BSC), or trifluridine-tipiracil which provided a 1.8 month OS benefit vs BSC in the refractory mCRC setting (10, 11). While additional therapies that target EGFR improve survival, these are only indicated for tumors with wild-type KRAS/NRAS genes and left-sided tumors. Clearly, options for patients who have failed initial therapy for metastatic disease are limited, especially for the subgroups with mutated KRAS or NRAS genes.

There have been no randomized comparisons of different anti-angiogenic agents such as ziv-aflibercept or ramucirumab plus combination therapy beyond first line bevacizumab-containing combinations with FOLFOX or FOLFIRI. Similarly, there are no randomized studies evaluating "second-line anti-angiogenic agents" such as ziv-aflibercept or ramucirumab in thesetting of bevacizumab use beyond progression in the second line setting where bevacizumab-containing combination therapies (after first-line bevacizumab-containing combination therapy in mCRC) are used.

Given the prior data that some patients who progressed on bevacizumab-containing combination therapy could derive some benefit from aflibercept plus FOLFIRI (VELOUR study) some patients in clinical practice have been offered such therapy beyond progression on bevacizumab containing combination therapy. While the original VELOUR study results suggested a modest benefit from the zFOLFIRI therapy beyond bevacizumab, there is little data on the use of zFOLFIRI beyond progression on bevacizumab-containing combination therapy regimens in general in mCRC. We therefore explored the real-world treatment outcomes in two cohorts of patients with mCRC

treated with aflibercept in this context. We have treated 18 mCRC patients with zFOLFIRI beyond progression on bevacizumab in the second line and have noted in some cases prolonged stable disease. A significant number of the patients had prior bevacizumab plus FOLFIRI (N=17), a clinical setting not previously evaluated in VELOUR or other trials. This case series therefore aims to summarize the patient demographics and clinical outcomes in patients that were treated with zFOLFIRI beyond progression on a bevacizumab-containing regimen. We also describe a separate cohort of patients in the Flatiron Health Database treated with zFOLFIRI after FOLFOX+bevacizumab and FOLFIRI+bevacizumab with similar findings. Our results provide a rationale for future exploration of zFOLFIRI beyond progression on bevacizumab plus FOLFIRI and bevacizumab plus FOLFOX. In the absence of additional clinical trial data, our observed clinically meaningful stable disease on zFOLFIRI beyond progression on bevacizumab plus FOLFIRI in mCRC suggests a possible further line of therapy for patients with otherwise limited options.

PATIENTS AND METHODS

Chart Review

A retrospective chart review was conducted after review and approval of a clinical protocol by both the Research Review Committee and the Institutional Review Board at Fox Chase Cancer Center. A cohort was identified of 19 stage IV patients (18 with mCRC) who received aflibercept plus FOLFIRI (zFOLFIRI) in a GI Oncology Clinic (W.S.E-D.) at Fox Chase Cancer Center during the period from 2014-2018. All collected data was de-identified.

Data was collected on patient demographics, the clinical setting in which zFOLFIRI was used, i.e. capturing prior lines of therapy, mutation status of the mCRC (KRAS/NRAS/BRAF/MSI), the duration of zFOLFIRI therapy, along with response data (progression free survival (PFS), response rate (RR), time on treatment, stable disease, progressive disease), changes in tumor marker carcinoembryonic antigen (CEA), and toxicities that were encountered in the course of therapy with zFOLFIRI. Serum CEA was measured over time while on treatment with zFOLFIRI. The CEA value measured at each time point was divided by the baseline measurement to calculate a ratio and then plotted on a graph of percent change from baseline over time. Patients were stratified by their progression free survival (less than or greater than 4 months). Disease progression was always determined radiographically at our tertiary care NCI-designated comprehensive cancer center as what would necessitate a change in therapy regimen in the heavily pretreated advanced mCRC population with limited life expectancy.

A secondary goal of our research was to examine available clinical outcomes in a nationwide real-world database. After obtaining Institutional Review Board approval, data were obtained from the Flatiron Health EHR-derived database, a database comprised of patient-level structured and unstructured data, curated via technology-enabled abstraction. These data represent a demographically and geographically diverse patient population derived from the electronic health records of 280 cancer clinics (∼2.1 million patients with cancer in the United States; https://flatiron.com/real-world-evidence/, August 2018, mortality v2.0). Data provided to third parties were de-identified and provisions were in place to prevent re-identification in order to protect patients' confidentiality.

The study population in the Flatiron Health cohort included patients with an ICD code for colorectal cancer (ICD-9 153.x or 154.x or ICD-10 C18x, or C19x, or C20x, or C21x), at least two documented clinical visits on or after January 1, 2013, pathology consistent with CRC (confirmed via abstraction), had evidence of stage IV or recurrent mCRC diagnosed on or after January 1, 2013, who received zFOLFIRI after prior use of (FOLFOX+Bevacizumab or CAPEOX+bevacizumab) and FOLFIRI+bevacizumab (**Figure 1B**).

Data Analysis

Given the pilot nature of this study, our emphasis was on collecting preliminary data, point estimation, and hypothesis generation to support future directions. We used standard descriptive statistics (e.g., means, medians, ranges, proportions, frequencies) to characterize the data collected. Overall survival (OS) and PFS time were characterized using the methods of Kaplan and Meier, and Fox Chase patients were analyzed separately from patients in the Flatiron Health cohort (12). OS time was defined as the number of days from first treatment with zFOLFIRI to death. PFS time was defined as the period from first treatment with zFOLFIRI to disease progression (determined by radiographic assessment in the FCCC cohort) or death, whichever occurred first. Patients who were alive, or alive and progression free at last contact were censored for OS and PFS analyses, respectively. Best response and treatment-related toxicities were characterized using frequency tables. A spider plot displaying the percent change in CEA from first measurement for each patient on the logarithmic scale was generated. All statistical analyses and plots were generated using the R statistical computing platform (https://www.r-project.org/).

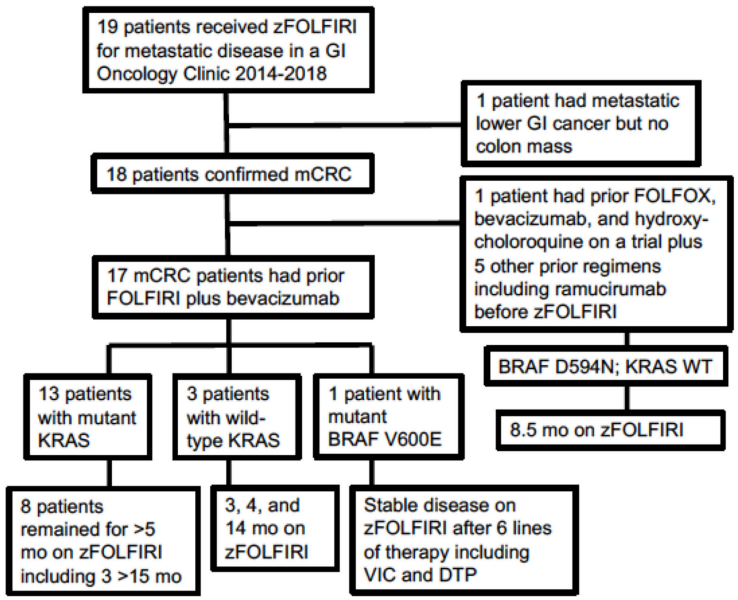
To assess the relationship between rapid vs. prolonged progression free interval with changes in longitudinal CEA measures we first estimated the rate of change in CEA per day by fitting a separate linear regression for each patient using the data from the first 4 months on zFOLFIRI treatment. To adjust for substantial differences in baseline CEA values across patients, we first scale normalized the longitudinal measures for each patient by dividing by their CEA measurement closest to and prior to treatment initiation with zFOLFIRI. We used a Wilcoxon rank sum test (two-sided, $\alpha = 0.05$) to compare slope estimates between early progressors and others.

RESULTS

Patient Demographics, Tumor Characteristics and Prior Therapies

The median age at diagnosis of the cohort that received zFOLFIRI was 51 years of age ($N = 19$). This includes 12 females and 7 male patients. Of the 18 patients with mCRC, 8 patients had rectal cancer, 10 had colon cancer (9 with sigmoid or rectosigmoid)

A



B

Criteria	Flatiron Health Patients
Diagnosed with CRC cancer (ICD-9 153.x or 154.x or ICD-10 C18x, or C19x, or C20x, or C21x); At least two documented clinical visits on or after January 1, 2013; Pathology consistent with CRC; Has evidence of stage IV or recurrent metastatic CRC diagnosed on or after January 1, 2013	18877
Has had at least one line of therapy	14830
Less than a 90-day gap from metastatic diagnosis to first structured activity*	13458
Three or more lines of therapy	3031
Any zFOLFIRI use	215
Prior to zFOLIRI line of therapy, use of (FOLFOX+Bevacizumab or CAPEOX+bevacizumab) and (FOLFIRI+bevacizumab)	29
Prior to zFOLIRI line of therapy, use of (FOLFOX+Bevacizumab) and (FOLFIRI+bevacizumab)	26

*structured activity is defined as any evidence of a physical visit to site of treatment (e.g. vital signs collection, chemotherapy administration, or any other hard data entered into an EHR). Patients were excluded if they had more than a 90 day gap between metastatic diagnosis and a structured activity so as to minimize the possibility of being treated at another site first.

FIGURE 1 | (A) Breakdown of the patient cohort treated with zFOLFIRI at Fox Chase Cancer Center leading to the 17 mCRC patients who had prior bevacizumab plus FOLFIRI. Details of tumor mutational status, some prior therapies, and disease control while on zFOLFIRI is shown. **(B)** Cohort selection of mCRC zFOLFIRI from patients in the Flatiron Health network. Out of the entire cohort of 18,877 patients diagnosed with CRC cancer, filters were applied to define a Flatiron Health cohort that most closely matched the FCCC cohort. Patients were filtered by increasingly strict criteria and excluded if they did not meet the criteria as defined in each row.

(**Table 1**). We also report the results of one patient who had a metastatic lower GI cancer without a colonic primary (Patient #17) who was treated with zFOLFIRI in the refractory setting. We included her in the descriptive analysis, but excluded her from the survival analysis. Of the 19 patients treated with zFOLFIRI, 8 had prior bevacizumab plus FOLFOX and 18 had prior bevacizumab plus FOLFIRI prior to receiving zFOLFIRI (**Figure 1A**). The median age at the time of zFOLFIRI treatment of the 18 mCRC patients ($N = 18$) was 54. The median number of systemic regimens prior to zFOLFIRI was 4 (range: 1–6). The full de-identified source patient data is provided as a **Supplementary Table 1**.

For the 17 mCRC patients who received zFOLFIRI after prior bevacizumab plus FOLFIRI, 13 had mutant tumor KRAS, 3 had

TABLE 1 | Patient characteristics at baseline.

	Fox chase cancer center zFOLFIRI cohort ($n = 19$)	Flatiron health zFOLFIRI cohort ($n = 26$)	CORRECT study ($n = 505$)	RECOURSE study ($n = 534$)
Age at time of diagnosis (years), Median (Range)	51 (32–68)	61 (27–81)		
Age at start of zFOLFIRI (years), Median (Range)	54 (40–74)	62 (42–83)	61	63
RACE				
Caucasian	15 (79%)	18 (69%)	392 (78%)	306 (57%)
African american	1 (5%)	2 (8%)	6 (1%)	4 (<1%)
Asian	1 (5%)	1 (4%)	76 (15%)	184 (34%)
Other/Not specified	2 (11%)	5 (19%)	31 (6%)	
SEX				
Male	7 (37%)	16 (62%)	311 (62%)	326 (61%)
Female	12 (63%)	10 (39%)	194 (38%)	209 (39%)
PRIMARY SITE OF DISEASE				
Colon	10 (53%)	22 (85%)	323 (64%)	338 (63%)
Rectum	8 (42%)	4 (15%)	151 (30%)	196 (37%)
Unknown	1 (5%)			
MUTATIONAL STATUS$^{\Sigma}$				
KRAS mutated	13 (68%)	19 (73%)	205 (41%)	262 (59%)
KRAS wild type$^{\#}$	3 (16%)	3 (12%)	273 (54%)	272 (51%)
BRAF mutated	1 (5%)	1 (4%)	14 (4%)	
PIK3CA mutated*	2 (11%)			
TP53 mutated*	8 (42%)			

$^{\Sigma}$*Patient with cancer of unknown primary excluded from mutational status.*

**6 patients with missing data.*

$^{\#}$*4 patients in the Flatiron-Health Cohort with missing KRAS status.*

WT KRAS, and one had BRAF V600E. For the single patient from the cohort of 18 mCRC patients who did not receive prior bevacizumab plus FOLFIRI, two VEGF-directed regimens were administered prior to zFOLFIRI, including FOLFOX plus bevacizumab as well as hydroxychloroquine as part of a clinical trial and irinotecan plus cetuximab plus ramucirumab as part of a separate clinical trial. 8 patients underwent a metastatectomy as part of their prior treatment (**Table 2**).

Overall, the average number of 2-week cycles of zFOLFIRI administered was 8.9 (range: 1–27 with 3 unknown precisely due to treatment primarily outside of our center and early disease progression). In patients treated at Fox Chase Cancer Center with mCRC, the median PFS while on zFOLFIRI was 7.1 months (214 days) and the OS from time of first treatment with zFOLFIRI was 13.8 months (416 days) (**Figures 2A,2B**).

The biochemical response to treatment with zFOLFIRI is shown in the **Figure 3A**. In patients who responded, CEA trends also tended to stabilize over time, correlating with stable disease in this cohort. Among the patients included in **Figure 3A**, the rate of change in normalized CEA measures over the first 4 months on zFOLFIRI treatment was significantly different ($p = 0.004$) between rapid progressors and other mCRC cases with PFS > 4 months (**Figure 3B**). All 4 early progressors had positive slopes as compared to only 1 of the mCRC patients with prolonged progression free survival intervals (**Figure 3B**). Some patients exhibited a durable response, with 3 patients who remained on therapy for >15 months (**Figure 4A**).

In the Flatiron Health cohort, 26 patients were identified as having received zFOLFIRI after previous lines of FOLFOX+bevacizumab and FOLFIRI+ bevacizumab (**Table 1**). The median age at time of treatment with zFOLFIRI in this cohort was 62 with an ECOG performance status of 0-2, and the majority of these patients had tumors that were KRAS-mutated (19/26). The median time-on-treatment for these patients was 4.4 months (132.5 days), and the median OS for these patients was 7.8 months. The longest duration of treatment with zFOLFIRI in this cohort was 266 days (**Figure 4B**). When combined with the FCCC cohort, the median time on treatment remained the same at 132.5 days.

Description of Adverse Events in 18 mCRC Patient Cohort Treated With zFOLFIRI

zFOLFIRI was well tolerated although 8/18 patients (44%) experienced a grade 3 or higher toxicity, including GI-related events (ileus, small bowel obstruction, or fistula formation) in 16% of patients as well as neutropenic fever in 22% of patients (**Table 3**). 15/18 patients (83%) experienced a toxicity of any grade. Of note, the rate of neutropenic fever and GI events was higher in our cohort vs. the original VELOUR study (fistula formation was 1.1 vs. 5% in our cohort and febrile neutropenia was 6.5 vs. 22%) however our patient cohort was small and a more heavily pretreated.

TABLE 2 | Therapies given to mCRC patients prior to zFOLFIRI.

	Fox chase cancer center zFOLFIRI cohort ($n = 19$)	Flatiron health zFOLFIRI cohort ($n = 26$)	CORRECT study ($n = 505$)	RECOURSE study ($n = 534$)
Lines of therapy in metastatic setting prior to zFOLFIRI (number of lines), Median (Range)	4 (1–6)	3 (2–4)		
Number of previous anticancer therapies (on or after diagnosis of metastatic disease)				
1–2	8 (44%)	15 (58%)	135 (37%)	95 (18%)
3	4 (22%)	8 (31%)	125 (25%)	119 (22%)
≥4	6 (33%)	2 (8%)	245 (49%)	320 (60%)
No. patients who received FOLFOX plus bevacizumab	8 (44%)	26 (100%)*		
No. patients who received FOLFIRI plus bevacizumab	17 (94%)	26 (100%)*		
No. of patients who received cetuximab	5 (28%)	3 (12%)		
No. of patients who received trifluridine-tipiracil	3 (17%)	4 (15%)		
No. of patients who received regorafenib	1 (6%)	5 (19%)		
No. of patients treated with metatastectomy	8 (44%)			

**These therapies were in the inclusion criteria for this cohort.*

Description of Specific Cases of Interest Treated With zFOLFIRI Within the 18-Patient mCRC Cohort

Patient 4; Mutated KRAS, Peritoneal Spread, Prior FOLFIRI Plus Bevacizumab, zFOLFIRI PFS > 1.5 year

Patient is a 43-year-old woman with KRAS mutated (G12V) metastatic mucinous adenocarcinoma who was first diagnosed at age 36 with widespread peritoneal disease. She subsequently underwent a debulking surgery followed by 4 cycles of FOLFOX-based chemotherapy, and then further debulking and hyperthermic chemotherapy (HIPEC) instillation. She completed a further 8 cycles of palliative FOLFOX with disease control for approximately 9 months. When her disease subsequently progressed, she was treated with FOLFIRI plus bevacizumab but suffered severe diarrhea requiring cessation of 5-FU at the time. After suffering a pulmonary embolism, irinotecan plus bevacizumab were stopped and the patient was treated with another round of cytoreductive surgery plus mitomycin-C based HIPEC.

Her disease remained stable for a subsequent 2 years until progression was noted on a PET-CT scan at which point she was started on zFOLFIRI. The patient stayed on therapy despite requiring a dose reduction for approximately 9 months after which time her scan showed stable disease. The decision was then made to treat her dominant pelvic mass with palliative XRT and she subsequently went onto maintenance capecitabine with the addition of bevacizumab for 17 months. Upon disease progression at that time, zFOLFIRI was restarted with stable disease for an additional 8 months with a continued response at the time of data censorship. The CEA trends observed in this patient showed reduction during both zFOLFIRI treatment periods (**Supplementary Figure 1**).

Patient 5; KRAS G12C Mutant, Prior FOLFIRI Plus Bevacizumab, zFOLFIRI PFS ∼1 Year

A 56-year-old man was diagnosed with KRAS G12C mutated stage IIIB rectosigmoid adenocarcinoma. Post-resection he was treated with adjuvant FOLFOX. 1.5 years later his disease recurred in the lungs and after a metastatectomy he was treated palliatively with FOLFIRI and bevacizumab for 11 months. At the point of disease progression, he was started on zFOLFIRI which he took for about 2 months. However, due to personal issues, the patient was lost to follow-up for roughly 3 months.

When he again presented to our clinic to reinitiate therapy, restaging scans taken at that time showed stable disease and he resumed treatment without issue. He remained on therapy for an additional 10 months after which he was lost to follow-up. His most recent restaging scans prior to him leaving our clinic showed ongoing disease stability.

Patient 8; BRAF D594N Mutant, Prior Bevacizumab, Ramucirumab and Cetuximab, zFOLFIRI PFS ∼1 year

A 42-year-old man was diagnosed with KRAS wild-type and BRAF D594N mutated stage III rectal carcinoma. He was started on capecitabine, oxaliplatin, and radiation and subsequently underwent an abdominal perineal resection followed by adjuvant capecitabine and oxaliplatin (XELOX) chemotherapy. After 1 year, the patient recurred with disease in the lungs, liver, and lymph nodes. He was started on a phase II trial of FOLFOX, bevacizumab and hydroxychloroquine but stopped after 6 months due to an oxaliplatin reaction and was treated with maintenance 5-FU plus bevacizumab and hydroxychloroquine.

His disease remained stable for approximately 18 months until he developed progressive disease in the lungs and liver, both of which were resected. However, his disease recurred in the liver, lungs, and retroperitoneum 4 months later and he was started on

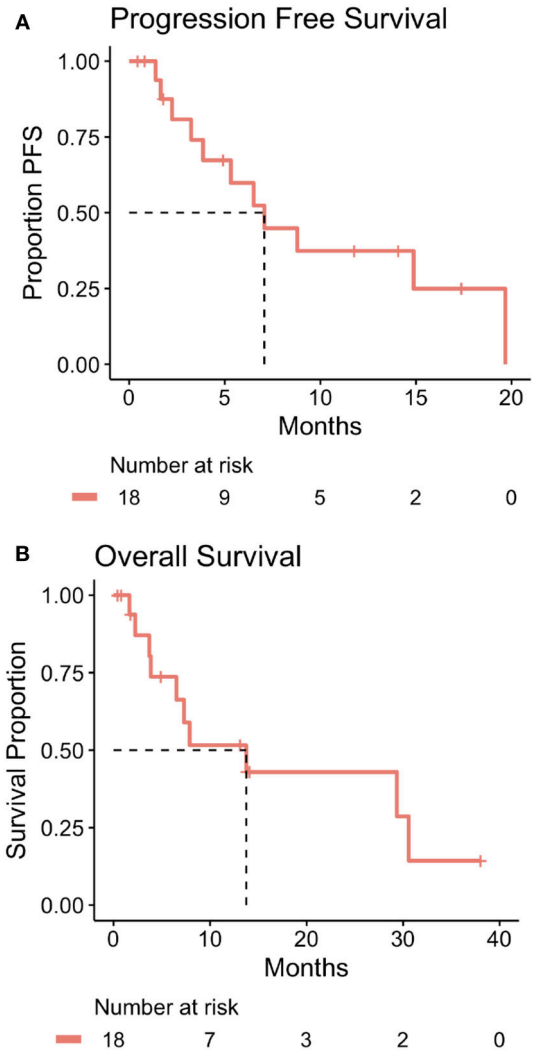


FIGURE 2 | (A) Kaplan-Meier plot of progression free survival time for mCRC patients treated at Fox Chase Cancer Center with zFOLFIRI including table of number of patients at risk over time. The dotted line indicates the estimated median progression free survival time which is 7.1 months (214 days). Patient #17 was treated with zFOLFIRI but was excluded from the PFS, OS, and swimmer plot as they did not have documented mCRC. **(B)** Kaplan-Meier plot of overall survival time for mCRC patients treated at Fox Chase Cancer Center with zFOLFIRI including table of number of patients at risk over time. The dotted line indicates estimated median patient overall survival time which is 13.8 months (416 days). Patient #17 was treated with zFOLFIRI but was excluded from the PFS, OS, and swimmer plot as they did not have documented mCRC.

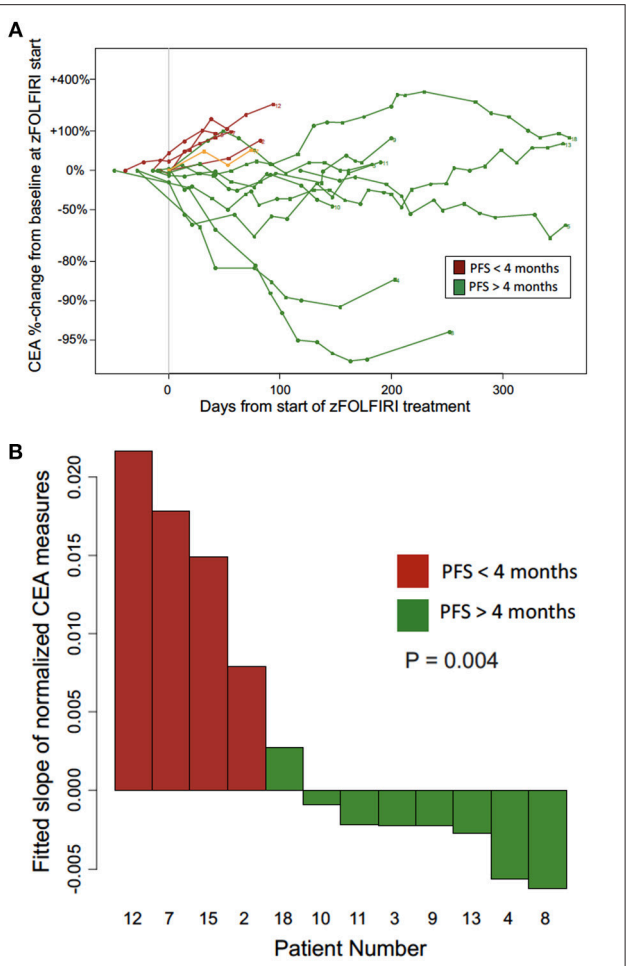


FIGURE 3 | (A) CEA trend over time from the Fox Chase Cancer Center cohort treated with zFOLFIRI. The biochemical response to treatment with zFOLFIRI is shown. Serum levels of carcinoembryonic antigen (CEA) were measured over treatment time, and values are represented as percent change from baseline. Each line represents one patient. Patients were included if they had at least one scan to monitor treatment response after initiating treatment, had CEA values recorded in the electronic medical record, and either progressed or were followed for at least 120 days. Patients represented in red (n = 5) progressed in less than 4 months, and patients represented in green (n = 9) had a progression free survival of 4 months or more. Patient #17 was included in the spider plot (shown in orange) as the patient who had a lower GI malignancy did not have a documented colorectal cancer primary but did experience stable disease. Patient #17 who also had treated brain metastases requested discontinuation of therapy due to an overall poor quality of life and treatment-related toxicities such as nausea and diarrhea. **(B)** CEA trends among patients with early progression (PFS < 4 months) vs. longer disease control (PFS > 4 months) on zFOLFIRI (patients from Fox Chase Cancer Center cohort). The rate of change per day in normalized CEA measures over the first 4 months on zFOLFIRI treatment is shown for progressors (red) and other mCRC cases with PFS > 4 months (green).

cetuximab, irinotecan, and ramucirumab on a separate clinical trial but disease unfortunately progressed after 2 months. He was then treated with trifluridine-tipiracil for roughly 1 year after which his disease progressed and he was started on zFOLFIRI. He had a grade 3 small bowel obstruction but once this resolved was able to stay on zFOLFIRI for 8 months prior to progression of disease. The patient survived for another 13.8 months from the time of initiation of zFOLFIRI.

Patient 10; BRAF v600E Mutated, Prior FOLFIRI Bevacizumab and EGFR Directed Therapy With VIC and DTP, zFOLFIRI PFS Ongoing at > 4 Months

A 44-year-old woman was diagnosed with sigmoid colonic adenocarcinoma with mesenteric adenopathy on CT scan,

hemicolectomy revealed a stage IIIB tumor (T3N1M0) that was moderately differentiated. Post-resection, the patient was treated with adjuvant FOLFOX for 6 months and entered surveillance. Three years later her CEA began to rise with CT scan revealing new bilateral ovarian metastases which were biopsy proven as metastatic colonic adenocarcinoma. Analysis at that time was significant for a tumor BRAF V600E mutation. She was then treated with FOLFIRI for 4 cycles initially with bevacizumab, however due to delayed wound healing bevacizumab was held.

Restaging scans after 4 cycles showed progression of disease and therefore she was treated for 4 months with FOLFIRI and cetuximab, however her disease then continued to grow. Given her BRAF V600E mutation, she was treated with vemurafenib, irinotecan, and cetuximab (VIC) (13) with disease control for 6 months. She was then treated with dabrafenib, trametinib, and panitumumab (DTP) due data showing effectiveness of this combination in these patients (14), however she progressed after 3 months. Given that she had never progressed on bevacizumab, she restarted FOLFIRI and bevacizumab which controlled disease for an additional 3 months. At time of progression, she was consented to zFOLFIRI. Despite grade 1 diarrhea, nausea, and vomiting she has tolerated therapy well and has ongoing stable disease at >4 months into treatment at time of censorship.

Patient 13; KRAS Wild-Type, Prior FOLFIRI Bevacizumab and zFOLFIRI PFS >14 Months

A 68-year-old man was first diagnosed with metastatic KRAS wild-type rectal cancer at the age of 65 and was treated with FOLFOX plus bevacizumab for 5 months and upon progression was treated with FOLFIRI plus cetuximab for a subsequent 8 months. He then underwent a resection of liver metastases and received radiation to the pelvic region for pain control. He then was continued on maintenance 5-fluorouracil plus capecitabine-based chemotherapy for 5 months after which time his cancer progressed and a liquid biopsy at that time revealed a KRAS Q61H mutation which was felt to be acquired from prior therapy with cetuximab. He was therefore consented to FOLFIRI plus bevacizumab. After 5 months of treatment on this regimen his cancer again progressed and he was consented to zFOLFIRI. On the zFOLFIRI regimen, the patient's disease initially regressed in the liver and then remained stable on multiple scans, allowing the patient to remain on treatment for 14 months with an ongoing response at time of data censorship.

Patient 18; KRAS Mutant, Prior FOLFIRI Plus Bevacizumab, zFOLFIRI PFS ∼18 Months

A 52-year-old woman was diagnosed with stage IV KRAS G12C mutated rectosigmoid colonic adenocarcinoma involving the liver and was started on treatment with capecitabine, oxaliplatin, and bevacizumab. She was treated for 6 months with treatment response and subsequently underwent a liver-directed metastatectomy followed by resection of the primary tumor via a low anterior resection. She was then started on FOLFIRI plus bevacizumab after imaging showed progression of disease in the liver on which she was maintained for 4 months. At the time of disease progression, she was started on zFOLFIRI which showed a partial response in the liver. The patient was ultimately able to stay on this treatment for 18 months before imaging showed progression of her liver disease requiring cessation of this line of therapy. The patient ultimately expired 30 months after starting treatment with zFOLFIRI.

DISCUSSION

Our results identify the potential for clinical benefit in two cohorts of advanced mCRC patients (a Fox Chase Cancer Center cohort, and a Flatiron Health EHR-derived cohort who received zFOLFIRI after prior bevacizumab+FOLFOX and bevacizumab+FOLFIRI). The FCCC cohort was a series of advanced mCRC patients ($N = 18$) who received zFOLFIRI in the third line after progression on FOLFIRI plus bevacizumab ($N = 17$) given as second line therapy. This is typically a population of patients with limited options and most of these patients had tumors that were KRAS-mutated, making their therapy options even more limited. The Flatiron Health cohort included 26 patients who received zFOLFIRI after prior bevacizumab+FOLFOX and bevacizumab+FOLFIRI. The latest available NCCN guidelines (Version 2.2018) state that there are no data to suggest activity of zFOLFIRI in a patient who has progressed on FOLFIRI-bevacizumab, and that ziv-aflibercept has only shown activity when given in conjunction with FOLFIRI in FOLFIRI-naïve patients. Our results in the combined population of 45 patients with advanced mCRC suggest potential for significant disease control with zFOLFIRI beyond prior FOLFIRI plus bevacizumab. CEA is often used to track response to treatment in patients with mCRC, with stabilization or a decline in CEA values correlating with response to treatment. In the FCCC cohort of patients treated with zFOLFIRI who had stable disease at the time of their first imaging, the CEA trend stabilized as would be expected. In patients who did not respond, the CEA predictably increased as illustrated in **Figure 3** for this cohort of patients. For the initial cohort of FCCC patients, PFS was extended by 5 months or greater in the majority of patients, and a subgroup of patients (23.5%) who experienced prolonged stable disease of greater than 1 year indicating significant activity of zFOLFIRI in this population.

While our patient cohort was slightly younger with a median age of 54 at time of zFOLFIRI vs. 61 in the VELOUR study, they were more heavily pretreated and had more refractory disease, and so their younger age may be counterbalanced by the advanced nature of their disease (9). For the 13 patients with mutated KRAS, 8 patients remained on zFOLFIRI for >5 months including 3 patients who remained on therapy for >15 months. The median PFS was 7.1 months (214 days) and the median OS was 13.8 months (416 days). The PFS prolongation compares favorably with the 2 month PFS prolongation observed in third line therapy with either regorafenib or trifluridine-tipiracil (10, 11).

To add to the FCCC cohort, we also explored clinical outcomes data from the national Flatiron Health EHR-derived database, a real-world data set which represents a nationwide sample from community and academic practices in the

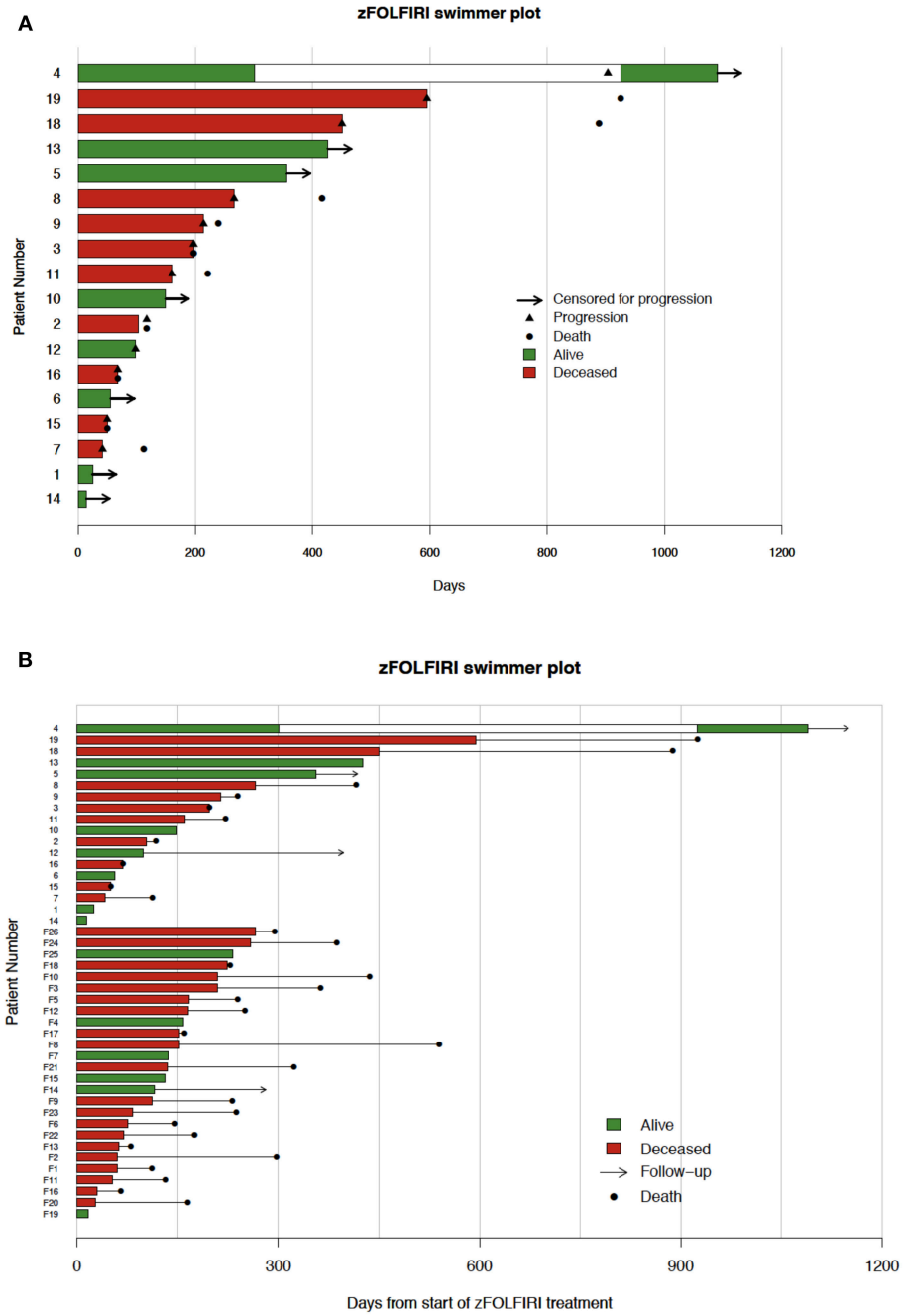


FIGURE 4 | (A) Fox chase cohort swimmer plot. Each row represents one patient. Bar lengths show time on zFOLFIRI with green and red indicating live and deceased patients, respectively. Black triangles indicate the point of progression, and black dots indicate patient deaths. The white region for patient 4 marks a zFOLFIRI treatment holiday. Patient #17 was treated with zFOLFIRI but was excluded from the PFS, OS, and swimmer plot as they did not have documented mCRC. **(B)** Swimmer plot of both fox chase cancer center and flatiron health data. Each row represents one patient. Bar lengths show time on zFOLFIRI with green and red indicating live and deceased patients, respectively. Black dots indicate patient deaths, arrows indicate length of follow-up. The top 18 patients represent patients treated at Fox Chase Cancer Center. Numbers preceded by an F represent patients in the Flatiron Health Database.

United States. From the Flatiron Health database we identified a cohort of 26 additional patients treated with zFOLFIRI after receiving FOLFOX and bevacizumab as well as FOLFIRI and bevacizumab. These patients had a median time on treatment of 4.4 months and an overall survival of 7.8 months. While overall survival of the cohort from the Flatiron Health database was less than in the Fox Chase patient cohort, the time on treatment was still longer than the historic PFS of either regorafenib or

TABLE 3 | Adverse events in all patients treated at fox chase cancer center with zFOLFIRI.

Grade 3 toxicities	No. of patients*	Grade 2 toxicities	No. of patients	Grade 1 toxicities	No. of patients
Neutropenic fever	4	fatigue	1	Hyperbilirubinemia	1
Ileus/Small Bowel Obstruction	2	TIA	1	Diarrhea	1
Fistula formation	1	Nausea/Vomiting	1	Myalgia	1
Syncope	1				

**1 patient experienced more than 1 grade 3 toxicity.*

trifluridine-tipiracil, each of which have a PFS of roughly 2 months. The fact that median age at time of zFOLFIRI treatment in the Flatiron Health cohort was 62, 8 years older than in the Fox Chase Cancer Center cohort, may explain the shortened overall survival although despite this difference in median age, OS was still longer than that reported in other nth line studies. When combined with our cohort, the median time-on-treatment was 132.5 days and did not change significantly. Whether median age at time of treatment impacts PFS or OS was not a goal of our study and would need to be investigated in the future.

Interestingly, 3 additional patients treated with capecitabine, oxaliplatin, and bevacizumab and also treated FOLFIRI with bevacizumab before zFOLFIRI were identified in the Flatiron Health database. One of these patients remained on zFOLFIRI for 20.5 months, indicating that this regimen may be effective, in the patients who experience prolonged disease control, regardless of the previous specific fluoropyrimidine used. Our combined data therefore lends support to our hypothesis that zFOLFIRI has activity in the refractory setting beyond FOLFIRI and bevacizumab and prior oxaliplatin-based combination chemotherapy plus bevacizumab.

Our goal was to test the hypothesis that ziv-aflibercept, which has a broad and potent anti-angiogenic activity, may be active beyond progression on bevacizumab (anti-VEGF) combination therapy in later lines of mCRC therapy and may offer a clinical benefit to patients while maintaining a similar toxicity profile to their previous bevacizumab-containing therapy. The finding of disease control with zFOLFIRI despite prior bevacizumab plus FOLFIRI suggests that ziv-aflibercept at least in combination with FOLFIRI has potential for clinical activity beyond bevacizumab. The results also suggest the clinical activity of ziv-aflibercept in combination with FOLFIRI can be observed beyond prior FOLFIRI including prior FOLFIRI plus bevacizumab.

Vascular endothelial growth factor (VEGF) is a major regulator of tumor angiogenesis (15) and a target for anti-cancer therapy development (16). Previous studies have shown that plasma VEGF levels increase after intravenous administration of bevacizumab (17). However, these are acute changes and the increase in VEGF may be due to impaired clearance (18). The use of VEGF as a predictive biomarker for bevacizumab therapy has remained inconclusive, however VEGF is a potent mediator of tumor angiogenesis and higher levels may be indicative of increased tumor angiogenesis and increased growth (19). Ziv-aflibercept is a more potent inhibitor of VEGF as compared to bevacizumab (20). As such, the question arises as to whether the disease control observed within our cohort with zFOLFIRI may be due to the more potent inhibition of VEGF signaling by ziv-aflibercept. On the other hand, other proangiogenic factors such as VEGF-C, PIGF or VEGF-D may contribute to the emergence of resistance to anti-VEGF therapy with bevacizumab and may in part explain subsequent sensitivity to ziv-aflibercept after progression (21).

In addition to the observed prolonged disease control in the FOLFIRI plus bevacizumab pretreated advanced mCRC population, it is noteworthy that one of our patients had an aggressive BRAF V600E mutated mCRC that achieved disease control with zFOLFIRI after prior sequential VIC (13) and DTP (14) targeted regimens, as well as multiple prior treatments. The setting in which zFOLFIRI was used in this patient represents a currently difficult clinical situation with a subset of mutant BRAF mCRC patients who have a very poor prognosis and extremely limited treatment options especially beyond the targeted therapy combinations.

We believe a strength of our report is that the population of mCRC patients from our Fox Chase Cancer Center case series that we treated is typical of the population of advanced mCRC patients treated at a tertiary care NCI-designated comprehensive cancer center. We note that the population we treated with zFOLFIRI at Fox Chase Cancer Center and the Flatiron Health database cohort with advanced mCRC treated with zFOLFIRI were both enriched for patients with aggressive refractory KRAS-mutant mCRC which currently presents a major challenge in clinical practice. The common theme in our Fox Chase Cancer Center cohort is that all 19 patients were heavily pretreated with prior chemotherapy regimens, 18 of the 19 patients had mCRC, and 17 of the 18 mCRC patients had prior FOLFIRI plus bevacizumab, a setting not predicted to respond to zFOLFIRI and not currently recommended for such treatment in the 3rd line setting. The Flatiron Health database-derived cohort with the observed outcomes further supports this notion. While future larger cohort studies and randomized trials can investigate in more detail the impact of genetic subtypes of mCRC, what is clear from our case series is that patients with various genetic subtypes were found to have disease control with zFOLFIRI despite prior therapies, and some in each subgroup had prolonged disease control. It will be important in the future to further investigate the basis for more durable disease control, and characterize the influence of underlying tumor biology and clinical natural history.

A number of mechanisms of resistance to antiangiogenic therapy have been recognized (22). These include growth factor

redundancy, effects of bone marrow-derived cells, stromal cells, vasculogenic mimicry, and a number of emerging molecular mechanisms or factors such as glycosylation, extracellular vesicles, or polymorphisms (22). A number of biomarkers have been identified and associated with prolonged OS on zFOLFIRI (23). Yoshino et al. found that the baseline levels of 8 biomarkers (TIMP-1, IL-8, EN-RAGE, SP-D, TN-C, IGFBP-1, Kallikrein 5, TNFR2) correlated with OS. This list is only partially overlapping with a prior study that identified the baseline levels of six markers (CRP, Gro-alpha, IGFBP-1, TF, ICAM-1 and TSP-2) correlated with PFS and OS after bevacizumab and everolimus (24). A prior study noted that Ras mutated as well as BRAF mutated mCRC can respond to zFOLFIRI (25) while another study identified SNPs in VEGFB as correlated with PFS and high IL8 with worse PFS (26). miR-21 has been found to be increased after bevacizumab therapy suggesting more complex interactions in signaling that may inform biomarker choice for efficacy or resistance to anti-angiogenic therapy (27).

zFOLFIRI was well tolerated in our patient cohort. ∼83% of patients treated with zFOLFIRI in the original VELOUR study experienced a grade 3 or 4 adverse event, including VEGF-specific events [i.e., arterial (1.8%) or venous (7.9%) thromboembolism, hemorrhage (2.9%), or hypertension (19%)] as well as chemotherapy related events, such as diarrhea (19%), thrombocytopenia (3.3%) and febrile neutropenia (5.7%) (9). In our patients 44% experienced a grade 3 adverse event, including GI-related events and neutropenic fever. While rates of some of these events were somewhat higher in our cohort vs. the original VELOUR study (febrile neutropenia: 6.5 vs. 22%, GI fistula formation:1.1 vs. 5% in our cohort), our patient cohort was small making absolute percentages difficult to interpret. They were also more heavily pretreated and thus may have been more prone to adverse events. Of note, these adverse events are in line with the original studies of bevacizumab in combination with chemotherapy. In the original trial by Hurwitz, et al. establishing bevacizumab as an active agent in colorectal cancer, 84.9% of patients experienced a $\geq$ grade 3 adverse event, including thrombotic events (19.4%), hemorrhage (3.1%), and hypertension (11%), similar to the rates in the VELOUR study and greater than rates of some of these toxicities seen in our cohort (2). Similarly, the RAISE trial of FOLFIRI-ramucirumab after treatment with oxaliplatin-based chemotherapy yielded a 79% rate of grade 3 or higher adverse events (7). Rates of febrile neutropenia among these trials are overall quite low, with 3% in the RAISE study and 2.9% in the VELOUR trial. Given the increased potency of ziv-aflibercept vs. bevacizumab or ramucirumab, efficacy does not seem to be compromised by increased toxicity (8).

Similarly, the rate of grade 3 adverse events in our cohort is in line with studies of other non-VEGF-based therapies conducted in patients with refractory mCRC. For example, in the RECOURSE study which led to the approval of trifluridine-tipiracil in this setting, 69% of patients experienced a grade 3 or higher adverse event (10). In the CORRECT trial of regorafenib vs. placebo for patients with refractory mCRC, 54% of patients had a $\geq$ grade 3 toxicity (10). Therefore, the rate of adverse events seen in our patient cohort is not unexpected or prohibitive.

Our results support the additional exploration of clinical activity of zFOLFIRI in later lines of therapy, in additional retrospective and exploratory prospective cohorts. Efficacy beyond FOLFIRI plus bevacizumab in addition to oxaliplatin-based combination chemotherapy plus bevacizumab is the mCRC population of interest. Patients with mutant KRAS are of interest and predicted to have potential for benefit based on our observations, although we also saw prolonged time to disease progression in other genetic subtypes such mutant BRAF, or wild-type KRAS and BRAF. In the absence of a clinical trial, our results support the feasibility and provide some indication of expected outcomes for zFOLFIRI in patients with advanced mCRC including those who have received bevacizumab plus FOLFIRI in the second line or later therapy settings in addition to oxaliplatin-based combination chemotherapy plus bevacizumab. Future studies will need to incorporate appropriate biomarkers and consider disease control as a clinically meaningful endpoint for this patient population.

It is important to note that the zFOLFIRI regimen beyond FOLFIRI plus bevacizumab was associated with disease control for over 1 year in several patients. We recognize that the PFS of 7.1 months with zFOLFIRI in the 3rd line beyond FOLFIRI plus bevacizumab is not significantly different from the PFS of 6.9 months with zFOLFIRI in the second line observed in the VELOUR study. We are certainly reassured that it is very similar despite the fact that 17 of the 18 mCRC patients treated with zFOLFIRI had prior FOLFIRI plus bevacizumab. The lesser PFS prolongation with zFOLFIRI observed in the Flatiron Health database-derived cohort is a real-world experience from a second older patient cohort and still supports our overall conclusions regarding potential benefit for zFOLFIRI beyond both FOLFOX plus bevacizumab and FOLFIRI plus bevacizumab. Both the Fox Chase Cancer Center and the Flatiron Health database-derived cohort outcomes with zFOLFIRI suggest benefit from zFOLFIRI in patients who were previously treated with bevacizumab plus FOLFIRI in addition to other regimens, and those outcomes were better than what has been observed with current standard of care such as regorafenib or trifluridine-tipiracil. It is noteworthy that both our Fox Chase Cancer Center cohort and the Flatiron Health database-derived cohort were enriched for patients with KRAS-mutant mCRC, but that within our Fox Chase Cancer Center cohort, disease control was also observed with wild-type KRAS/NRAS or mutant BRAF. Future studies can focus more on the impact of driver mutations such as KRAS/NRAS or BRAF on disease control with zFOLFIRI.

In summary, we observed meaningful stable disease on zFOLFIRI beyond progression on bevacizumab plus FOLFIRI in a total of 44 mCRC patients from two real-world cohorts (Fox Chase Cancer Center as well as the Flatiron Health database) suggesting that additional exploration of the clinical benefit of this therapeutic approach is warranted. An appropriate next step could be a comparative effectiveness study of zFOLFIRI vs. other third-line treatments in real-world retrospective cohorts or perhaps a prospective pragmatic clinical trial.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00142/full#supplementary-material

Supplementary Figure 1 | CEA trends in Fox Chase Cancer Center patient #4 are shown during the time the patient was treated with zFOLFIRI. The grey region represents time off of zFOLFIRI when the patient was treated with maintenance capecitabine.

Supplementary Table 1 | De-identified patient data for 19 patients treated at Fox Chase Cancer Center with zFOLFIRI.

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CASE REPORT
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Circulating Tumor DNA Detection in the Management of Anti-EGFR Therapy for Advanced Colorectal Cancer

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Background: Anti-EGFR antibodies are a standard care for advanced *KRAS*-wild type colorectal cancers. Circulating tumor DNA (ctDNA) monitoring during therapy can detect emergence of *KRAS* mutant clones and early resistance to therapy.

Case Presentation: We describe a 61-years-old man presenting a metastatic and recurrent rectal cancer treated with different chemotherapy regimens. His tumor was *KRAS* wild-type based on tissue analysis and he was treated sequentially with cetuximab-based chemotherapy, chemotherapy alone and panitumumab-based chemotherapy. We performed sequential analysis of ctDNA using droplet digital PCR (ddPCR) and a commercial assay designed for the detection of frequent *KRAS* mutations during his clinical follow-up. Prior to the first cetuximab-based chemotherapy ctDNA analysis demonstrated an absence of *KRAS* mutations. Emergence of *KRAS* mutations in ctDNA occurred ∼3 months after treatment initiation and preceded clinical and imaging progression in about 2 months. Fractional abundance of *KRAS* mutation rapidly increased to 70.7% immediately before a chemotherapy alone regimen was initiated. Interestingly, *KRAS* mutation abundance decreased significantly during the first two months of chemotherapy, reaching a fractional abundance of 3.0%, despite minimal clinical benefit with this therapy. Re-challenge with a different anti-EGFR antibody was attempted as later line, but high levels of *KRAS* mutations in ctDNA before therapy correlated with an absence of clinical benefit.

Conclusions: The monitoring of resistance mutations in *KRAS* using ctDNA during the treatment of *KRAS* wild-type advanced colorectal cancers can detect the emergence of resistant clones prior to clinical progression. Dynamics of resistant clones may alter during periods on and off anti-EGFR antibodies, detecting window of opportunities for a re-challenge with these therapies.

Keywords: liquid biopsies, anti-EGFR therapy, colorectal cancer, drug resistance, monitoring, circulating tumor DNA

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BACKGROUND

Over the past years, substantial advances in cancer genomics established a new era in which information on the genetic background of tumors moved from laboratories to the clinic. For many solid tumors, certain genetic alterations proved to be prognostic, predicting treatment sensitivity, and resistance, especially to targeted therapies. Much of the recent data about somatic genetic alterations were generated based on tissue analysis, obtained in a fixed time point during tumor evolution. Nonetheless, it is well-known that solid tumors change over time and space as a result of clonal evolution, leading to significant intra-tumor genetic heterogeneity (1). In this setting, liquid biopsies are gaining relevance as a tool to capture genetic tumor evolution and intra-tumor genetic heterogeneity more precisely.

Broadly, liquid biopsies consist of diagnostic methods based on the detection of circulating tumor material such as cells, nucleic acids, proteins, and extracellular vesicles in a minimally invasive manner through the sampling of blood or other body fluids. Nevertheless, the detection of circulating tumor DNA (ctDNA) in the plasma is the most clinically useful modality of liquid biopsies due to its high specificity and sensitivity, relative low cost, and straightforward analysis (2). Using ctDNA to characterize genetic alterations is appealing, due to its minimally invasive nature, possibility to represent a background from multiple tumor sites and facility to repeat tests during treatment (3). There are a number of applications for liquid biopsy, but the only clinically approved uses are to monitor treatment response and detect the emergence of drug resistance in a few tumor types (3).

Monoclonal antibodies that specifically target Epidermal Growth Factor Receptor (EGFR) are frequently used as monotherapies or in combination with chemotherapy to treat advanced colorectal cancer. Their benefit in increasing response rate and prolong survival is restricted to patients with *KRAS* wild-type tumors (4, 5). Both drugs currently approved for this setting, namely Cetuximab and Panitumumab, require tissue testing for *KRAS* alterations. More recently, sidedness of the tumor was also implied as a potential predictive factor, as left-side tumors seem to derive a pronounced benefit with anti-EGFR therapies (6).

Despite its efficacy, some patients will not respond to Cetuximab or Panitumumab and the majority of responders will develop resistance at some point during treatment. Since these drugs are commonly used in combination with chemotherapy, it is difficult to discern if disease progression implies resistance to both anti-EGFR and chemotherapy or just one of these agents. Some authors suggested that resistance can occur only with the chemotherapy component and, in this scenario, maintaining the EGFR blockade while changing the chemotherapy backbone could be a strategy (7). On the other hand, it has been demonstrated that, in patients developing resistance to anti-EGFR therapies, a period free of therapy targeting this receptor could re-sensitize tumors by reducing the clonal selection pressure. In this setting, re-challenge with an anti-EGFR therapy would be able to produce further tumor regression (8).

Resistance to anti-EGFR antibodies is mainly driven by the emergence of mutations in certain genes during treatment, especially in *KRAS, NRAS, BRAF,* and *EGFR* (9). Recent studies suggested that some of these alterations may be multi-clonal, and, thus, associated with intra and/or inter-lesions heterogeneity (10). In this context, longitudinally monitoring the landscape of genetic alterations during treatment with anti-EGFR antibodies could help to detect the emergence and dynamics of the mutations associated with resistance and guide the decision-making process when choosing between anti-EGFR therapy continuation vs. re-challenge (11).

Here, we describe a case of a patient with advanced *KRAS* wild-type colorectal cancer treated with anti-EGFR therapy in combination with chemotherapy that was monitored with sequential analysis of ctDNA using droplet digital PCR (ddPCR) and a commercial assay designed for the detection of frequent *KRAS* mutations (*KRAS* G12/G13 Screening Kit—BioRad). We evaluated clinical response during sequential systemic therapies including two different anti-EGFR antibodies, along with dynamics of *KRAS* status in ctDNA.

CASE PRESENTATION

KLM, a North American white man, was 61 years old in August 2010 when he was diagnosed with a distal rectal cancer, clinically staged as T3N1M0. His initial therapeutic approach included neoadjuvant radio/chemotherapy followed by close surveillance, since digital rectal examination, proctoscopy and pelvic MRI, at the end of treatment, were normal.

In September 2011, an increase in serum levels of Carcinoembryonic Antigen (CEA) was noted. A local relapse and a 3 cm lesion in liver segment VIII were simultaneously diagnosed. Some suspicious, but undetermined, small lung nodules were also observed at that time. Patient was initially submitted to a full-thickness transanal excision and then to neoadjuvant (perioperative) chemotherapy with FOLFOX followed by hepatectomy and adjuvant FOLFOX. Intensive proctologic follow up was still maintained. Molecular analyses of the tumor obtained from liver metastasis showed *KRAS* and *BRAF* wild-type status.

On December 2013, lung metastases became clear and first line chemotherapy with FOLFIRI/bevacizumab was initiated. Patient was treated with this regimen until June 2015, when new hepatic lesions were detected and chemotherapy changed to irinotecan with cetuximab (CPT11/CTX). At that time, the patient agreed by written consent to have his blood periodically collected for molecular testing. He was informed that results of these tests would be, however, kept unrevealed until at least the end of treatment with anti-EGFR.

Blood samples were collected periodically from June 2015 until April 2017 and the emergence and dynamics of KRAS mutations in ctDNA was monitored using ddPCR as previously described (12). Briefly, 15 ml of blood were collected using

Abbreviations: CEA, carcinoembryonic antigen; ctDNA, circulating tumor DNA; cfDNA, cell free DNA; ddPCR, digital droplet PCR; MRI, magnetic resonance imaging; RECIST, response evaluation criteria in solid tumors; CT, computed tomography; mCRC, metastatic colorectal cancer.

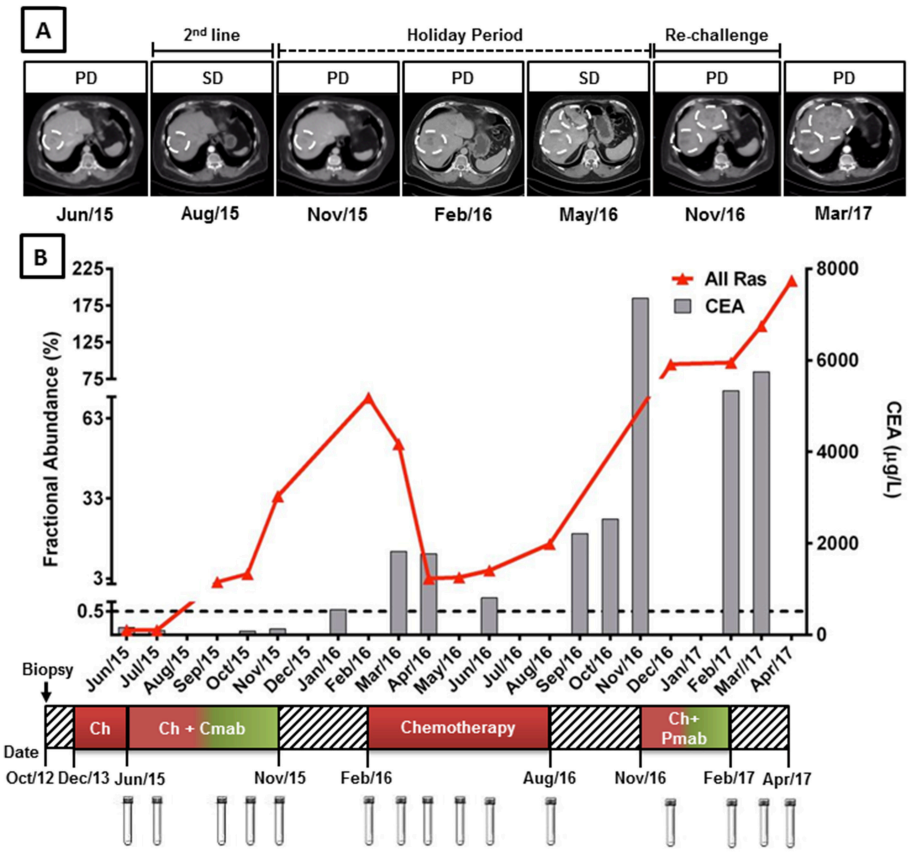

FIGURE 1 | *KRAS* G12/13 longitudinal monitoring and clinical response to anti-EGFR mAb plus chemotherapy in cell-free circulating DNA of a mCRC patient. **(A)** Computed tomography (CT) images of liver metastasis during anti-EGFR mAb treatment, holyday and mAb re-challenge. **(B)** Red line indicates the fractional abundance of *KRAS* mutation (percentage of mutated alleles in a total DNA background) detected in ctDNA at treatment course (X-axis) by ddPCR. Approximately 3,000 haploid genome equivalents were analyzed for each time point (*KRAS* limit of detection 0.5%). Gray columns represent CEA levels. *KRAS* mutation detection was able to anticipate clinical progression, preceding CEA elevation. **(A,B)** *KRAS* levels decreased during anti-EGFR holyday and chemotherapy switch coinciding with stable disease. High levels of *KRAS* mutations after chemotherapy progression were able to predict poor response to anti-EGFR re-challenge. Cmab, cetuximab; Pmab, panitumumab; Chemo, chemotherapy; SD, stable disease; PD, progressive disease.

tubes containing EDTA. Plasma was separated from blood by centrifugation within 2 h after collection and plasma was stored at −80°C. Cell free DNA was isolated using the QIAamp MinElute Virus Vacuum Kit and stored at −80°C. We used a RNase P Copy Number Reference Assay to determine the total amount of DNA in plasma samples and a commercial assay designed for the detection of frequent KRAS mutations (KRAS Screening Kit BioRad – G12V, G12D, G12A, G12C, G12R, G12S e G13D). A total of 3000–3500 genome equivalents were analyzed per reaction for a detection sensitivity between 0.1 and 0.5%. ddPCR was performed on the QX200 Droplet Digital PCR System and data were analyzed using QuantaSoft software. ctDNA detection results are presented as fractional abundance (proportion of the mutant allele in total circulating DNA) for comparison between different time points.

At the beginning of CPT11/CTX treatment, in June 2015, blood samples were negative for the presence of *KRAS* mutations (**Figure 1**). First evaluation of response was performed after 6 cycles in August 2015 and showed stable disease by RECIST, with an expressive decline in serum CEA (from 162 to 80 µg/L). Treatment was maintained until clinical progression was observed in November 2015 at expenses of significant skin and gastrointestinal toxicities. *KRAS* mutations became detectable in September 2015, anticipating clinical disease progression, and raised considerably over the next 2 months reaching a fractional abundance of 33.8% in November 2015 (**Figure 1**).

From November 2015 to February 2016 patient remained in a "drug holiday period," during which no chemotherapy was administered. Rapid CEA elevation and CT scans denoting progression in pulmonary and liver metastasis have induced a new treatment to begin. A significant increase in *KRAS* mutation fractional abundance was also observed during this period (from 33.8 to 70.7% in February 2016) (**Figure 1**). He was then re-challenged with FOLFOX, achieving again an initial clinical benefit (small reduction in tumor sizes and CEA response) followed by progression of disease on August 2016. *KRAS* mutation abundance decreased significantly during the first two months of FOLFOX treatment, reaching a fractional abundance

of 3.0% in April 2016. However, *KRAS* mutation abundance started to increase steadily thereafter, anticipating, once more, clinical disease progression (**Figure 1**).

A fourth line of palliative chemotherapy, combining irinotecan with panitumumab was also tried from November 2016 to February 2017 without success. High levels of *KRAS* mutations in ctDNA were detected in December 2016, remaining relatively stable until February 2017 and anticipating poor response to palliative treatment and disease progression. The best supportive care was offered up to patient's death in June 2017. A significant increase in *KRAS* mutation abundance in ctDNA was observed after the interruption of palliative treatment (**Figure 1**).

DISCUSSION

Monoclonal antibodies anti-EGFR in combination with chemotherapy is one of the standard treatments for RAS wild-type metastatic colorectal cancer. However, many RAS wild type patients do not respond to anti-EGFR therapies and even those who initially respond to therapy will ultimately progress, at least in part, because of the emergence of *KRAS* mutations.

Retrospective studies including a small number of patients with mCRC patients treated with anti-EGFR therapy have shown that ctDNA analysis in plasma samples can detect acquired mutations in *KRAS* leading to therapy resistance (8, 13, 14). In this scenario, longitudinal monitoring of *KRAS* status using liquid biopsies during anti-EGFR therapy may allow the early detection of acquired resistance and guide clinical decision to switch to a subsequent line of therapy, increasing the likelihood of the patient to derive maximal benefit from sequential therapy. Longitudinal monitoring of *KRAS* status may be particularly useful to guide the decision-making progress when choosing between anti-EGFR therapy continuation vs. re-challenge (7).

Although the use of liquid biopsies to monitor cancer patients is a technically feasible and affordable procedure, our current knowledge of ctDNA detection in cancer patients must be expanded before liquid biopsies can be routinely implemented into clinical practice (15). In this case study, liquid biopsies were used to monitor the emergence and dynamics of *KRAS* mutations in a patient with advanced *KRAS* wild-type colorectal cancer treated with anti-EGFR therapy in combination with chemotherapy. It is important to highlight that ctDNA analysis was performed retrospectively and that ctDNA detection results did not influence the therapeutic decisions for this patient.

The clinical decision to select an anti-EGFR as part of the therapy is driven by a *KRAS* wild-type mutational testing in the tissue and a variety of clinical factors. In this case study, Cetuximab was associated to CPT11 as a second line therapy. Comparative studies showed no overall survival difference between anti-EGFR and bevacizumab-based regimens (which was the choice for initial palliative therapy for this patient) as a first line therapy for *KRAS* wild-type colorectal cancers (16, 17). For this patient, *KRAS* testing was performed in a liver metastasis prior to initiation of first-line therapy. In accordance with tissue genotyping, our ctDNA analysis before initiation of the anti-EGFR therapy demonstrated an absence of *KRAS* mutations. Prior studies established over 90% agreement between plasma and tissue *KRAS* status, (18, 19) which reinforces that, in fact, this patient was wild-type by current clinical guidelines. Interestingly, higher sensitive techniques for *KRAS* plasma detection may identify a higher number of patients with mutations that were actually negative by tissue testing (20). It is unknown, however, if such a finding may alter initial clinical management of patients, as survival for patients receiving first line Cetuximab-based therapy is similar compared to patients submitted to tissue and plasma biopsies (21).

Our results corroborate previous studies that have shown that ctDNA detection can be used to track the emergence of tumor resistant subclones during anti-EGFR therapy, allowing early detection of drug resistance and disease progression. Marked increases in *KRAS* mutation abundance was detected in blood samples from our patient 2 months before clinical progression of the disease after the first exposure to anti-EGFR therapy. Others authors reported that the allelic frequency of mutations in plasma from CRC patients (including *KRAS*) may be an indicator of response or resistance to systemic therapy (8, 13, 14). Thus, ctDNA can be helpful for monitoring response, also considering that up to 30% of patients with CRC do not show alterations in CEA blood levels (22). Our case also illustrates that elevation in the allelic frequency of *KRAS* mutations also preceded CEA elevation.

Finally, our results also suggest that ctDNA analysis can be efficiently used to monitor the dynamics of *KRAS* mutated resistant clones during systemic treatment of mCRC and to identify patients eligible for anti-EGFR therapy continuation or re-challenge. Interestingly, marked decreases in *KRAS* mutation abundance were not observed in our patient immediately after anti-EGFR was withdrawn (November 2015 and February 2017), but were detected readily after the chemotherapy switch. Recent data demonstrated that *KRAS* mutant clones might decline after stopping an anti-EGFR therapy, similarly to *KRAS* allelic fraction in plasma (8, 23). This finding suggests that the RAS-resistant phenotype may be reversible, leading to new opportunities to use anti-EGFR therapies. On the other hand, high levels of *KRAS* mutations in ctDNA were able to predict poor response to anti-EGFR re-challenge, as illustrated in our case report. It is plausible to hypothesize that re-challenge with an anti-EGFR therapy might be better offered to patients with no resistance mutations on ctDNA.

CONCLUSIONS

In conclusion, our results demonstrate that colorectal tumor genomes adapt dynamically to intermittent anti-EGFR treatment and indicates that liquid biopsy is a promising tool to monitor acquired resistance to anti-EGFR therapy and guide second line treatment strategies.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

ETHICS STATEMENT

Patient has signed an informed consent form to participate in the study. This study was approved under number # 2015–22 by the ethics committee of the Instituto de Ensino e Pesquisa do Hospital Sírio Libanês.

AUTHOR CONTRIBUTIONS

FK and FB carried out the ctDNA analysis. JS was responsible for patient clinical management. FK, FB, AC, and JS conceived and designed the analysis. All authors analyzed the data, wrote and revised the manuscript, and read and approved the final version for submission.

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Detection of *ERBB2* (HER2) Gene Amplification Events in Cell-Free DNA and Response to Anti-HER2 Agents in a Large Asian Cancer Patient Cohort

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Background: HER2 antagonists have marked activity and are approved for the treatment of HER2 overexpressing breast and gastric cancers. Recent studies have shown that *ERBB2* (HER2) gene amplification and overexpression may also be actionable in other tumor types. Inter- and intratumoral heterogeneity in HER2 status, however, poses a significant challenge in identifying patients that may benefit from HER2-targeted therapies. *ERBB2* amplification as identified by circulating cell-free DNA (cfDNA), which circumvents tissue heterogeneity issues, is emerging as a robust biomarker predictive of response to anti-HER2 agents. Here, the prevalence and genomic landscape of *ERBB2* alterations detectable by next-generation sequencing (NGS) of cfDNA was evaluated in a large cohort of Asian patients with advanced solid tumors.

Methods: Results were queried for consecutive patients (n = 469) tested by a comprehensive 70/73-gene cfDNA NGS assay (Guardant360®) between November 2015 and June 2018. Patients with *ERBB2* gene alterations including copy number amplifications (CNAs), single nucleotide variants (SNVs), and insertion-deletions (indels) were identified.

Results: *ERBB2* alterations were detected in 52 patients (11.1%); *ERBB2* SNVs, CNAs, and indels were found in 27 (5.8%), 27 (5.8%), and 10 (2.1%) patients, respectively. *ERBB2* amplification was most frequently identified in gastric (21.4%; 6/28), colorectal (11.1%; 5/45), lung (3.9%; 9/231), and breast (3.2%; 1/31) cancer patients. *ERBB2* amplification was often mutually exclusive with other oncogenic alterations in gastric (83.3%; 5/6) and colorectal (60%; 3/5) cancer patients. *ERBB2* copy number gains were also highest in gastric and colorectal cancers (median 4.8 and 6.6, respectively). We further report two cases of advanced gastric cancer patients, one treatment naïve, and the other having failed four lines of therapy, whose *ERBB2* CNAs were identified by cfDNA and derived clinical benefit from HER2-based therapies.

Conclusion: Our data indicate that *ERBB2* amplification is a common event in solid tumors among Asian cancer patients. High *ERBB2* incidence and copy number gains were observed in gastric and colorectal cancer patients, often in the absence of other oncogenic mutations, underscoring its likely role as the driver alteration in those settings. Finally, we show the potential of comprehensive cfDNA testing in identifying patients who are most likely to benefit from HER2-targeted therapies.

Keywords: cfDNA, ERBB2, HER2, liquid biopsy, NGS

INTRODUCTION

The human epidermal growth factor receptor-2 (HER2) is a receptor tyrosine kinase, belonging to the ErbB family (EGFR/HER1, HER2, HER3, and HER4), involved in signal transduction pathways that mediate key cellular processes including cell proliferation, differentiation, and survival (1). HER2 deregulation via gene mutation, amplification, and post-transcriptional upregulation has been observed in a wide array of human cancers. Importantly, it has been well-established that its aberrant expression and activation is sufficient to drive cell transformation and oncogenesis in preclinical models; an observation consistent with clinical and etiological findings in certain cancer indications (2, 3).

Among the clinical evidence supporting its oncogenic driver role, HER2 antagonists have had demonstrable clinical activity in patients with HER2 overexpressing breast and gastric cancers. Clinical trials evaluating trastuzumab, a monoclonal antibody targeting HER2, when combined with chemotherapy in patients with HER2-positive breast and gastric cancers yielded objective response rates (ORR) of ∼50% and median overall survivals (mOS) surpassing 1–2 years (4, 5). More recently it has been shown that addition of pertuzumab, an antibody targeting a different HER2 epitope, to trastuzumab/chemotherapy increased ORR to 80% and mOS to 56.5 months in patients with metastatic breast cancer (6, 7). Based on these encouraging results, HER2 therapies have been approved in the first-line setting for the treatment of metastatic breast and gastric cancer patients.

Similarly, impressive response rates and clinical benefit have been reported in clinical trials evaluating anti-HER2 agents in patients with heavily pretreated HER2-positive colorectal cancer (CRC). Among 33 patients enrolled in the phase II HERACLES trial testing trastuzumab and lapatinib, a small molecule HER2/EGFR tyrosine kinase inhibitor, in patients with *ERBB2* (HER2) amplified/*KRAS* wild-type CRC, the ORR was 30% with disease stabilization achieved in 70% of patients (8). Importantly, the observed responses were durable; half of the patients remained on therapy for more than 6 months and one patient for over 3 years (9). Notably, patients with higher tissue *ERBB2* copy numbers (CN; ≥10 copies) derived the most clinical benefit and had a significantly longer time to progression compared to those with lower copy number gains (53.1 vs. 34 weeks, respectively). These findings compare favorably to the response rates observed with immunotherapy (ORR 35%) in patients with treatment-refractory CRC and underscore *ERBB2* gene amplification and overexpression as a targetable oncogenic driver in advanced CRC (10, 11).

Historically, *ERBB2* gene amplification and protein overexpression in tumor biopsy material, as measured by *in situ* hybridization (ISH) or immunohistochemistry (IHC), has been used to select patients most likely to benefit from HER2-based therapeutic strategies. A major challenge with this approach is the described inter- and intratumoral heterogeneity in HER2 status, particularly in cancers of gastrointestinal (GI) origin and breast cancers, due to tissue sampling bias. *ERBB2* gene amplification identified by next-generation sequencing (NGS) of tissue DNA or cell-free DNA (cfDNA) in the blood is emerging as a robust biomarker predictive of response to HER2 targeted agents and offers an alternative to IHC/ISH (12). We recently reported that among a cohort of HER2-positive gastric cancer (GC) patients treated with a combination of lapatinib and capecitabine/oxaliplatin, the response rate for those with detectable *ERBB2* copy number amplification in their blood was 100% (12, 13). Moreover, it has been shown that cfDNA captures tumor heterogeneity in GC patients, allowing for the identification of the 10–20% HER2-negative primary tumors with synchronous HER2-positive metastatic lesions (12, 14–17).

Here, we examined the prevalence and genomic landscape of potentially actionable *ERBB2* gene amplification-positive advanced solid tumors as detected by NGS of cfDNA in a large cohort of patients from Asia. We further present case reports of patients with metastatic and treatment-refractory GC that had meaningful clinical responses to HER2-based therapies initiated upon detection of *ERBB2* gene amplification by cfDNA testing.

METHODS

cfDNA Sequencing Platform

The Guardant360® panel is a CLIA-certified, College of American Pathologists (CAP)-accredited, New York State Department of Health (NYSDOH)-approved test that detects single nucleotide variants (SNVs) (70 or 73 genes), copy number amplifications (CNAs) (18 genes), insertion-deletion alterations (indels) (23 genes), and fusions (6 genes). Briefly, cfDNA is barcoded for digital sequencing library preparation. This library is amplified and enriched for the target genes using biotinylated custom baits. Each of the cancer-related genes is paired-end sequenced on an Illumina NextSeq 500 and/or HiSeq 2,500 at a 15,000x average coverage depth per base pair. After sequencing, algorithmic reconstruction of the digitized sequencing signals is used to reconstruct the cfDNA fragments.

The absolute number of unique DNA fragments at a given nucleotide position is quantified, enabling measurement of circulating tumor DNA (ctDNA) as a percentage of the total cfDNA. The mutant allele frequency (MAF) for a given somatic alteration is calculated as the fraction of cfDNA molecules harboring the variant of interest divided by the total number of unique cfDNA molecules mapping to the variant position. The reportable range for SNVs, indels, fusions, and CNAs in cfDNA by the Guardant360 assay is ≥0.04%, ≥0.02%, ≥0.04%, and ≥2.12 copies, respectively. Plasma copy number is reported by centiles with 2+ being between the 50th to 90th-centile in the Guardant Health database and 3+ being greater than the 90th-centile. Focal gene amplifications are determined bioinformatically as those with statistically higher copy numbers relative to other genes across the same chromosome arm.

Analytic and clinical validation of the assay has been previously reported (18, 19).

cfDNA *ERBB2* Landscape

The Guardant Health clinical results database was queried for consecutive ctDNA-positive patient ($n = 469$) samples originating from 15 Asian centers tested between November 2015 and June 2018 (70- or 73-gene panel versions). Patients with non-synonymous *ERBB2* gene alterations (CNA, SNV, and indel) were identified. The prevalence of *ERBB2* alterations among patients with GI ($n = 129$) and non-GI ($n = 340$) cancers were compared. The cfDNA genomic landscape of patients exhibiting *ERBB2* amplification was further assessed. Variants of unknown significance were excluded from the analysis of co-occurring alterations. In addition, the prevalence of *ERBB2* amplification and copy number gains by cancer type (gastric, breast, colorectal, pancreatic, and lung cancer) was compared between Asian and non-Asian patient cohorts. The latter cohort included ctDNA-positive patient samples received from non-Asian centers and tested during the same time period ($n = 31{,}412$ patients) This research was conducted as per a protocol approved by the Quorum Institutional Review Board (IRB) for the generation of de-identified data sets for research purposes.

Statistical Analyses

A two-tailed Student *t*-test was applied to determine the difference between the means of two groups. Population proportions were evaluated using a z-score test to determine the associated *P*-values.

RESULTS

cfDNA and *ERBB2* Gene Alteration Detection in Asian Patient Cohort

The Guardant Health clinical database was queried for Asian patients that had received Guardant360 cfDNA testing between November 2015 and June 2018 ($n = 567$ samples; $n = 539$ unique patients). Patient samples were received from Hong Kong, Singapore, Japan, Thailand, South Korea, and Taiwan (**Supplementary Figure 1**). The average age in this patient cohort was 60 (range 26–116) with equivalent gender representation (53% female/47% male; **Supplementary Table 1**). Of the samples tested, ctDNA was detected in 86.4% (490/567; **Figure 1A**). Similar rates of ctDNA detection were observed among patients with GI (listed in **Supplementary Table 1**) and non-GI cancers (85.4 vs. 86.8%; $P = 0.664$).

A

	All	GI	Non-GI
# Samples tested	567	157	410
# ctDNA-positive	490	134	356
Percentage (%)	86.4%	85.4%	86.8%

B

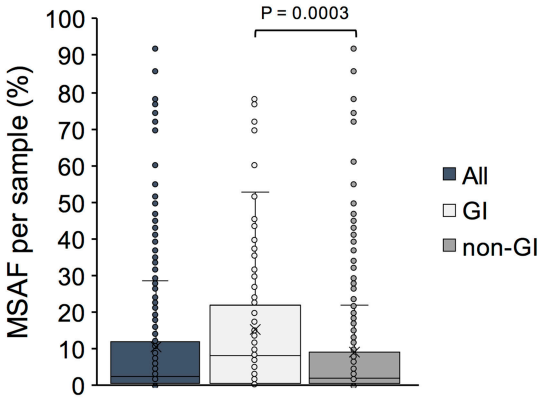


FIGURE 1 | Detection of circulating tumor DNA (ctDNA) and degree of DNA shedding as measured by maximum somatic mutant allele fraction (MSAF) in an Asian patient cohort. **(A)** The number and percentage of ctDNA-positive cancer patient samples in the entire cohort vs. a subset of patients with gastrointestinal (GI) and non-GI tumors. **(B)** The MSAF per patient sample plotted for the entire cohort and by indication. The mean and median values are denoted by an x and a bar, respectively.

We next examined the maximum somatic mutant allele frequency (MSAF) identified per sample which can serve as a proxy for the degree of tumor DNA shedding into circulation. The median MSAF across all SNV, indel, and fusion variants detected in patient samples was 2.7% (range 0.03–92%; **Figure 1B**). A wide range of values has been previously reported and can be attributed to various biological factors affecting ctDNA shedding including, but not limited to, the degree of metastatic disease burden and proximity to the vasculature (19, 20). A significantly higher median MSAF was observed in patients with GI cancers (7.9%; range 0.1–78%) compared to those with non-GI cancers (1.8%; range 0.03–92) ($P = 0.0003$). This finding is consistent with prior reports indicating that GI tumors are high shedders and thus optimal candidates for cfDNA testing (21).

ERBB2 Gene Amplification Prevalence in Asian GI Patients

Non-synonymous *ERBB2* alterations were detected in 52 of the 469 ctDNA-positive patients (11.1%) (**Figure 2**); *ERBB2* CNA, SNV, and indel mutations were found in 27 (5.8%), 27 (5.8%), and 9 (1.9%) patients, respectively. Activating *ERBB2* gene mutations

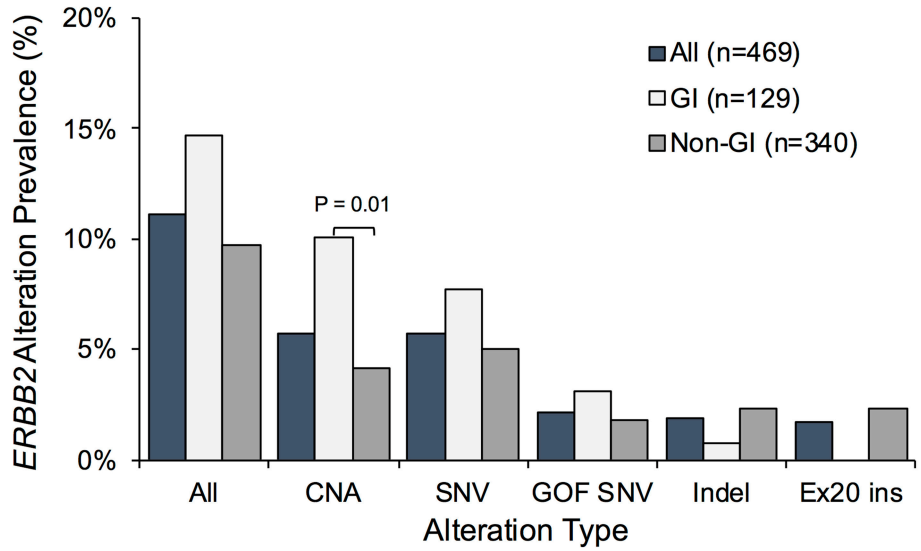


FIGURE 2 | Prevalence of non-synonymous *ERBB2* gene alterations identified in cell-free DNA in the full Asian patient cohort vs. patients with gastrointestinal (GI) or non-GI cancers. Percentage of patients exhibiting *ERBB2* copy number amplification (CNA), single nucleotide variants (SNV), and/or insertion deletion variants (indels) was plotted. Patients with gain-of-function (GOF) SNVs and activating exon 20 insertions (Ex20 ins) represent subsets of those with SNVs and indels, respectively.

including gain-of-function SNVs and exon 20 insertions were observed in 2.1% (10/469) and 1.7% (8/469) of patients. While the prevalence of *ERBB2* amplification was higher in patients with GI cancers ($P = 0.013$), no exon 20 insertion mutations were observed in that subset of patients but rather were exclusively identified in patients with lung cancer (**Figure 2**).

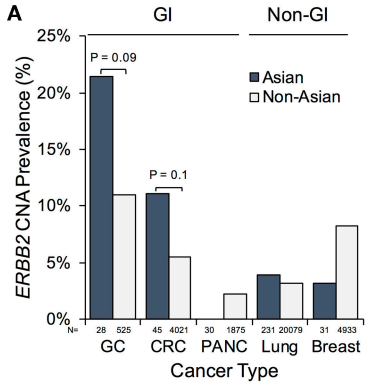
ERBB2 gene amplification was most frequently identified in patients with gastric (21.4%; 6/28), colorectal (11.1%; 5/45), lung (3.9%; 9/231), and breast (3.2%; 1/31) cancers (**Figure 3A**, **Supplementary Figure 2**). The *ERBB2* gene amplification prevalence observed in Asian patients with GC and CRC exceeded that observed in non-Asian patients (11% [58/525; $P = 0.093$] and 5.5% [223/4021; $P = 0.103$], respectively). In contrast, the frequency of *ERBB2* gene amplification in pancreatic, lung, and breast cancers was similar or lower compared to non-Asian patients. The absolute *ERBB2* copy number in plasma was equivalent among Asian and non-Asian gastric (median CN = 4.8 vs. 4.1), lung (median CN 2.8 vs. 2.5), and breast (median CN 3.3 vs. 2.9) cancer patients but higher in Asian CRC patients (median CN 6.6 vs. 2.7; $P = 0.146$; **Figure 3B**). Notably, the observed plasma *ERBB2* CNs were higher in GI compared to non-GI cancers, indicating that they may be more biologically relevant in these disease settings.

Detection of Focal vs. Aneuploidy-Related *ERBB2* Copy Number Gains

ERBB2 gene amplifications can be focal or may be due to chromosome/arm level copy number gains. While the clinical implications of these different mechanisms have not been thoroughly probed, studies indicate that focal amplifications typically affect candidate oncogenic drivers (22). Thus, patients harboring focal *ERBB2* CNAs may be more likely to respond to its therapeutic targeting. Unlike IHC/ISH, NGS testing is capable of distinguishing focal gene amplification events from aneuploidy-associated copy number gains. We therefore reanalyzed the samples to establish the incidence of focal *ERBB2* gene amplifications, as identified in cfDNA, in this patient cohort. All of the GC (6/6) and 80% (4/5) of CRC *ERBB2* CNAs were focal (**Figure 4A**). Most of the non-focal calls were observed in patients with lung cancer, which exhibited more simultaneous gene CNAs on average compared to other cancer types (median 4.5 vs. 3, respectively) indicative of aneuploidy (**Figure 4A**, **Supplementary Table 2**). The majority of *ERBB2* copy number gains across cancer types, however, were in fact focal; accounting for 85 and 71% of calls in GI and non-GI cancers, respectively (**Figure 4B**). cfDNA genomic landscape in *ERBB2*-amplification positive GI patient samples

In addition to focality, the mutual exclusivity with other driver mutations is indicative of a more significant role for an amplified gene in cancer development and progression. *ERBB2* amplification was often mutually exclusive with canonical oncogenic alterations in GC (83.3%; 5/6) and CRC (60%; 3/5) cancer patients, reinforcing its likely importance in these cancer types (**Figure 5**, **Supplementary Table 2**). In contrast, co-occurring oncogenic mutations, including *BRAF* V600E, *EGFR* L858R/exon 19 deletion, multiple *K/HRAS* G12/13 variants, and *PIK3CA* E542K/E545K, were detected in 50% (7/14) of *ERBB2* amplified non-GI cancers (**Figure 5**, **Supplementary Table 2**). Consistent with the reported prevalence in Asian patients, 44.4% (4/9) of the lung cancer patients exhibited activating *EGFR* mutations.

Copy number gains in multiple genes accounted for the majority of co-occurring somatic variants identified in both *ERBB2*-amplified GC (66.6%; 4/6) and CRC (100%; 5/5) patient samples (**Figure 5**). Co-occurring *CCNE1* gene amplification was observed in 66.6% (4/6) of GC while potentially actionable



B

Cancer Type	Median *ERBB2* CN (range)	
	Asian	Non-Asian
GC	4.8 (2.6 - 16.6)	4.1 (2.1 - 78.1)
CRC	6.6 (2.3 - 78.0)	2.7 (2.1 - 147.9)
PANC	-	2.3 (2.1 - 3.2)
Lung	2.8 (2.2 - 3.9)	2.5 (2.0 – 117.6)
Breast	3.3 (n/a)	2.9 (2.1 - 93.1)

FIGURE 3 | *ERBB2* gene amplification prevalence and copy number gains in Asian vs. non-Asian patients by cancer type. **(A)** Percentage of patients exhibiting *ERBB2* gene amplification as identified in cell-free DNA. Total patient numbers tested per indication listed below bars. **(B)** Median blood *ERBB2* copy number and range among patients with *ERBB2* gene amplification. GI, gastrointestinal; GC, gastric cancer; CRC, colorectal cancer; PANC, pancreatic cancer; CN, copy number.

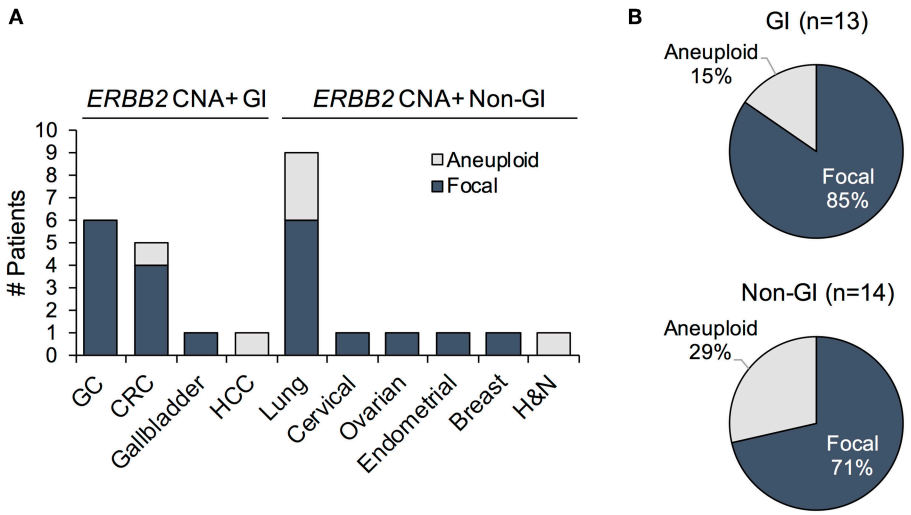


FIGURE 4 | (A) Prevalence of patients exhibiting *ERBB2* gene amplification with focal vs. aneuploidy-related copy number gains plotted by cancer type. **(B)** Proportion of focal vs. chromosome/arm level *ERBB2* amplification events among *ERBB2*-amplified gastrointestinal (GI) and non-GI cancer patients. GC, gastric cancer; CRC, colorectal cancer; HCC, hepatocellular cancer; H&N, head and neck cancer.

receptor tyrosine kinase gene (*EGFR*, *MET*, and *FGFR1*) copy number gains were observed in 100% (5/5) CRC patient samples. The latter finding suggests that combination therapies geared at targeted both EGFR and HER2 (i.e., trastuzumab + cetuximab or lapatinib) may be beneficial in CRC that display amplification of both genes.

Case Reports

To assess the reliability of cfDNA testing in correctly identifying actionable *ERBB2* gene amplifications, in the absence of co-occurring mutations that may impact therapeutic response, the following case studies were considered.

Patient #1—A 70-year old male was diagnosed with advanced gastric cancer with multiple liver metastases. The pathologic examination revealed that the tumor was a tubular adenocarcinoma with moderate differentiation in grade. The HER2 IHC of the primary tumor showed HER2 overexpression (3+) in all tumor areas examined. Blood-based cfDNA genomic profiling was simultaneously performed using the Guardant360 73-gene panel. Consistent with the IHC result, the Guardant360 test demonstrated a focal *ERBB2* gene amplification of 15.47 copies in plasma (3+) in the absence of any other oncogenic variants. As a result, the patient was subsequently treated with capecitabine/cisplatin (XP) in addition to trastuzumab. After two treatment cycles, the liver metastases, and gastric cancer decreased in size by >80% from baseline per RECIST criteria which was maintained for eight cycles of XP/trastuzumab. Multiple liver metastases had nearly disappeared within four cycles of XP/trastuzumab (**Figure 6A**).

Patient #2—A 56-year old male initially diagnosed with gastric cancer was treated and experienced disease progression after 12 cycles of FOLFIRI, four cycles of docetaxel, four

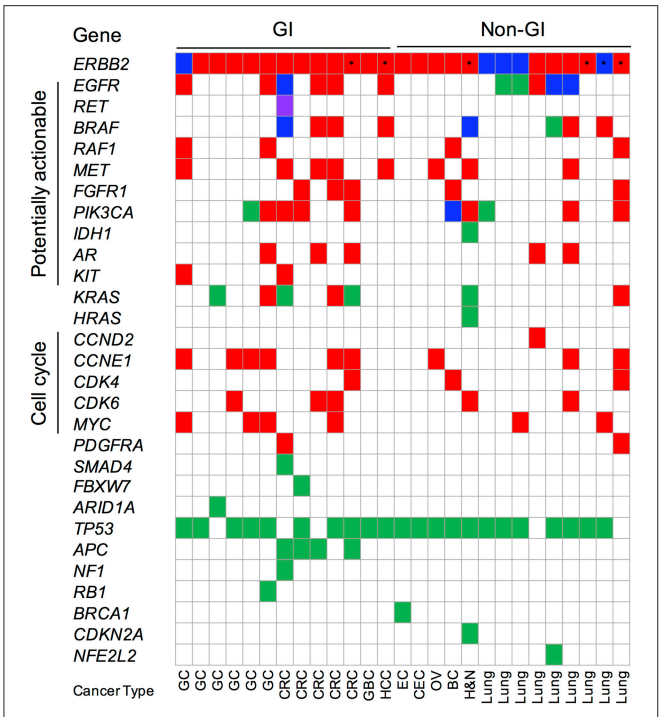


FIGURE 5 | Oncoprint of cfDNA genomic landscape of *ERBB2*-amplified gastrointestinal (GI) and non-GI Asian cancer patients. Red fill denotes copy number amplification (CNA); green fill denotes single nucleotide (SNV) or insertion-deletion variants; blue fill denotes concurrent CNA and SNV; purple fill denotes gene fusions; and asterisk denotes patients with aneuploidy-related *ERBB2* CNA events. Synonymous alterations and variants of unknown significance excluded from analysis. GC, gastric cancer; CRC, colorectal cancer; GBC, gallbladder cancer; EC, endometrial cancer; CEC, cervical cancer; OV, ovarian cancer; BC, breast cancer; H&N, head and neck cancer.

cycles of nivolumab, and four cycles of taxol/ramucirumab. There was no evidence of *ERBB2* amplification or HER2 overexpression in the primary tumor specimen taken at the time of diagnosis; tissue genotyping demonstrated only *CCNE1* gene amplification when DNA was extracted from baseline tumor biopsy. During treatment with taxol/ramucirumab, the patient received Guardant360 cfDNA profiling which revealed a high level focal *ERBB2* gene amplification (4.75 copies in the blood; 3+). Consistent with the primary tissue biopsy result, a *CCNE1* gene amplification was also detected by cfDNA analysis. By the time the *ERBB2* amplification was identified, the patient had disseminated metastases to the liver, peritoneum, lung, and brain. The patient received five cycles of FOLFOX/trastuzumab which stabilized the disease for 3 months with significantly reduced cancer pain which was indicated by reduced opioid medication following chemotherapy (**Figure 6B**). Unfortunately, the patient died of aspiration pneumonia.

DISCUSSION

While it is well-appreciated that *ERBB2* (HER2) gene amplification and protein expression is a bona fide therapeutic target in the treatment of patients with breast and gastric cancers, more recent trials have drawn attention to its actionability in other cancer types (23, 24). We report here that focal *ERBB2* gene amplification, as identified by cfDNA analysis, is common in various solid tumor types in a large cohort of patients from Asia. Strikingly, the prevalence of *ERBB2* gene amplification in Asian GC (21.4 vs. 11%) and CRC (11.1 vs. 5.5%) patients is higher than in non-Asian patient samples (**Figure 3A**). The former observation is consistent with a large study that identified HER2 overexpression, by IHC/ISH, in 9.7% of all Asian GC patients ($n = 5{,}301$) and 18.1% when China was excluded from the analysis, suggesting regional differences in prevalence (25). In the case of primary CRC, however, another study reported an *ERBB2* amplification/overexpression frequency of only 3.3% in an Asian patient cohort ($n = 4913$). A potential explanation for the divergent findings is that the patient population reported here is biased toward heavily pre-treated patients such that they may have acquired *ERBB2* gene amplification during the course of disease progression or as a mechanism of resistance to prior therapies (26). Interestingly, the absolute *ERBB2* gene copy number gains as measured in plasma are highest in GI cancers, in particular in Asian CRC patients (median CN 6.6 vs. 2.7 in non-Asian patients; **Figure 3B**). While additional studies in larger Asian patient cohorts would be required to corroborate these findings and achieve statistical significance, they clearly demonstrate that a substantial portion of GC and CRC cancer patients present with or acquire high-level focal *ERBB2* amplification.

Further supporting a pathogenic role for HER2 in patients with GC and CRC is the finding that *ERBB2* gene amplification is rarely detected in conjunction with a canonical oncogenic mutation (**Figure 5**). Only 2 of 5 (40%) *ERBB2* amplified CRC patients displayed concurrent oncogenic alterations; the first patient displayed multiple *subclonal* driver variants (*BRAF* V600E, *KRAS* Q61H, and a *CCDC6-RET* fusion) as well as *EGFR* ectodomain mutations (G465R, S464L) suggestive of the acquisition of several on- and off-target resistance mutations in response to standard-of-care cetuximab therapy while the second patient had a co-occurring *subclonal KRAS* G12R mutation (**Figure 5**, **Supplementary Table 2**). Recent cfDNA results from the HERACLES study (lapatinib plus trastuzumab in *ERBB2*-amplified metastatic CRC) found that the majority of non-responders (6/7) harbored concomitant *clonal RAS/RAF* gene mutations at baseline while *subclonal* RAS/RAF/PI3K-AKT pathway alterations emerged at progression after initial response or disease stabilization. The presence of *subclonal RAS/RAF* mutations co-occurring with *ERBB2* amplification in this cohort of CRC patients begs the question if targeted HER2 therapy may still achieve benefit and/or if a combination approach might be warranted (27, 28). One *ERBB2* amplified GC patient had a co-occurring *subclonal PIK3CA* E545G variant (1/6; 16.6%); one other had an atypical *KRAS* V14I loss-of-function mutation although its clinical significance remains to be elucidated. This is in stark contrast to non-GI cancers where half display co-occurring oncogenic alterations (**Figure 5**).

The most common co-occurring alterations observed in *ERBB2* amplified GI patients were copy number gains in other

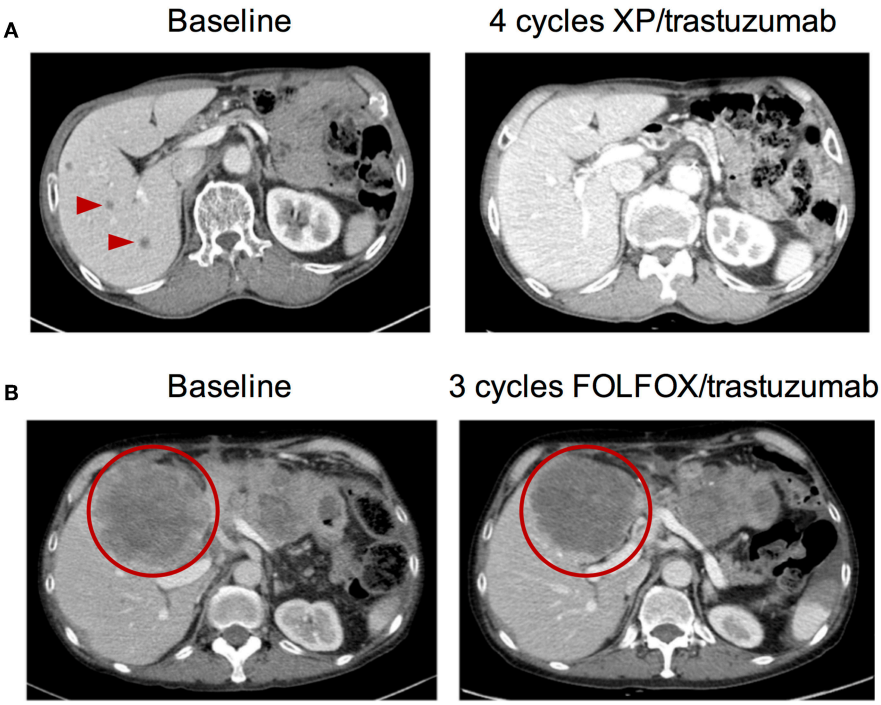


FIGURE 6 | Pre- and post-anti-HER2 therapy abdominal CT images from two *ERBB2*-amplified gastric cancer patients identified by cell-free DNA. **(A)** Patient #1 at baseline and after four cycles of capecitabine/cisplatin (XP) combined with trastuzumab. **(B)** Patient #2 at baseline and after three cycles of FOLFOX combined with trastuzumab. Arrowheads and circles denote malignant lesions.

genes, including other receptor tyrosine kinases (ex. *MET*, *EGFR*, *FGFR1*) and cell cycle genes (ex. *CCNE1*, *CDK4/6*). Many of these can, in principle, serve as bypass mechanisms of resistance to not only standard-of-care but HER2-directed therapies and are potentially targetable. Understanding the genomic landscape of various GI tumor types or in individual patients may facilitate the determination of optimal combination therapies in specific genetic contexts. For instance, two-thirds of *ERBB2*-positive patients in the CRC cohort had evidence of *EGFR* co-amplification providing potential rationale for combining a HER2 targeted agent with cetuximab/chemotherapy in these patients. Alternatively, treatment with a dual HER2/EGFR inhibitor, such as lapatinib, may be warranted in this scenario.

Here we presented two patient case reports of patients with metastatic GC with high-level focal *ERBB2* amplifications as detected by cfDNA. These reports provide examples of how cfDNA testing can inform treatment decision both at diagnosis and at progression by (1) identifying the actionable alteration/capturing intertumoral heterogeneity and (2) confirming a lack of co-occurring mutations that can confound response to targeted therapy. Neither patient had evidence of alternative driver alterations in the plasma and both benefited from combined trastuzumab + chemotherapy therapeutic regimens (**Figures 6A,B**). Critically, *ERBB2* amplification and overexpression was identified earlier in the first patient's clinical journey and hence he experienced a partial and a more durable response to therapy. Unfortunately, the second patient had already failed several lines of therapy but remarkably still had disease stabilization and symptom improvement when treated with trastuzumab. In addition to these case reports, several others have been published demonstrating that patients with advanced cancers treated based on a positive cfDNA test result derive clinical benefit from the corresponding molecularly-targeted therapy (summarized in **Table 1**). An important consideration is that Asian patients may have differential sensitivity to specific drugs and combination therapy approaches. It has been reported that a subset of Asian patients with GC derived benefit from treatment with lapatinib and trastuzumab in the LOGiC trial, while this combination was not effective in Caucasian patients (33). Hence optimal treatment strategies may need to be independently established for patients from different geographical regions and racial/ethnic backgrounds.

Nonetheless, an important advantage of cfDNA testing is the ability to capture both inter- and intratumoral heterogeneity to obtain a holistic view of the genomic landscape in primary and metastatic lesions. In the PANGEA trial of personalized antibodies for gastroesophageal patients, profiling of both primary and metastatic tumor tissue specimens led to treatment reassignment in almost a third of patients, many of which had HER2-positive metastases (14). Importantly, cfDNA and metastatic tissue genotyping results were 87.5% concordant while substantive discordance was seen between primary and metastatic tissue genotyping and between primary tissue and cfDNA genotyping results. Similarly, in a recent

TABLE 1 | Summary of publications demonstrating clinical validity and utility of Guardant360® cell-free DNA test in gastrointestinal cancers.

	Cancer type	Genomic target	ctDNA role in study	Tissue concordance	Therapeutic regimen	Key findings
Hong et al. (29)	mCRC	*BRAF* V600E	Correlative	N/A	Vemurafenib + irinotecan + cetuximab	• 35% (6/17) objective response rate | 88% (15/17) disease control rate • Median PFS 7.7 months • Near perfect correlation between Guardant360-detected and ddPCR-detected $BRAF^{V600E}$ • $BRAF^{V600E}$ ctDNA trends over time correlated with radiographic changes • ctDNA analysis identified mutations in genes reactivating MAPK signaling at progression
Montagut et al. (30)	mCRC	*EGFR* extracellular domain mutations	Exploratory secondary objective	N/A	Sym004 + investigators choice	• Sym004 did not improve overall survival in an *unselected* population of mCRC patients and acquired anti-EGFR resistance • Guardant360 analysis defined a triple negative subgroup (*RAS*/*BRAF*/*EGFR* ECD negative) with improved median OS (12.8 vs. 7.3 m)
Siravegna et al. (27)	mCRC	*ERBB2* (HER2) amplification	Correlative	98% (*ERBB2* amp detected in 51/52 samples)	Trastuzumab + lapatinib	• 24% (8/32) objective response rate • Guardant360 identified *clonal RAS/RAF* mutations in 86% of primary resistance cases and acquired *subclonal RAS/RAF*, *ERBB2*, *EGFR*, *PIK3CA,* etc. mutations at progression
Kim et al. (13)	Gastric/GE	*ERBB2* (HER2) amplification (and others)	Treatment selection	N/A	Lapatinib + Capecitabine + Oxaliplatin	• Multiple parallel cohort, open-label, clinical trial using ctDNA-guided matched therapy when tissue was insufficient, or unobtainable for NGS • 80% (4/5) objective response rate among *ERBB2* (HER2) amplified cases • 67% (6/9) objective response rate including *ERBB2* amplified (4/5), *MET* amplified (1/1), *FGFR2* amplified (0/1), and *PIK3CA* mutant (1/2)
Pectasides et al. (14)	Gastric/GE	*ERBB2* (HER2) amplification (and others)	Correlative	85% concordance between cfDNA and metastases	FOLFOX or FOLFIRI + targeted agent when applicable	• Significant discordance between primary GE tumors and metastases based on tissue testing in 36% (10/28) of patients leading to treatment change in nine patients (32% of 28) • In five discordant cases, no actionable genomic alteration was detected in the primary, yet the metastasis and Guardant360 both revealed actionable copy number amps in *ERBB2* (2), *MET* (1), *EGFR* (1), or *FGFR2* (1)
Kim et al. (12)	Gastric/GE	*ERBB2* (HER2) amplification	Exploratory secondary objective	67% (6/9) among all; 86% (6/7) among responders	Lapatinib + Capecitabine + Oxaliplatin	• 69% (22/32) objective response rate • Significant *ERBB2* (HER2) amp discordance, 40% (4/10), between primary tumor and metastasis based on tissue testing • Detectable *ERBB2* copy number amplification in plasma at baseline was predictive to the response (100% response rate) and changes in plasma-detected genomic alterations were associated with lapatinib sensitivity and/or resistance
Maron et al. (31)	Gastric/GE	*EGFR* amplification	Correlative	86% (6/7 *EGFR* amplification)	EGFR mAB ± chemotherapy	• Treatment details: 3 FOLFOX + ABT-806; 1 FOLFORI + cetuximab; 3 cetuximab • 57% (4/7) objective response rate | 100% disease control rate • Median PFS 10.0 months • Serial ctDNA and tissue NGS identified mechanisms of primary and acquired resistance in all patients
Kim et al. (32)	Gastric/GE	TMB-high or PD-L1 >1% or MSI-high	Exploratory secondary objective	See key findings	Pembrolizumab	• Good concordance ($r^2 = 0.54$) between ctDNA and tissue exome TMB with one outlier who had high ctDNA TMB/low tissue TMB; second tissue biopsy showed high TMB (i.e., tumor heterogeneity captures by ctDNA) • 25% (15/61) objective response rate overall; a Guardant360 "digital" tumor mutation burden score in the top tertile predicted improved ORR (83 vs. 7.7%, $p = 0.0014$)

trial of pembrolizumab as salvage therapy in advanced GC patients, cfDNA analysis identified a tissue microsatellite stable (MSS)/tumor mutation burden (TMB)-low patient with pronounced intratumoral heterogeneity as being TMB-high. A second biopsy at the same site confirmed MSI/TMB-high status consistent with the cfDNA result, further highlighting that plasma testing allows for the enrichment and analysis of DNA shed from various heterogeneous tumor sites (32). Such inter- and intratumoral heterogeneity is certainly not limited to GC but is also observed in CRC and other cancer types. It has been reported that CNAs are the major source of tumor heterogeneity in CRC development (34). The same authors found that *ERBB2* amplification is acquired in 6–7% of metastases. Given that distal metastases are a major cause of morbidity in cancer patients, targeted treatment of the secondary lesion(s) is strongly advisable if not recommended, as in the case of breast cancer. cfDNA genotyping provides a non-invasive solution that can drive therapy selection in metastatic patients where tissue sampling multiple metastatic lesions is not practical or in some cases possible.

A limitation to our study is that the relative patient numbers by cancer type are skewed based on standard-of-care ordering practices. For instance, lung cancer patients are over-represented in the Guardant Health patient cohort given that testing for actionable alterations at diagnosis, or progression on a targeted therapy, is routine. In the same vein, we observed a lower prevalence of *ERBB2* amplification in this breast cancer patient cohort compared to that in a general population (3 vs. 10–15%, respectively) but a higher incidence of *ESR1* mutations (**Supplementary Figure 3**) associated with progression on hormone-based therapies. Another important limitation is the lack of clinically annotated Asian patient cfDNA samples (i.e., disease stage, lines of therapy, tissue test results, and clinical outcomes). Establishing the genomic landscape of *ERBB2* amplified Asian patients in more defined clinical contexts will be key in identifying and defining appropriate treatment strategies, and their sequencing, by disease stage and patient treatment history. While an impressively high degree of concordance (97.9%) has been described between cfDNA and tissue *ERBB2* amplification status of CRC patients enrolled in the HERACLES trial (27), a head-to-head comparison of tissue and blood-based test results in larger patient cohorts will be necessary to define and develop HER2 positivity criteria. Indeed, "low" expressers have been described to derive some clinical benefit from anti-HER2 agents such that a more extensive study will be necessary to define cut-offs, particularly in the absence of alternative therapies.

CONCLUSION

Here we demonstrate, for the first time, that high level, focal *ERBB2* gene amplification, as identified by cfDNA testing, is a common event in Asian patients with advanced cancers. In particular, higher *ERBB2* incidence and CN gains were observed in GC and CRC patients in the absence of other oncogenic alterations, indicating that HER2 may be the dominant driver of tumor proliferation in those settings. Given its established role as an oncogene in certain contexts and the availability of HER2 antagonists, both approved and in clinical development, *ERBB2* amplification is an attractive therapeutic target. Comprehensive cfDNA testing represents a non-invasive method of assessing HER2 status in the metastatic disease setting. It not only captures inter- and intratumoral heterogeneity but allows for assessment of co-occurring somatic mutations and the identification of patients most likely to benefit from HER2-based therapeutic strategies.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

JL, AF, VR, and RL conceived and designed the study. AF and YS collected, assembled, and analyzed the data. AF, KB, VR, and RL interpreted the data. JL, SK, and K-MK treated the patients and provided clinical samples. AF, JL, and RL wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00212/full#supplementary-material

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Role of Circulating Tumor DNA in Gastrointestinal Cancers: Update From Abstracts and Sessions at ASCO 2018

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Background: The promising aspect of circulating tumor DNA (ctDNA) is its rapid turnaround and non-invasive nature. According to the American Society of Clinical Oncology (ASCO) and College of American Pathologists joint ctDNA review published in March 2018, there is not sufficient evidence to support the use of ctDNA in practice for GI cancers. However, there were numerous studies presented at ASCO Annual Meeting supporting its value. We aimed to summarize on its role in the management of gastrointestinal cancers based on the studies presented recently, and future directions.

Methods: We limited our search to keywords "ctDNA," "circulating tumor DNA," "cell-free DNA (cfDNA)" and/or "liquid biopsy," at the 2018 ASCO Annual Meeting library abstracts and presentations.

Results: There were 35 studies that revolved around ctDNA as a diagnostic tool, prognostic marker and/or a measure of tumor heterogeneity in gastrointestinal cancers. Depending on the assay used, the results of several studies showed that ctDNA was able to identify relevant mutations or fusions including *RAS, HER2/Neu, BRAF, MET, BRCA2, APC, TP53, ALK, ROS1, PTEN,* and *NF1*. The prognosis in terms of tumor mutation burden, objective response rate, metastasis and survival were also estimated by various studies based on ctDNA. The findings showed that higher baseline ctDNA levels and/or increased number of mutations detected in ctDNA were associated with poor survival and multi-site metastasis. Right-sided colon cancer was associated with higher number of mutations in ctDNA than left-sided colon and rectal cancers. Similarly, tubular adenocarcinoma subtype of gastric cancer was more likely to have higher ctDNA levels than signet-ring cell subtype. The feasibility of assessing response to therapy and residual metastatic disease by using ctDNA which was otherwise not detected on imaging was also presented.

Conclusions: The studies presented at ASCO 2018 report on the many ways ctDNA is of value in patients with gastrointestinal malignancies. Experts and discussants at the meeting argued that this may well indeed be ready for prime time for certain GI malignancies including colorectal cancers, especially in the metastatic setting. These findings alongside ongoing studies showing its feasibility into practice would likely lead to revision of the current guidelines for metastatic GI cancers.

Keywords: circulating tumor DNA, ctDNA, liquid biopsy, ASCO, gastrointestinal cancer, colorectal cancer

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INTRODUCTION

Circulating tumor DNA (ctDNA), considered as cancer biomarker, is the free DNA found predominantly in plasma and derived from tumor cells. Analysis of ctDNA, also referred to as "liquid biopsy," is a non-invasive and cheaper technique allowing for serial oncologic assessments, though several controversies exist. In March 2018, American Society of Clinical Oncology (ASCO) and the College of American Pathologists published a joint review on ctDNA. They reported on the value in terms of clinical utility and validity for few types of late stage cancers, but not for the majority of late stage cancers and not at all for early stage cancers (1). The consensus statement therefore narrated that routine ctDNA testing is not yet ready to be considered as an integral part of the management of cancers. Since the publication of this review in March 2018, the 54th ASCO Annual Meeting was convened at Chicago, Illinois on June 1–5, 2018. The research abstracts and the discussions, however, at ASCO conference argued otherwise.

In this article we aim to highlight the recent key updates and advances related to ctDNA presented at 2018 ASCO Annual Meeting that was held after the consensus statement. A literature search of 2018 ASCO Annual Meeting library, by using the key words "ctDNA," "circulating tumor DNA" "cell-free DNA (cfDNA)" or "liquid biopsy" was done; and the most relevant abstracts pertaining to GI malignancies were the ones selected to be summarized/included in this review.

ctDNA AS A DIAGNOSTIC TOOL

Tissue biopsy remains the gold standard test for the diagnosis of cancers. Furthermore, genetic testing done on tissue is what is used to find actionable genes or aberrations. The yield depending on site and procedure often is enough to make the diagnosis but not enough to run additional genetic tests. There were an array of studies reporting on the feasibility and specificity of liquid biopsies aiding and/or corroborating findings noted on tissue biopsies in gastrointestinal cancers including colorectal cancers (CRC) and non-colorectal cancers. Hu et al. investigated the role of ctDNA in CRC patients and reported that the number of DNA mutations detected in tissue biopsy correspond with that found in liquid biopsy (2). Huang et al. did a study on 30 CRC patients who underwent surgery and presented that the majority of patients (83%) had at least one mutation detected in both tissue biopsy as well as liquid biopsy (3). A study conducted on anal cancer patients demonstrated the ability of ctDNA in terms of sensitivity (89%) to help diagnose the cancerous disease (4). An Australian study reported the detection ctDNA in 62.2% pre-operative and 37.1% post-operative plasma samples of patients with early stage pancreatic cancer (5). Mody et al. conducted a study on 104 cholangiocarcinoma patients and reported that at least one gene mutation was identified in ctDNA sample in 77% of patients (median number of mutations per patient = 3; range = 1–15) (6). Another study revealed high mutational concordance between liquid and tissue biopsy for biliary tract cancer (74%) and intrahepatic type (92%) (7). In another study of gastroesophageal cancer patients, researchers were able to detect at least one mutation (median number of mutations per patient = 2; range = 0–15) in ctDNA in 66% of patients (8).

Many clinical trials, mostly ongoing, have demonstrated that the mutations in oncologic genes responsible for carcinogenesis can be identified in ctDNA. These ctDNA mutations correlate well with tissue biopsy results. In the REVERCE phase II trial studying early exposure to regorafenib vs. anti-EGFR in patients with CRC, Tsuji et al. identified mutations in several genes including *RAS, BRAF, EGFR, HER2,* and *MET* using ctDNA samples of patients with metastatic CRC (Clinical trial: UMIN000011294) (9). What was interesting was that the arrays of mutations acquired were different depending on the sequence of therapy (Regorafenib anti-EGFR vs. anti-EGFR Regorafenib). Similarly, in the HERACLES study, a phase II trial of trastuzumab and lepatinib in *HER-2* positive metastatic CRC, researchers were able to correctly identify the *HER-2* amplifications in 96% of samples using ctDNA (10). In another study, researchers reported that *HER-2 (ERBB2) amplifications* were identified in 61% of gastroesophageal cancer patients using ctDNA samples (11). Iqbal et al. detected a variety of mutations in different genes including *HER2, BRCA2, TP53, APC, ROS1, PTEN, KRAS, CCEN1, GNAS, NF1, CTNNB1, PIK3CA,* and *ARID1A* using ctDNA in gastroesophageal adenocarcinoma patients (12). Jia et al. demonstrated the practicality of employing cfDNA for the detection MET amplification in patients with RAS wild-type metastatic CRC (Clinical trial: NCT02008383). They enrolled the patients in two groups i.e., one receiving cabozantinib plus panitumumab and the second receiving cabozantinib alone; and found detectable cfDNA levels and MET amplification in 98 and 18% of patients, respectively (13). In WJOG7112G study, Sukawa et al. studied gastric or gastroesophageal cancer patients who had disease progression despite receiving chemotherapy, and identified HER2 amplifications mutations in ctDNA in 60% of patients (14).

In summary, multiple studies show that GI malignancies in general shed DNA that can be detected and the current technologies available corroborate and correlate well with tissue based genetic testing.

ctDNA AS A PROGNOSTIC BIOMARKER

Prediction of Response to Therapy

The behavior of cancer in response to therapy can be predicted by determining the type and number of ctDNA mutations. The research presented at ASCO conference does establish ctDNA as an independent prognostic marker in many cancers. Zhang et al. investigated ctDNA of 43 esophageal squamous cell carcinoma patients, and reported the role of ctDNA in predicting response to therapy. Their results showed that the patients who did not respond to neoadjuvant chemotherapy were associated with higher driver gene molecular mutation burden compared to those who responded well ($p < 0.01$) (15). Yang et al. studied 88 rectal cancer patients and reported that ctDNA levels became undetectable during neoadjuvant chemoradiotherapy in 65.5% of the patients which were congruous with the imaging and histological changes (16). Another study examined various driver mutational genes detected by ctDNA in CRC patients

and reported a noticeable reduction in tumor mutation burden following surgery (3).

There were also studies that reported on ctDNA as a biomarker of the efficacy of specific chemotherapeutic agents in different cancers. Catenacci et al. investigated the response of margetuximab plus pembrolizumab in ERBB2-positive gastroesophageal cancer patients, and demonstrated that the response to therapy was predicted based on ctDNA sample results. They calculated objective response rate and disease control rate using ctDNA which were 57 and 86%, respectively, for their cohort. They also reported that a lot of cancer patients lost their ERBB2 amplifications as detected by ctDNA after receiving trastuzumab (11). Chen et al. conducted a phase II study in China and assessed the clinical rationale of apatinib in chemotherapy-refractory metastatic CRC patients (Clinical trial: NCT03190616). They reported that tumor mutation burden calculated by ctDNA is the main factor determining prognosis (17). In HERACLES study discussed earlier, the researchers reported that ctDNA precisely anticipated the response to HER-2 receptor inhibitor therapy in HER2-positive CRC (10).

The conclusions one can draw from these studies are that in general if there is a decrease in the variant allele fraction or the number of mutations (or lack of detection of any ctDNA) after receiving therapy, it affirms an improvement in terms of reduction in tumor size. Studies noting a decline in ctDNA as early as 2 weeks could predict response to therapy months later on imaging studies. Unrelated, but utilizing ctDNA testing is also helpful in patients having pseudoprogression in their imaging on immunotherapy. These studies presented at ASCO conference do strongly support ctDNA as a reliable cancer biomarker and favor its use in the management of cancer patients in prognosticating and as a dynamic early biomarker predicting response to therapy (**Figure 1**).

Prediction of Survival

Researchers estimated and reported survival rates of CRC based on the findings of ctDNA. Siveke et al. studied the role of ctDNA in determining the prognosis of metastatic CRC and reported that high baseline ctDNA is associated with multi-site metastasis, increased levels of carcinoembryonic antigen, propensity of having right-sided origin and poor overall performance compared to low baseline ctDNA. They further demonstrated that baseline ctDNA is the predictor of progression free survival (HR = 2.01; 95% CI: 1.25–3.22, $p = 0.0033$) (18). Poulsen et al. showed the association of ctDNA mutations and overall survival in patients with metastatic CRC. They reported that the patients who received Sym004 (anti-EGFR antibody mixture) and were negative for *RAS* and *BRAF V600E* mutation in ctDNA had an overall survival advantage of 3.5 months compared to those who received standard therapy (HR: 0.71; $p = 0.134$). They further estimated the overall survival of patients who were negative for EGFR mutations in addition to *RAS* and *BRAF V600E* mutation in ctDNA (HR: 0.59; $p = 0.044$) (19). Kehagias et al. investigated 141 patients with advanced CRC in a multicentric trial, and measured cfDNA levels at baseline and at day 14 after starting regorafenib (Clinical trial: NCT01929616). The patients having cfDNA levels $\geq 1\,\mu$g/ml at day 14 were associated with poor progression-free (HR: 2.50, 95% CI: 1.73–3.63) and overall survival (HR: 3.83, 95% CI: 2.52–5.71) compared to those having levels $<1\,\mu$g/ml (20). A Japanese study showed similar results and reported that the finding of *KRAS* mutated ctDNA in post-chemotherapy metastatic CRC patients is associated with reduced progression free survival (21). The high levels of ctDNA and high tumor mutation burden correlated with poor survival rates of CRC.

Survival of non-colorectal cancers including esophageal and biliary tract cancers were also estimated based on the numbers of mutations detected in ctDNA. A study of esophageal squamous cell carcinoma patients who underwent radiation therapy reported ctDNA as one of the main factors determining prognosis. Their results revealed that the patients who had mutations in ctDNA after undergoing radiation therapy were associated with poor overall survival ($p = 0.005$) and a trend of a decrease in disease free survival ($p = 0.068$) compared to those who did not have any mutations in ctDNA (22). A German study showed the association of variant allele frequency detected by ctDNA and progression-free survival of intrahepatic biliary tract cancer (Spearman, $r = -0.5878$, $p = 0.0288$) (7).

Detection of Residual Disease, Progression, and Recurrence

Cancer patients are evaluated after completion of therapy to make sure there is no possible tumor left behind. Imaging and/or tumor markers are usually checked to rule out any residual disease. The feasibility of using ctDNA as a biomarker of residual disease was reported in various studies at ASCO 2018. Tie et al. conducted a study on 95 stage III colon cancer patients and reported that the response to therapy and the residual metastatic disease which is otherwise not identified on imaging can be detected by ctDNA. Their results showed that the finding of positive ctDNA post-surgery (HR: 3.52; $p = 0.004$) and post-chemotherapy (HR: 7.14; $p < 0.001$) is associated with poor recurrence free survival (23). Murray et al. did a prospective study on 172 post-surgery CRC patients, and demonstrated that ctDNA is a marker of residual disease and recurrence (Clinical trial: 12611000318987). Their results showed that the patients who were positive for ctDNA after surgery were at an increased risk of recurrence (HR: 3.8, 95% CI: 1.5–9.5) (24).

Furthermore, studies have shown the reliability of the use of ctDNA in ascertaining the disease progression in terms of metastasis. A study conducted on CRC patients demonstrated the association of post-surgery detection of ctDNA mutations and increased risk of disease progression. The researchers found that higher proportion of ctDNA mutation positive patients (27.8%) experienced disease progression compared to those who were ctDNA mutation negative (4.4%) after surgery (25). Another study showed the reliability of ctDNA in predicting the response to regorafenib or TAS-102 in patients with metastatic CRC using different PCR based methods (26). Cabel et al. conducted a study on 36 anal cancer patients in France and reported that post-chemoradiotherapy detection of ctDNA is associated with worse outcomes. Their results showed that 17% of the patients had metastatic relapse, and these were the only patients who

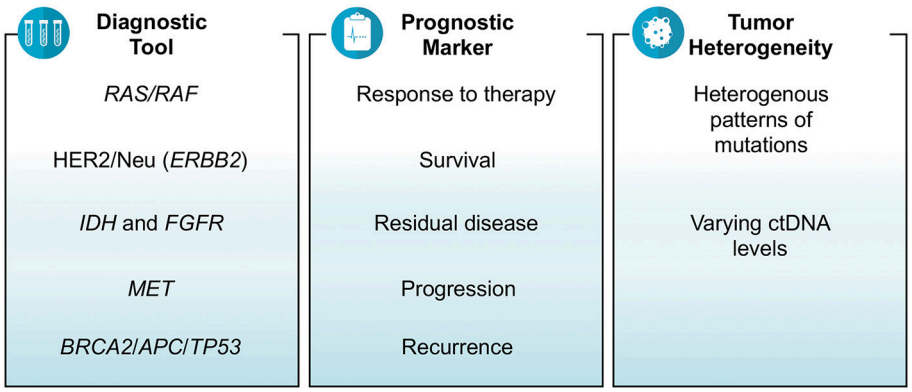

FIGURE 1 | Summary of studies reporting on use of ctDNA in GI malignancies at ASCO 2018.

were positive for ctDNA after receiving chemoradiotherapy (4). Parseghian et al. demonstrated the usefulness of ctDNA in monitoring the decay of clones which were resistant to anti-EGFR agents in metastatic CRC. They calculated the median relative mutant allele fraction (10.5 vs. 10.6%) and the decaying half-life of clones (3.4 vs. 6.9 months) for *RAS* and *EGFR*, respectively, while on anti-EGFR therapy (27). Acquisitions of these mutations are known mechanisms of secondary resistance in patients with metastatic CRC. What is indeed intriguing is that these clones can be lost over time allowing for potentially "rechallenging" some of these therapies leading to development of multiple trials employing this strategy.

Finally, recurrence of cancers is a common phenomenon and ctDNA was noted in multiple studies to be helpful in predicting the risk of recurrence after being treated for the primary cancer (**Figure 2**). Murray et al. studied the association of ctDNA and recurrence of CRC, and reported that post-surgery detection of ctDNA is associated with an increased risk of recurrence (HR = 3.8; $p = 0.004$) (Clinical trial: 12611000318987) (28). A Chinese study, conducted on hepatocellular carcinoma patients who underwent transcatheter arterial chemoembolization, reported that high mutational burden of 10 genes identified in ctDNA might be associated with the recurrence of the disease. The 10 genes detected in their study were *NRAS, BRAF, PIK3CA, KRAS, ARID1A, AXIN1, ARID2, TERT, TP53,* and *CTNNB1* (29). Lee et al. demonstrated that ctDNA is an indicator of prognosis in terms of determining the risk of recurrence and guiding treatment decisions in patients with early stage pancreatic cancer (Clinical trial: ACTRN12612000763842). Their analysis showed that detection of ctDNA both before and after surgery is associated with poor recurrence free and overall survival; and 100% of patients showed recurrence who had measurable ctDNA after surgery despite of being on adjuvant chemotherapy (5).

TUMOR HETEROGENEITY AND ctDNA

Gastrointestinal cancers including CRC are heterogeneous in nature in terms of histology, presenting symptoms, progression, response to therapy, and outcomes that can lead to a difference in the management of various types of cancers (30). Tumor heterogeneity can be evaluated by measuring the wide-ranging levels of ctDNA and the identification of diverse patterns of mutations. A study reported that right-sided colon cancer is associated with an increased number of mutations in ctDNA than left-sided colon and rectal cancers (2). Another study demonstrated higher median cfDNA levels (14.2 vs. 8.94 ng/ml) and higher mutation concordance rate identified via liquid and tissue biopsies (94.7 vs. 50%) for colon cancer patients than rectal cancer patients (31). Similarly in another study, researchers identified a heterogeneous pattern of genomic alterations in ctDNA of patients with metastatic CRC including mutations in *BRAF, KRAS, NRAS, MAP2K1, PIK3CA, ERBB2, MET,* and *EGFR* genes (19). A Chinese study on patients with rectal cancer indicated that the most common mutations found in ctDNA were of *TP53, APC, and KRAS* genes (16). A study of cholangiocarcinoma patients reported the detection of mutations in ctDNA in a variety of genes including *TP53, KRAS, FGFR2, ARID1A, APC, PIK3CA, BRAF, CCND1, CCND2, CCNE1, CDK4, CDK6, EGFR, ERBB2, FGFR1, MET, MYC,* and *PDGFRA* (6). The same study revealed the finding of FGFR2 fusions and the actionable mutations in 3 and 61% of patients, respectively (6). Clifton et al. did a study for the identification of actionable gene fusions in CRC and reported that the main fusions detected in ctDNA samples were *RET, FGFR3, ALK, NTRK1, ROS1,* and *FGFR2* (32).

Several clinical trials have demonstrated the role of ctDNA in detecting tumor heterogeneity of different gastrointestinal cancers. In CRITICS phase III trial, Leal et al. investigated cfDNA of stage Ib-IVa resectable gastric cancer patients ($n = 115$) and showed that the level of ctDNA varies between different histologic types of cancer (Clinical trial: NCT00407186). They found higher ctDNA levels for tubular adenocarcinoma (mutant allele fractions: 0.25%) than signet-ring cell subtype (mutant allele fractions: 0.16%) of gastric cancer (33). Yaung et al. demonstrated the ability of ctDNA, by using next-generation sequencing based methods, to detect tumor heterogeneity in a clinical trial (STEAM) evaluating the outcomes of FOLFOXIRI-bevacizumab vs. FOLFOX-bevacizumab as first-line treatment

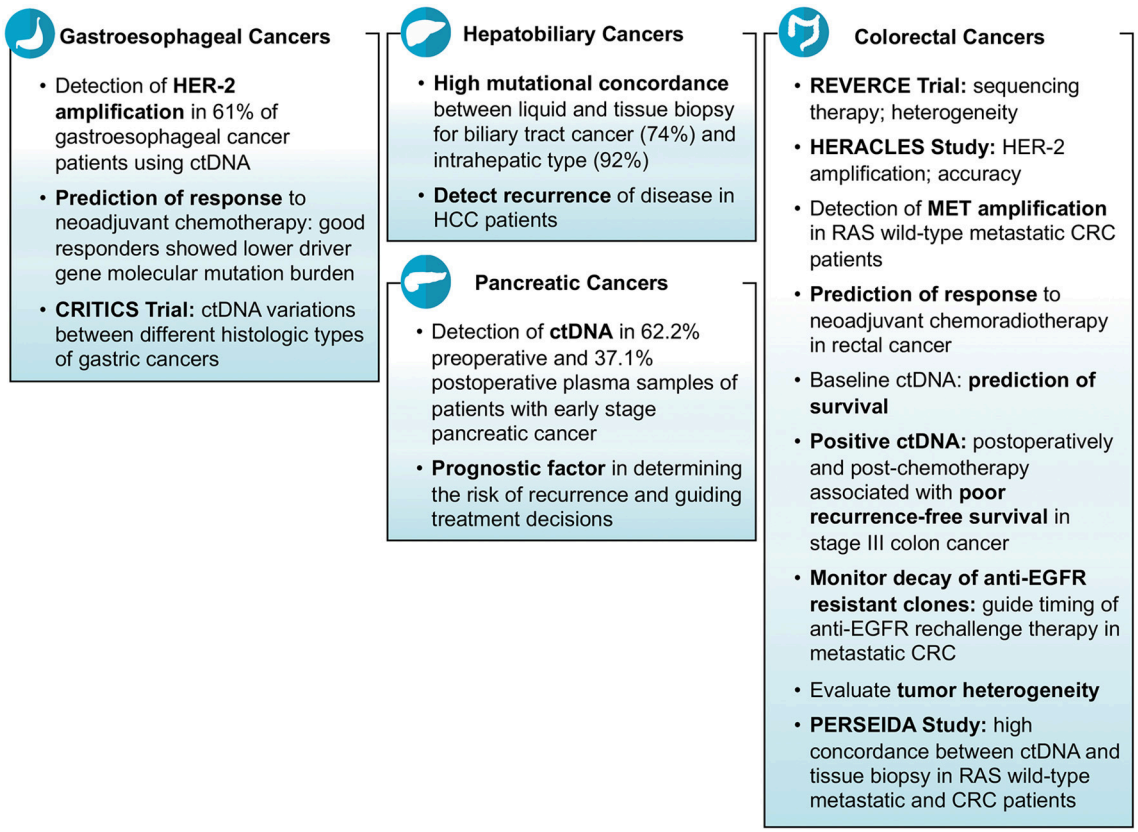

FIGURE 2 | Findings of ASCO 2018 studies about the use of liquid biopsy in gastrointestinal cancers.

of metastatic CRC (Clinical trial: NCT01765582). Their results showed that high mutant-allele tumor heterogeneity is associated with poor outcomes (34). Okamura et al. examined the ctDNA of gastroesophageal carcinoma patients ($n = 55$) and found different arrays of genomic alterations (Clinical trial: NCT02478931). The most frequent mutations found in their study were *TP53, PIK3CA, ERBB2* and *KRAS* in 50.9, 16.4, 14.5, and 14.5% of patients, respectively (8). In another clinical trial, the mutated genes on ctDNA in patients with advanced CRC were found to be *APC, TP53, KRAS,* and *PI3KCA* in 73, 72, 66, and 23% of ctDNA samples, respectively (20). PERSEIDA study which is an ongoing trial in Spain evaluated the concordance in mutation profile between ctDNA and tissue biopsy. The researchers analyzed the data of tissue biopsy-proven *RAS* wild type metastatic CRC patients ($n = 119$) from 20 centers who were later assessed with liquid biopsy, and reported that there was high concordance between the two but new *RAS* mutations were also detected in ctDNA majority of which were at low mutant allele fraction limit (Clinical trial: NCT02792478) (35).

FUTURE DIRECTIONS AND CONCLUSION

While the joint statement issued earlier in March 2018 was reasonable, the array of studies presented and discussed at ASCO 2018 argue more in favor of integrating ctDNA in practice for many gastrointestinal malignancies. For some e.g., CRC, gastroesophageal cancers as well as biliary duct cancers, it is helpful in not only identifying relevant actionable mutations but also in helping identifying secondary mechanisms of resistance. Furthermore, it helps in guiding therapy, as the appropriate chemotherapy drugs can be added to the treatment regimen depending on the emerging clones of resistance detected on ctDNA testing. It also aids in monitoring the response to treatment and predicting survival depending on the level of ctDNA detection in serial analyses. While the technology and the methods may not be ready for use in all stages in all cancers, for patients with metastatic GI cancers, particularly CRC, we would argue similar to discussants at colorectal cancer sessions at ASCO that this is indeed ready for primetime because of its feasibility, rapid turn around and non-invasive nature allowing for serial oncologic assessments. Reevaluation of the literature is needed which could result in an update in the current guidelines for use of ctDNA particularly in late stage gastrointestinal cancers.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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High Circulating Methylated DNA Is a Negative Predictive and Prognostic Marker in Metastatic Colorectal Cancer Patients Treated With Regorafenib

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Background: Regorafenib improves progression free survival (PFS) in a subset of metastatic colorectal cancer (mCRC) patients, although no biomarkers of efficacy are available. Circulating methylated DNA (cmDNA) assessed by a five-gene panel was previously associated with outcome in chemotherapy treated mCRC patients. We hypothesized that cmDNA could be used to identify cases most likely to benefit from regorafenib (i.e., patients with PFS longer than 4 months).

Methods: Plasma samples from mCRC patients were collected prior to (baseline samples $N = 60$) and/or during regorafenib treatment ($N = 62$) for the assessment of cmDNA and total amount of cell free DNA (cfDNA).

Results: In almost all patients, treatment with regorafenib increased the total cfDNA, but decreased cmDNA warranting the normalization of cmDNA to the total amount of circulating DNA (i.e., cmDNA/ml). We report that cmDNA/ml dynamics reflects clinical response with an increase in cmDNA/ml associated with higher risk of progression (HR for progression = 1.78 [95%CI: 1.01–3.13], $p = 0.028$). Taken individually, high baseline cmDNA/ml (above median) was associated with worst prognosis (HR for death = 3.471 [95%CI: 1.83–6.57], $p < 0.0001$) and also predicted shorter PFS (<16 weeks with PPV 86%). In addition, high cmDNA/ml values during regorafenib treatment predicted with

higher accuracy shorter PFS (<16 weeks with a PPV of 96%), therefore associated with increased risk of progression (HR for progression = 2.985; [95%CI: 1.63–5.46; $p < 0.0001$).

Conclusions: Our data highlight the predictive and prognostic value of cmDNA/ml in mCRC patients treated with regorafenib.

Keywords: regorafenib, DNA methylation, metastatic colorectal cancer, cell free circulating DNA, liquid biopsy, digital PCR, biomarkers, prognosis

BACKGROUND

Colorectal cancer (CRC) remains the third most common cancer in men and women worldwide (1). The advent of new therapeutic agents has enhanced the median overall survival (OS) up to 30 months for metastatic CRC (mCRC) patients (2, 3). Among the new lines of treatment added to the therapeutic armamentarium of mCRC, regorafenib is a multikinase inhibitor targeting angiogenic and oncogenic activities in the tumor and its stroma. It has demonstrated single agent efficacy in preclinical cancer models (4, 5) and in patients with chemo-refractory mCRC (6). In 2013, the phase III CORRECT study (7) showed a median OS improvement of 1.4 months, leading to the approval of regorafenib by the EMA. While mCRC patients treated with regorafenib achieved a response rate of 1% and a 16-week disease control rate in 19% cases, up to 54% individuals experienced grade 3 or 4 treatment related adverse events such as hand-foot skin reaction, fever, and fatigue, which severely impair quality of life (8). Consequently, the overall clinical benefit from regorafenib remains rather limited. While no validated biomarkers are available to prospectively identify individuals who could benefit from this drug, several studies have previously explored the use of circulating biomarkers.

A retrospective exploratory analysis of the CORRECT trial showed that baseline circulating total cell free DNA concentration was prognostic rather than predictive for clinical outcome; since both placebo and regorafenib provided a consistent survival benefit in a subset of patients based on low amount of tumor mutation and plasma protein biomarker concentrations (9). Another retrospective analysis by Komori et al. demonstrated that an early decrease in serum CA19–9 protein levels could predict for regorafenib efficacy and was associated with better progression free survival (PFS) in mCRC (10). In a phase II study, a profound decrease of *RAS* mutant clones in circulating tumor DNA (ctDNA) was associated with better PFS in 21 mCRC patients after 8 weeks of treatment with regorafenib, together with modification at dynamic contrast-enhanced MRI (11). Finally, Vandeputte et al. showed the prognostic value of monitoring genetic alterations in the ctDNA of a small cohort of 20 patients. This approach implied next generation sequencing of patient primary tumors and optimization of personalized assays for mutation tracking in plasma (12).

The above-mentioned circulating biomarker studies in mCRC patients undergoing regorafenib treatment were either based on a single parameter or on panels of genetic alterations requiring expensive and time-consuming personalized assay design. Nevertheless, simpler and more universally applicable biomarker would be desirable to improve cost-effectiveness of regorafenib treatment.

We and others recently demonstrated that cancer specific DNA methylation could represent a promising analyte for circulating tumor markers. Thanks to its stability and its specificity to cancer, CRC epigenetic alterations could be detected in plasma cell free DNA at higher prevalence and with a higher allelic ratio than genetic alterations. Some of these methylated loci were also identified as early events in the carcinogenesis process, representing promising cancer specific tools for early diagnosis using blood tests (13–20).

We previously identified a panel of five methylated genes (*EYA4*, *GRIA4*, *ITGA4*, *MAP3K14-AS1*, *MSC*) and used it in a liquid biopsy (LB) test to monitor mCRC tumor burden over the course of conventional chemotherapy regimens (21). Yet, the dynamics of ctDNA under treatment with regorafenib remains to be investigated.

Inspired by data of two recent studies (11, 12) with limited number of patients and using exclusively genetic alterations, we hypothesized that dynamics of circulating methylated DNA (cmDNA) may stratify mCRC patients treated with regorafenib according to their clinical outcome sparing a subgroup of unresponsive patients from prolonged drug exposure.

PATIENTS AND METHODS

Patient Selection and Study Design

We selected 76 mCRC patients who received regorafenib at Niguarda Cancer Center, NCC (Milano) or Istituto Oncologico Veneto, IOV (Padova) from December 2012 to August 2017 (**Supplementary Table S1**). LB samples were collected prospectively and analyzed retrospectively in double blind fashion for patient outcome (**Figure 1**).

Eligible patients had histologically confirmed metastatic adenocarcinoma of the colon or rectum, performance status (PS) 0–1, adequate organ function, age >18 years, life expectancy of at least 12 weeks (based on physician's prognostication according to patient age, ECOG performance status, general conditions, comorbidities, and lab tests including renal and liver

Abbreviations: CEA, carcinoembryonic antigen; cfDNA, cell free circulating DNA; ctDNA, cfDNA of tumor origin; cmDNA, circulating methylated DNA; CRC, colorectal cancer; HR, hazard ratio; LB, liquid biopsy; LDH, lactate dehydrogenase; mCRC, metastatic CRC; OR, odds ratio; OS, overall survival; PFS, Progression free survival; RECIST, Response Evaluation Criteria in Solid Tumors.

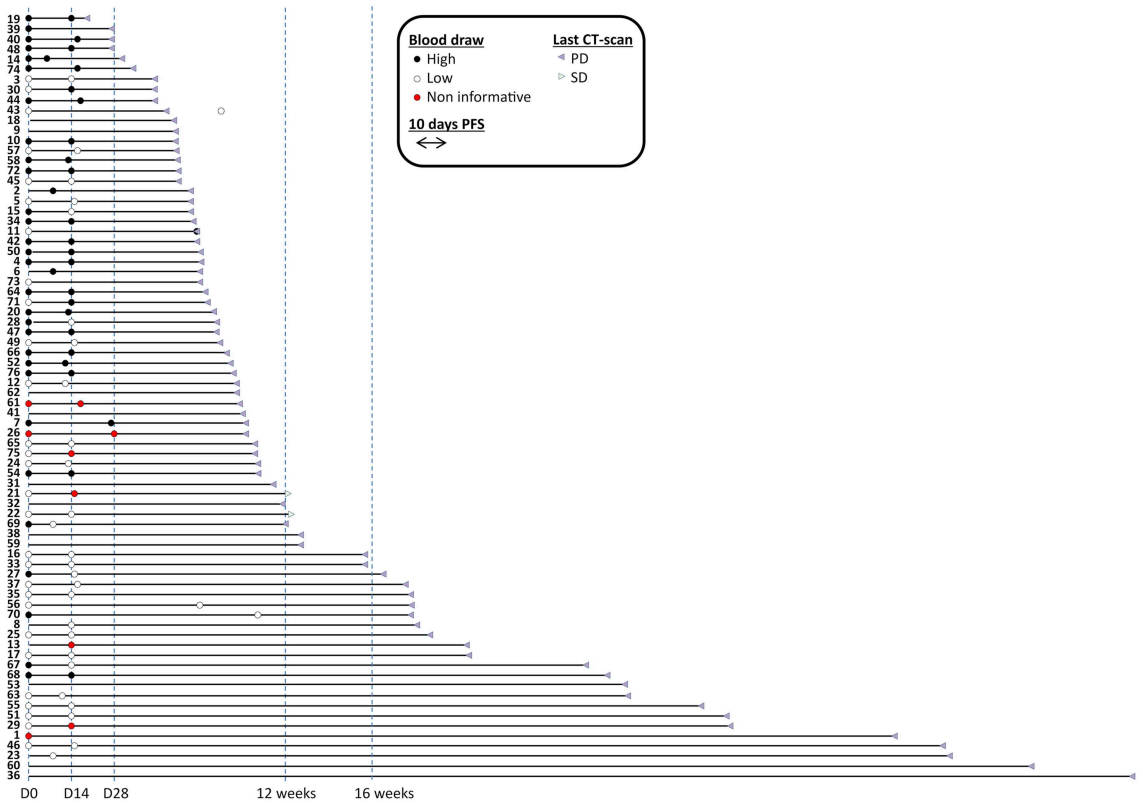

FIGURE 1 | Time to progression of patients enrolled in the study with sample availability and informativeness. Blood draws are represented with filled circles and colored in black when cmDNA/ml was above median, depicted in white when cmDNA/ml was below median; red circles label samples that were not informative (i.e., no positive markers). Patients were sorted by duration of progression free survival. Sixty baseline (among which 57 informative) and 62 under treatment blood draws (among which 56 informative) were available. Fifty seven patients had both types of blood draws available (among which 52 informative in both cases). Two cases were censored for follow-up. Imaging CT-scans are also indicated.

function), and were refractory or intolerant to conventional 5-FU-based chemotherapy treatments. Patients received 160 mg of regorafenib orally once daily for the first 3 weeks of each 4-week cycle, with dose reduction according to physician's discretion and current guidelines (**Supplementary Tables S1, S2**).

Blood was collected at baseline (prior to regorafenib start) and/or then biweekly or at any subsequent access to the hospital, during regorafenib treatment.

All the patients provided written informed consent to LB collection before and during regorafenib treatment. Protocol for blood collection and analysis was approved from NCC and IOV institutional Ethics Committees.

Assessment of Cell Free Circulating DNA Markers

Analysis of cell free circulating DNA (cfDNA) via a panel of methylated CRC-specific genes was carried out on blood as previously described (21).

We defined a value of cmDNA compiled as the methylation average of the markers demonstrating positivity above the previously published thresholds (21), supported by number of methylated events above the limit of detection. In subsequent longitudinal LB, for each patient, calculation was based exclusively on the loci used to compile the cmDNA in the baseline.

Additionally, blood samples from carriers of tumors with *KRAS*, *NRAS*, or *BRAF* mutations were also analyzed by digital droplet PCR commercial assay for the QX200 system (Biorad).

All PCR amplifications were performed in duplicate.

Data Collection

The following data were collected from medical records: patient characteristics, PS, presence of tumor *in-situ*, number and sites of metastases, mutational status of *KRAS*, *NRAS*, and *BRAF*, amplification of *HER2*, and MSI status, baseline lactate dehydrogenase (LDH), carcinoembryonic antigen (CEA).

Tumor response was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. CT scan was performed every 8 weeks from treatment start. PFS was defined as the time from initiation of regorafenib treatment to either radiological or clinical disease progression or death from any reason.

OS was defined as the time from initiation of regorafenib treatment to death from any reason. The cut-off for analyses and follow-up for survival status was 1st March 2018.

Information about regorafenib associated toxicities and dose reduction can be found in **Supplementary Table S1** and are summarized in **Supplementary Table S2**.

Statistical Analysis

All analyses were carried out using the STATA (22) and R software (23). Univariate and multivariate analysis were carried out in all evaluable patients (**Figure 1**). Hazard ratios and 95% CIs for PFS and OS were calculated using the stratified Cox model. Kaplan–Meier curves and comparison were computed using GraphPad 7 (Prism). All other analyses were descriptive. All *P*-values are two-sided.

RESULTS

Patient characteristics are summarized in **Table 1**. A total of 39 patients were male, and the median age was 60 years old. A majority of patients exhibited a good PS (57% with ECOG PS = 0). The median number of previous chemotherapy line was two (range from one to seven). The median number of metastatic sites was three, involving in the majority of cases liver, lung and lymph nodes. Eighty three percent of patients ($n = 63$) had prior resection of the primary tumor. Tumor molecular profiling was retrieved from clinical documentation when available: 62% ($n = 44/71$) were *KRAS* mutant, 3% ($n = 2/58$) *NRAS* mutant, 4% ($n = 3/68$) *BRAF* mutant and 20% ($n = 6/30$) HER2 amplified.

In this cohort no partial response was seen according to RECIST criteria, and the best response was stable disease (SD) in 32.9% ($n = 25/76$) of patients. At a median follow-up time of 5.5 months (1.25–56.5 months), median OS was 5.0 months (range 1–52 months) and median PFS was 10 weeks (range 3–52 weeks).

Main toxicities upon treatment were hand-foot syndrome (46%), hypertension (27%) and skin rash (22%) (summarized data are reported in **Supplementary Table S2**). Dose reduction was required in 41 patients (55%) and 10 patients stopped treatment due to toxicities (13%).

Sixty five patients (86%) had blood draws available for subsequent cfDNA analyses. Fifty-seven patients had both baseline and on-treatment samples, while three cases only had baseline plasma and five patients only under treatment.

TABLE 1 | Patient characteristics.

Characteristic	(*n = 76*)
Male gender–no. (%)	*39* (51%)
Age–year	
Median	60
Range	30–84
WHO performance status–no. (%)	
0	*43* (57%)
1	*33* (43%)
Previous CT line	
Median	2
Range	1–7
Primary tumor resected–no. (%)	*63* (83%)
Number of metastatic sites	
Median	3
Range	1–10
Metastases–no. (%)	
Peritoneum	*20* (26%)
Liver	*50* (66%)
Lung*	*49* (65%)
Nodes	*35* (46%)
Bone§	*9* (12%)
Other§	*18* (24%)
Molecular profile–no. (%)	
KRAS mutation	
Available in	*71* (93%)
Mutated	*44* (62%)
NRAS mutation	
Available in	*58* (76%)
Mutated	*2* (3%)
BRAF mutation	
Available in	*68* (89%)
Mutated	*3* (4%)
Her 2 amplification	
Available in	*30* (39%)
Mutated	*6* (20%)
MSI	
Available in	*44* (58%)
Mutated	*4* (9%)
OS–months	
Median	5
Range	1–52
PFS–weeks	
Median	10
Range	3–52
Alive–no. (%)	*19* (25%)

**75 observations; § 74 observations.*

Circulating DNA Markers at Baseline

Quantity of total cfDNA was successfully determined in all 60 patients from whom baseline LB was available. cfDNA concentration ranged from 4,750 to 4,541,672 genome equivalents per milliliter (GE/ml). Assessment of cmDNA was successful in all samples and positivity was observed in 95% ($n = 57/60$). In the three negative samples (5%), the assay was unable to detect any methylation signal above the limit of detection or limit of blank (**Figure 2A**). This suggests a lack of sensitivity of the assay, possibly due to very limited DNA release from these specific tumors or technical issues related to the DNA extraction process. Another explanation could be the specific site of metastases in those patients. In two out of the three cmDNA negative cases, metastases were limited to lung and lymph node or lung only. A correlation was observed between the cmDNA fraction and the total amount of circulating DNA (expressed in log(GE/ml) in baseline samples: 0.54 (95%CI: [0.33–0.70]; $p = 1.24e\text{-}05$; **Figure 3A**).

Forty one patients with baseline LB sample presented archival tumor tissue mutated for *KRAS*, *NRAS*, or *BRAF* (68.3%), and

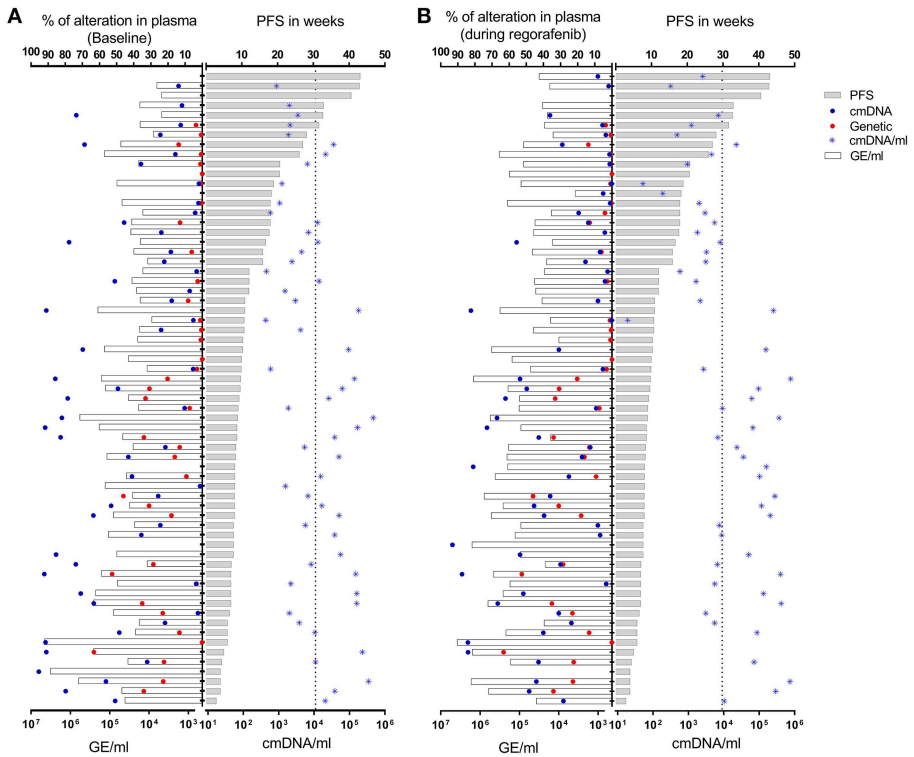

FIGURE 2 | Total amount of cfDNA (expressed as GE/ml), absolute percentage of mutant (genetic) or methylated (cmDNA) alleles, normalized fraction of methylated cfDNA (cmDNA/ml) in plasma samples drawn at baseline **(A)** or under regorafenib treatment **(B)**. Patients are stratified according to their PFS. Vertical dotted lines correspond to median value for cmDNA/ml for each time point.

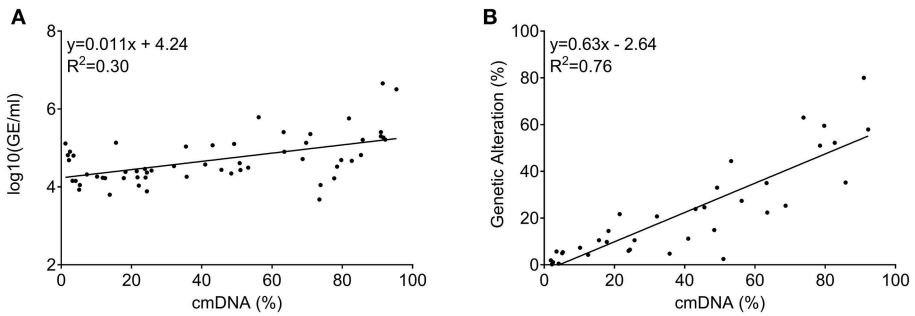

FIGURE 3 | Correlation among circulating DNA markers measured at baseline (prior to regorafenib treatment). **(A)** Correlation between total amount of circulating DNA (measured as GE/ml) and cmDNA. **(B)** Correlation between cancer specific genetic alteration levels (RAS/BRAF mutant alleles) and cmDNA when both were positive.

38/41 (92.6%) demonstrated concordant status for the expected patient specific mutations, as supported by more than one mutational event out of two replicates (**Figure 2A**). Of note, out of the 3/41 LB samples found to be negative for mutations, two were instead positive for cmDNA, while the remaining case was negative for both genetic and epigenetic alterations. The cmDNA fraction was informative in all *19* RAS/BRAF wild type patients, therefore bypassing the need to extensively sequence cancer tissue and design individualized assays in this subpopulation.

Concordance between positive genetic allelic-ratio and cmDNA for baseline samples was 0.87 (95%CI: [0.76–0.93]; $p =$ 6.2e-12; **Figure 3B**), demonstrating the interchangeability of both marker types when both are informative.

Circulating DNA Markers During Treatment and Their Dynamics

Sixty two blood samples drawn after regorafenib start were available (median blood draw time: 14 days (6–75).

Quantity of total cfDNA could be determined in all 62 samples and ranged from 3,764 to 3,810,759 GE/ml. Assessment of cmDNA was successful in all samples and positivity was observed in 90% ($n = 56/62$). Forty patients presented mutated

tumor (66.7%) and *38/42* (90%) demonstrated positivity in the on-treatment blood draw (**Figure 2B**).

We previously observed that changes in circulating DNA markers during chemotherapy treatment were shown to be correlated with patient outcome (21). We therefore investigated the relation between cfDNA markers and outcome upon regorafenib treatment by comparing cfDNA values at baseline (before treatment) and at the first blood draw (day 6–75, median: 14) after treatment initiation. Fifty-two patients had blood draw for both time points.

Contrary to what we previously observed with other drug regimens, regorafenib induced a significant decrease in cancer specific markers (genetic or cmDNA; **Figures 4A,B**) in most patients regardless of the response status, while total amount of cfDNA (measured by GE/ml) usually increased upon treatment (**Figure 4C**). In order to shed light on this unexpected finding, we tested whether regorafenib was able to differentially and directly modulate active release of cfDNA from normal or malignant cells. However, *in-vitro* treatment of either non-malignant or cancer cell lines showed that regorafenib did not significantly affect cfDNA release (data not shown). One alternative hypothesis is that the total amount of cfDNA could originate mostly from non-neoplastic cells due to drug on target and off-target broad effects on several cell types, tissues, and organs. Partially supporting this hypothesis, we observed a non-significant increase in total cfDNA amount (GE/ml) change in patients who required a dose reduction (due to toxicities) while the cmDNA (expected to be solely of tumor origin) remained similar between the two subgroups (**Supplementary Figure 1**). Therefore, we reasoned that the cmDNA fraction should be normalized to total amounts of cfDNA by taking into account the GE/ml, resulting in cmDNA/ml (calculated by multiplying the cmDNA by GE/ml). Compared to baseline, we found that this parameter significantly increases under treatment in patients with fast relapse while it significantly decrease under treatment in patients achieving ≥16 weeks disease control with regorafenib (**Figure 4D**).

In a Cox regression model, we confirmed that an increase in cmDNA/ml from baseline was associated with worst PFS (**Figure 5A**) ($p = 0.028$, HR = 1.78 [95% CI: 1.01–3.13]).

Survival Analysis for Clinico-Pathological Characteristics and Circulating DNA Markers

In the univariate analysis, factors significantly associated with shorter PFS were a high cmDNA/ml fraction at baseline ($p = 7.73e-06$), baseline LDH ($p = 0.0143$), and regorafenib dose reduction ($p = 0.036$).

There was no statistically significant association between PFS and either ECOG PS ($p = 0.11$) or the presence of primary tumor ($p = 0.11$) or drug-related toxicity. Age, previous CT line, baseline CEA and peritoneal metastasis were not significantly associated with PFS ($p = 0.43$, $p = 0.92$, $p = 0.42$, and $p = 0.85$, respectively). In the multivariate analysis, adjusted on baseline LDH, the amount of cmDNA/ml (as log transformed) at baseline remained significant (HR: 1.50 [95%CI: 1.23–1.82], $p = 6.03e-05$).

By univariate analysis, high cmDNA/ml at baseline ($p = 3.61e-08$) and increased bilirubin ($p = 0.003$) were associated with worst OS, while presence of hand foot syndrome associated with better OS ($p = 0.019$). There was no statistically significant association between OS and baseline CEA ($p = 0.117$), baseline LDH ($p = 0.079$), presence of primary tumor ($p = 0.106$), peritoneal metastasis ($p = 0.186$), and regorafenib dose reduction ($p = 0.083$). Age, previous CT line and ECOG PS were not significantly associated with OS ($p = 0.43$, $p = 0.84$, $p = 0.96$, respectively). In the multivariate model, the log transformed cmDNA/ml at baseline remained statistically significant (HR = 1.53 [95%CI: 1.25–1.87], $p = 2.72e-05$).

We performed in parallel a Cox-regression for OS using median as a cut-off value for baseline, and we found an association between high cmDNA/ml at baseline and worst survival (**Figure 5B**; HR for death = 3.471, $p < 0.0001$, [95% CI: 1.83–6.57]). Since in mCRC PFS is a surrogate endpoint for OS (24), further analyses were conducted to explore differences in terms of PFS. Using median as cut-off on both time points, we found an association between higher risk of relapse during regorafenib treatment and high cmDNA/ml at baseline (**Figure 5C**; HR for progression = 2.196, $p = 0.0015$, [95% CI: 1.26–3.84]) and during treatment (**Figure 5D**; HR for progression = 2.985, $p < 0.0001$, [95% CI: 1.63–5.46]).

DISCUSSION

Due to its stability and cancer specificity, cmDNA could be a promising source for tumor biomarkers. Recent studies analyzed the correlation between the methylation status of specific genes and response to therapy and prognosis in colon and rectal cancers (25–27).

In the present study, we have shown that by using a universal five-gene panel, cmDNA was detectable in 57/60 cases, corresponding to 95% of the whole patient population. In contrast, in the same cohort through the analysis of candidate hotspot mutations in RAS/BRAF genes by LB, we were able to detect mutations in plasma cfDNA in only 38/60 patients, corresponding to 95% of cases known to carry RAS/BRAF mutations in tumor tissue samples, but only to 63.3% of the entire cohort. This confirmed the universality of methylation markers for liquid biopsy and their possible better suitability for large cohort analyses such as epidemiologic studies.

To overcome the caveats and costs of assessing genetic alterations in cfDNA by large panels, several studies have proposed the use of targeting sequencing in archival tissue to identify cancer patient-specific genetic markers (12, 28–30). While such approach can certainly improve the specificity of LB assays, it would undoubtedly increase the costs as well as the sample processing time (due to optimization of single variation assay). This is of clinical importance since absence of candidate mutation would require NGS analysis of primary tumor and subsequent personal assay design, impairing the clinical turnaround, and the application of

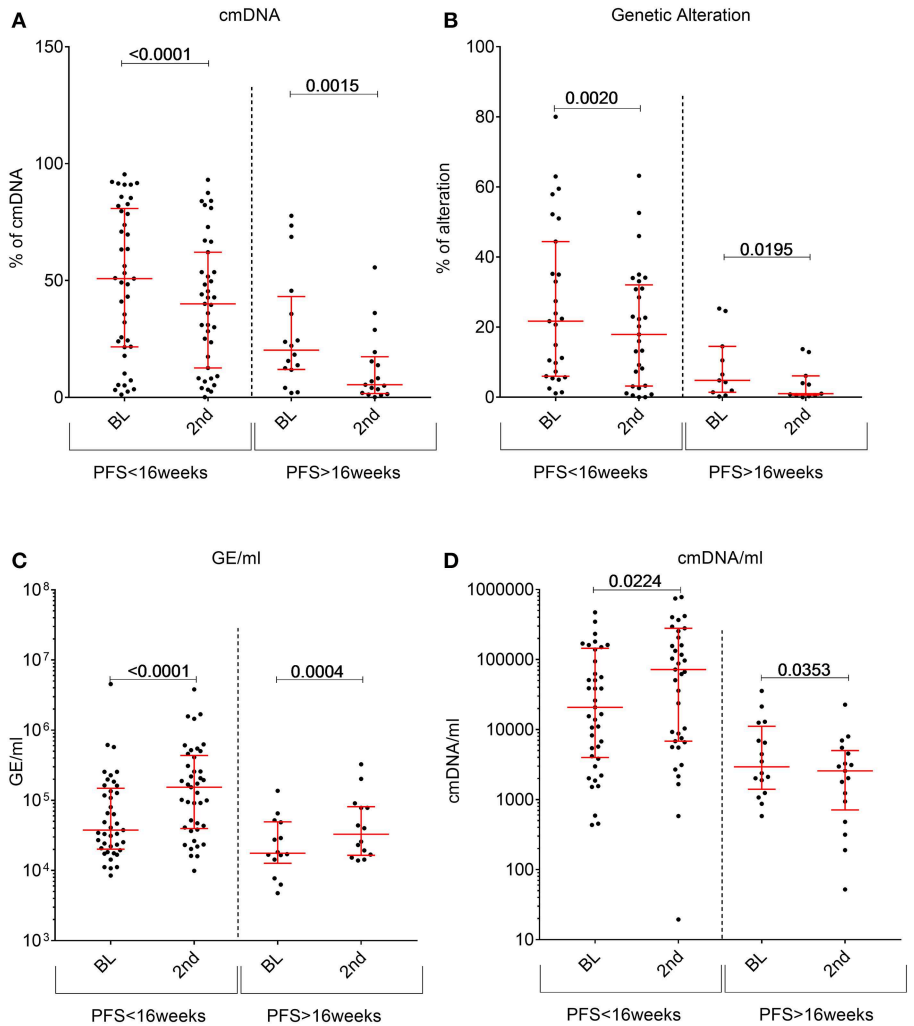

FIGURE 4 | Changes in circulating DNA features between baseline plasma samples (abbreviated as BL) and the second plasma time point collected during regorafenib treatment (labeled as 2nd). For each feature patients were stratified according to their PFS status at 16 weeks. **(A)** cmDNA, **(B)** Genetic alterations, **(C)** total cfDNA amount in GE/ml, **(D)** cmDNA/ml. *p*-values compiled using two-tailed *u*-test.

cfDNA in prospective analyses. Consequently, methylation assays might be more prone to be developed into routine clinically applicable tests for disease monitoring purposes. In this regard, we note that methylation based assays of cfDNA have already been proposed for early detection and cancer classification (31, 32).

We and others demonstrated that cmDNA reflects tumor burden; since its level correlated with the presence of unresected primary tumor, of bulky disease or of multiple metastatic lesions, whereas age and mutational status did not influence the cmDNA (21, 33, 34). We speculated that longitudinal assessment of cmDNA during chemotherapy could reflect the dynamics of tumor burden with a decrease potentially preceding response and an increase anticipating progression.

In the 52 mCRC patients with both evaluable baseline and under treatment plasma samples from present study, we observed a decline in cancer specific cfDNA markers (genetic or cmDNA) upon treatment with regorafenib that unexpectedly took place in most cases, while total amount of cfDNA increased. This behavior of cancer specific markers was different from what we previously observed using other anticancer therapies (5FU based chemotherapy, panitumumab or temozolomide). We therefore speculate that the observed decrease in cancer specific markers during regorafenib treatment could be due to a diluting effect by normal DNA shedding from healthy tissues (possibly due to cytotoxicity) as previously suggested by a report on a cohort of 20 regorafenib treated mCRC patients (12). This observation highlights the need for careful validation of LB assay according to the treatment used, and warrants fundamental research to improve our understanding of the factors influencing release of cfDNA by human cells.

Nevertheless, after normalization, a cmDNA/ml an increase upon treatment was associated with progressive disease while

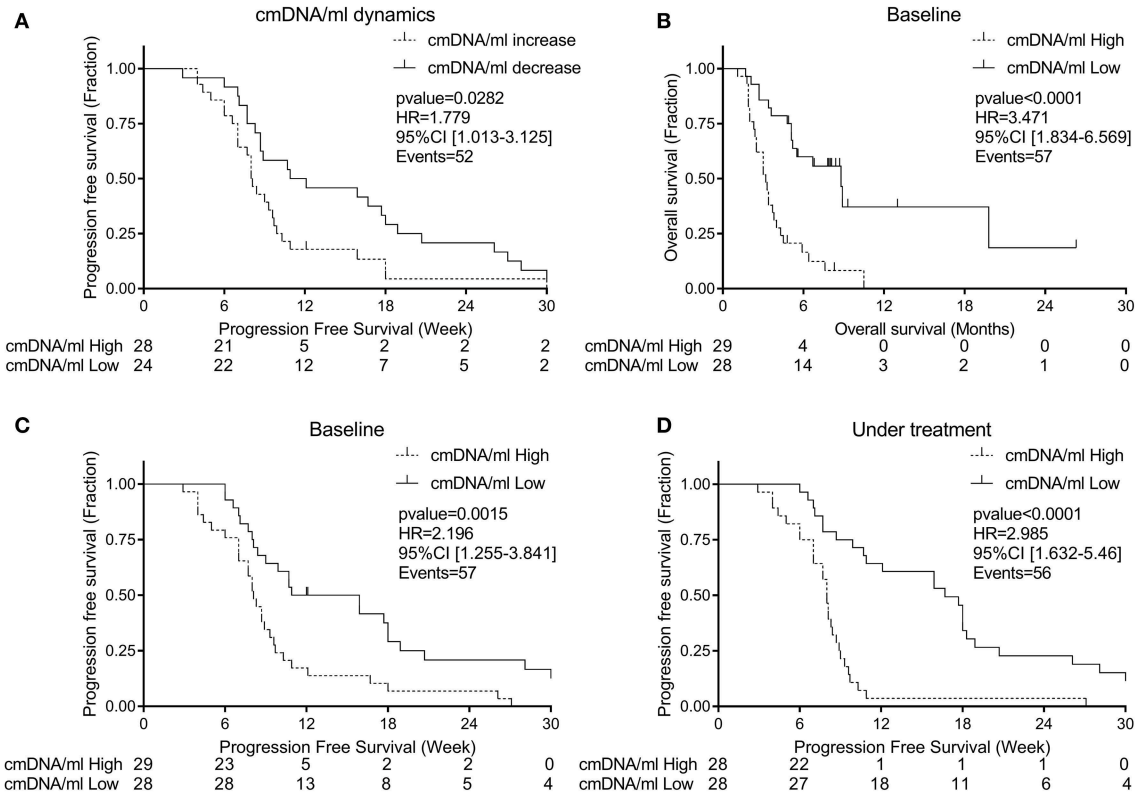

FIGURE 5 | (A) Progression free survival according to cmDNA/ml dynamics (decrease dash line; increase solid line), **(B)** Overall survival according to baseline cmDNA/ml, **(C)** Progression free survival according to baseline cmDNA/ml, **(D)** Progression free survival according to level of cmDNA/ml under treatment (Low ctDNA level dash line; High ctDNA solid line).

a decrease was associated with clinical benefit and improved PFS showing that despite being confounded by normal DNA release, dynamics of cmDNA was associated with drug activity. Contrary to conventional chemotherapy or targeted therapy based regimens which often trigger significant tumor burden changes, regorafenib seldom induces RECIST responses. The lack of tumor burden dynamics given by regorafenib is likely to influence the dynamics of ctDNA levels.

We identified cmDNA/ml at baseline as a prognostic marker. Through a multivariate cox-regression analysis, cmDNA/ml maintained a significant impact on both PFS and OS which was higher than other clinical variables such as age, previous CT, ECOG PS, presence of primary tumor, peritoneal metastases, CEA, and LDH. Consequently, integration of cmDNA/ml as auxiliary staging parameter might improve patient disease classification (35).

High values of cmDNA/ml (above median) during treatment were significantly associated with a higher probability for disease progression, suggesting that abrogation of ctDNA release needs to be achieved soon after treatment initiation in order to observe better PFS. This is in accordance with the literature demonstrating that early circulating biomarkers change is associated to clinical benefit (10, 12).

We acknowledge that our study is limited by its retrospective nature. Recent studies (36, 37) suggested that toxicity might be related to better prognosis upon regorafenib treatment. In our dataset, no specific toxicity was associated with improved PFS, however the need for dose reduction (surrogate for toxicity related comorbidity) was associated with improved response duration.

We found a difference in cmDNA/ml levels between progressing patients and those who achieved clinical benefit. Nevertheless, the establishment of an optimized threshold to clearly distinguish between these populations will be needed to stratify individual patients. Unfortunately, the number of patients with clinical benefit was relatively small due to the modest efficacy shown by regorafenib in this setting. Therefore, modeling an optimal threshold in our cohort was not possible due to the lack of statistical power. As a consequence, we preferred to use the median as a natural and unbiased cut-off for this work. We acknowledge that our study could not provide validation of such a cut-off in a separate cohort. Future efforts in this direction will require multicenter enrollment and long term follow-up to reach a large number of cases with clinical benefit. This may validate the application of cmDNA/ml for predicting regorafenib response in individual patients. Nevertheless, this is the first report investigating the correlation between survival

and a methylated gene panel in mCRC treated with regorafenib. Furthermore, data from the present cohort confirmed the general predictive value of cmDNA in mCRC reported in our previous work (21). However, the dynamics of the cmDNA may be affected by different treatments, since we observed a peculiar decrease in cancer specific markers (genetic or cmDNA) during regorafenib treatment in most of patients regardless of the response status warranting its normalization by total amount of cfDNA.

In conclusion, cmDNA/ml is of prognostic value and is a dynamic biomarker which longitudinal assessment could be used relatively early during the treatment of mCRC patients, before radiological assessment, to identify the patients with a negative prognosis.

CONCLUSIONS

To conclude, DNA methylation in cfDNA is a cancer specific biomarker that could be employed to track response during therapy in mCRC, enabling non-invasive monitoring of tumor burden. It could be used to select patients with poor survival who are not likely to benefit from regorafenib treatment and might allow faster therapeutic reorientation avoiding overexposure to the drug and possible side effects.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Niguarda Cancer Center (Milano) and Istituto Oncologico Veneto (Padova) Institutional Ethics Committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Niguarda Cancer Center (Milano) and Istituto Oncologico Veneto (Padova) Institutional Ethics Committee.

AUTHOR CONTRIBUTIONS

AA, MS, FT, SL, EB, LPa, DP, EP, FB, GA, RI, LPr, VZ, SS, FL, and AS-B participated in the patients recruitment and follow-up and in the acquisition of clinical information and blood draws. LB and BM processed the blood samples (cfDNA extraction) and performed the molecular experiments. AA, MS, FT, and LB reviewed the data. MN, AA, and LB performed the statistical analyses. MA provided preclinical samples and participated in functional analyses design. AA, MA, MS, SM, VZ, SS, AB, FL, FD, AS-B, and LB designed the study. FD, AB, and LB designed the molecular experiments. AA, MS, FD, AS-B, and LB wrote the manuscript. All authors revised the manuscript critically for important intellectual content and approved its final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00622/full#supplementary-material

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Conflict of Interest Statement: AA participated to advisory boards for Amgen and Bayer. AS-B participated in advisory boards for Amgen, Bayer, and Sanofi.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer RG declared a past co-authorship with several of the authors MS, FL, FB, SL, and VZ to the handling editor.

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Precision Medicine Tools to Guide Therapy and Monitor Response to Treatment in a HER-2+ Gastric Cancer Patient: Case Report

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Trastuzumab, has played a major role in improving treatment outcomes in HER-2 positive gastric cancer. However, once there is disease progression there is a paucity of evidence for second line therapy. Patient-derived xenografts (PDXs) in combination with liquid biopsies can help guide individual therapeutic decisions and have now started to be studied. In the present case we established a PDX model from a metastatic HER-2+ gastric cancer patient and after the first engraftment passage we performed a mouse clinical trial to test T-DM1 as an alternative therapy for the patient. The PDX tumor response served as a guide to administer T-DM1 therapy to the patient who responded to treatment before relapsing 6 months later. Throughout out the clinical follow up of the patient, ctDNA levels of HER-2 copy number and a PIK3CA mutation were monitored and we found their correlation with drug response and disease progression to outperform that of CEA levels. This study highlights the utility of applying precision medicine tools combining PDX models to guide therapy with circulating tumor DNA (ctDNA) to monitor treatment response and disease progression.

Keywords: precision medicine, patient derived xenograft, ctDNA, gastric cancer, HER-2+, T-DM1

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BACKGROUND

Precision Medicine focuses on tailoring treatment for the tumor specific characteristics of individual patients (1). This implies moving beyond using large phase 3 trials of unselected, though superficially similar patients, to select the best therapy for one patient. The potential is enormous both for patients and for the healthcare system. Important clinical and biological challenges still stand in the way, including the fact that intra-tumoral heterogeneity may limit the ability of tumor biopsies to capture the complete biological portrait of the tumor (2). Moreover, next generation sequencing frequently identifies multiple variants, sometimes more than one that can be matched to an available drug, and distinguishing driver from passenger variants remains elusive (3, 4). Efforts are underway to address this latter point by including transcriptomic and proteomic analyses (5–7), but a more patient-focused and direct approach is presented here. An attractive solution to the dearth of actionable mutations is to directly test tumor response on patient- derived tissue samples. Patient-derived xenografts (PDX) have provided new insights to efficiently test clinical samples with various drug combinations. This allows for correlations between PDX tumor response and patient clinical response (8). Although the use of PDX models to guide therapeutic decision has been

challenging because of the lengthy process of tumor engraftment, expansion, and drug testing (9), this approach has been studied in many solid tumors such as breast (10), colon (11), renal cell (12), and duodenal cancer (13). Another possible solution to the problem of intra-tumor heterogeneity is the use of circulating tumor DNA (ctDNA), which may be a broader method to assess tumor molecular composition (14). We present a case of a patient with metastatic human epidermal growth factor receptor (HER-2) positive gastric carcinoma in which treatment decisions were guided by PDX data and treatment response was monitored with ctDNA markers on serial blood samples. This is a prime example of the power of cutting-edge personalized precision medicine based on PDX testing and ctDNA measurements in changing clinical care in oncology.

CASE PRESENTATION

Patient is a 76-year-old male diagnosed with metastatic HER-2 positive moderately differentiated gastric adenocarcinoma in April 2011. Staging scans demonstrated metastatic lesions in the lung and liver at time of diagnosis. The patient was started on carboplatin and paclitaxel followed by Xeloda along with Trastuzumab. He had a dramatic response, with disappearance of all evidence of disease, except for modest PET uptake in the gastric primary site. Since he was asymptomatic, he was maintained on single agent Trastuzumab from June 2012–2016. Serial surveillance PET CT scans demonstrated no FDG-avid disease up until he began to experience increasing symptoms of post-prandial dysphagia and epigastric pain in 2015. In July 2015, a gastroscopy showed a large ulcerative lesion in the lesser curvature of the stomach, which was significantly larger than on previous examinations. In December 2015 he underwent open subtotal gastrectomy with a Roux-en-Y anastomosis. Pathology confirmed the same intestinal type adenocarcinoma G3 poorly differentiated, pT4pN3b, with 27 out of 35 lymph nodes involved. HER-2 status was positive by immunohistochemistry (IHC) and copy number analysis using Cytoscan HD (**Figures 1A,B**). A piece of this tumor was collected for molecular analysis and PDX engraftment for drug testing. In February 2016, he developed melena, and was found to have a bleeding ulcer, which was biopsy proven invasive adenocarcinoma. He underwent palliative radiation therapy with concurrent low dose Capecitabine chemotherapy. In August 2016, new hypermetabolic lung nodules were detected on another PET scan. Capecitabine was discontinued in November 2016 and a CT scan performed in December 2016 showed the appearance of new sub-pleural hypermetabolic nodules. By this time, PDX results showed excellent response of PDXs to T-DM1. We had difficulty in obtaining off-label T-DM1, based on the negative results of a randomized study of T-DM1 in this setting (15). Nevertheless, since there was no obvious alternative, we persisted, and he finally began treatment with T-DM1 in March 2017. The patient did not receive any medication between November 2016 and the beginning of T-DM1. The patient completed 3 cycles of treatment and a CT scan in early May 2017 demonstrated treatment response and the patient reported complete disappearance of coughing symptoms and felt more ease at breathing (**Figure 1C**). T-DM1 was interrupted for 3 weeks to confirm that the imaging was indeed demonstrating tumor and not infection in the lung and treatment resumed in July 2017. The patient was maintained on T-DM1 with no reported side-effects until disease progression in September 2017 (**Figure 1C**) and he passed away from CNS disease in January of 2018.

PDX DEVELOPMENT AND DRUG TESTING

Pieces of tumor from the gastrectomy were engrafted subcutaneously in one immune-deficient mouse (NOG). Adenocarcinoma was confirmed by histology in the PDX tumor and HER-2 amplification was confirmed by copy number analysis and IHC and matched the original patient tumor (**Figures 1A,B**). An initial drug study was performed at the first PDX generation passage (F1) in May 2016. Interestingly, of the 4 mice treated with Trastuzumab, only 1 demonstrated drug resistance while treatment with T-DM1 resulted in tumor regression in all mice tested (**Figures 2A,B**). A second drug study was performed in October 2016 on PDXs (F3) generated from the Trastuzumab resistant PDX (mouse 712). This study showed emergence of resistance to Trastuzumab and validated the rapid and durable response to T-DM1 (**Figures 2C,D**).

DEVELOPMENT OF PERSONALIZED ctDNA ASSAYS AND MONITORING OF RESPONSE TO TREATMENT

The gastrectomy tumor and PDX tissues underwent targeted MiSeq for the analysis of somatic mutations and copy number analysis. Four different somatic mutations were identified (**Table 1**) and digital droplet PCR (ddPCR) assays were developed for the PIK3CA variant and the HER-2 amplification. ddPCR analyses were performed on serial bloods collected at each clinical follow up. We observed an increase in ctDNA levels in January 2017 as resistance to Trastuzumab was setting in clinically, with new lesions appearing in the lungs. We noted that ctDNA levels had already begun to fall significantly in April 2017, 3 weeks after starting T-DM1 treatment. Interestingly this fall in ctDNA levels of both HER-2 copy numbers and mutated PI3KCA correlated with clinical response while CEA levels were markedly increasing (**Figure 3**). After a short interruption in T-DM1, a second cycle was given, not before some rise in ctDNA levels. The administration of this second T-DM1 cycle resulted in a prompt decrease in ctDNA levels, until progression, which occurred in September 2017 (**Figure 1C**). At this point, ctDNA levels had markedly risen, coincident with disease progression, while, in contrast CEA levels had decreased and did not reflect disease status (**Figure 3**).

DISCUSSION

The era of Precision Medicine is highlighting new technologies that help identify and predict therapies that may benefit patients,

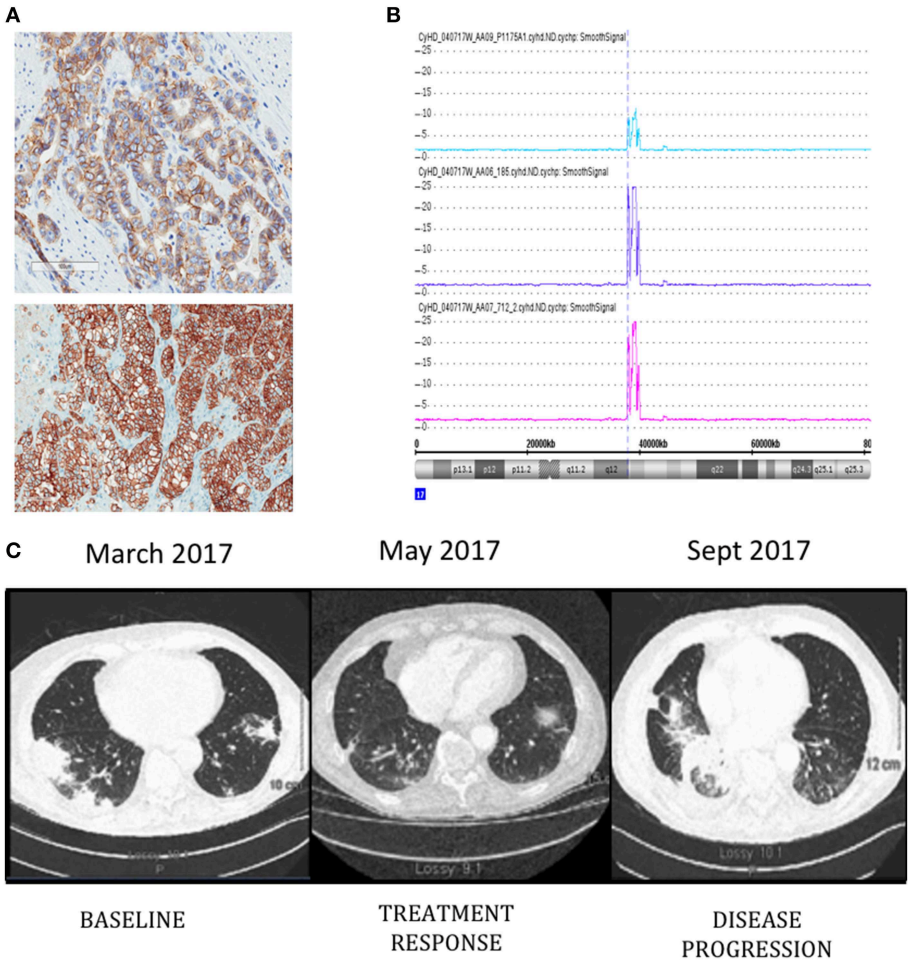

FIGURE 1 | ERBB2 expression detected by immunohistochemistry **(A)** in original patient tumor (top) and PDX 712 tumor (bottom). Visualization of Chromosome 17 region containing the ERBB2 amplicon **(B)**, the number of ERRB2 copies are shown in original patient tumor (blue) and PDX tumors F0 (purple) and F1 (pink). Computed tomography (CT) images of the patient's thorax **(C)** before T-DM1 treatment (left panel), at the time of treatment response (middle panel), and progression (right panel).

in the absence of phase 3 supportive data for an unselected but similar patient group. In this case, the PDX model predicted response to T-DM1 in second line for HER-2+ gastric cancer, despite negative phase 3 data in similar but otherwise unselected patients. Our case highlights how patient treatment planning can be tailored based on PDX models and how treatment response can be monitored with ctDNA. Even after HER-2 functional blockade with Trastuzumab was no longer clinically effective, the persistence of HER-2 in the tumor served both as an effective passive target for the antibody-dependent cytotoxic approach, as well as a tumor-related cell-free DNA biomarker that appears to be significantly more effective than current standard blood CEA test in predicting disease status. The ability to quantitate ctDNA over time has tremendous implications both for clinical progress of tumor volume and the biological study of tumor evolution over its entire trajectory, while avoiding serial tumor biopsies that are often difficult for patients to accept (16, 17). In our case, a PDX was generated from the gastric cancer as it became resistant to Trastuzumab. Interestingly, Trastuzumab resistance was observed in only one of 4 mice initially tested, highlighting the heterogeneity of PDX samples with respect to treatment response. Further testing with Trastuzumab of PDXs generated from the Trastuzumab resistant mouse showed the emergence of resistance after 2 months of treatment suggesting an enrichment for drug resistant clones. This highlights the limitations of using a single PDX mouse for treatment decisions. On the other hand, response to T-DM1 was uniform in all PDX mice tested. Ideally, to minimize the effects of genomic drift on drug response, drug testing should be performed at early PDX passages. Here we performed drug studies at passages F1 and F3 with similar responses to T-DM1. Although we obtained PDX evidence of T-DM1 response in May 2016, the patient started T-DM1 treatment in March 2017 after a long process to access T-DM1 off label. Interestingly, the PDX model established from the primary gastric tumor could predict patient response of the lung metastatic lesions, and

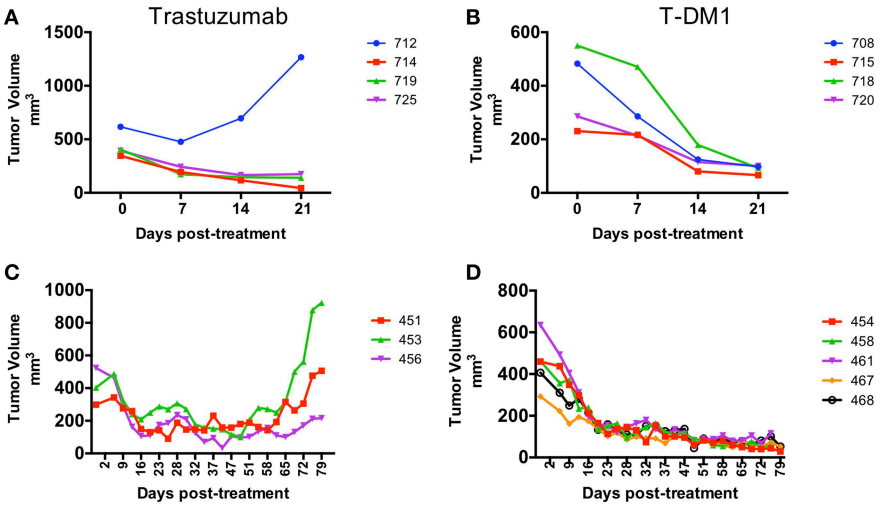

FIGURE 2 | Response to treatment in PDX models. PDX drug efficacy of Trastuzumab and T-DM1 in two consecutive studies performed on NOG mice engrafted with F0 PDX tumor **(A,B)** and with Trastuzumab resistant PDX tumor from mouse 712 (F1) **(C,D)**. Data are presented as tumor volume (mm^3) during treatment days (x axis). Each colored line represents a single mouse PDX.

this, months later, and despite the tumor evolution that could have taken place after capecitabine treatment. Although many reports have correlated clinical drug response with response in PDXs, the majority of these studies have done so in a retrospective manner (8, 18–21). The use of PDXs to guide clinical treatment decisions in a prospective manner or in real-time is challenging and very few cases have been published (22–24). Our study demonstrates the feasibility of using PDXs to identify personalized effective therapies in patients with limited therapeutic options. Circulating tumor DNA (ctDNA) is an emerging method to monitor treatment response oftentimes in advance of imaging evidence. The value of ctDNA testing in gastric cancer patients has been studied using targeted panels or specific ddPCR assays to detect somatic mutations and gene amplifications (25–27). In particular, the measurement of HER-2 copy number levels in ctDNA holds great promise for its potential clinical use to identify gastric cancer patients who may benefit from Trastuzumab and to monitor response to treatment and disease progression (27–29). In our study we used ctDNA assays to detect the HER-2 amplification and a PIK3CA variant in the peripheral blood and monitor drug response. We observed that changes in both ctDNA markers paralleled each other very tightly and that ctDNA levels were sensitive predictors of response and disease progression and outperformed CEA testing. These findings are consistent with other reports in which increase in ctDNA levels coincide with recurrence of gastric cancer (14, 28, 29) and those of Garcia-Murillas et al. (30), in which ctDNA level changes anticipated imaging evidence of tumor progression by almost 8 months. Our case illustrates the direction that cancer care is evolving toward. We created PDX models that guided our patient's care, and followed tumor response using ctDNA, based on sequencing of the tumor. This case highlights the power of combining 2 cutting edge tools, PDXs and ctDNA, which together allow us to more successfully apply precision medicine in metastatic gastric cancer.

TABLE 1 | Somatic mutations identified in original patient tumor engrafted in PDX (F0) and the Trastuzumab resistant PDX 712 (F1).

Gene	Variant	Allele frequency F0	Allele frequency F1 (712)
PIK3CA	c.317G>T	0.44	0.35
PIK3R1	c.428-21G>A	0.99	0.99
CDHI	c.687+1_687+2delGT	0.93	0.93
TP53	c.733G>A	0.99	0.99

METHODS

(For a detailed description of the methods, see **Supplementary Methods**).

Ethics

The patient provided written informed consent for sample collection and use as part of the JGH Central Biobank, the biobank protocol (#10–153) was approved by the Jewish General Hospital research ethics committee. We have obtained written informed consent from a participant's relative for the publication of this case report and any potentially-identifying images/information.

PDX Development

Two pieces (2 ×2 mm) of tumor were implanted sub-cutaneously into both flanks of one 5-week old NOG (NOD.Cg-Prkdcscid Il2rgtm1Sug/JicTac) mouse (Taconic Labs). Tumor growth was monitored with calipers and 3 months later, when the tumor reached 1,500 mm^3, fresh pieces of tissue were engrafted into one flank of 12 NOG mice. Mice were treated with vehicle saline, Trastuzumab (5 mg/kg, i.p once a week) or T-DM1 (10 mg/kg, i.p, once a week). A second drug study was carried out using the trastuzumab resistant PDX mouse 712 which was transferred into both flanks of 3 NOG mice. Pieces of tumors from 2 of these mice were used to

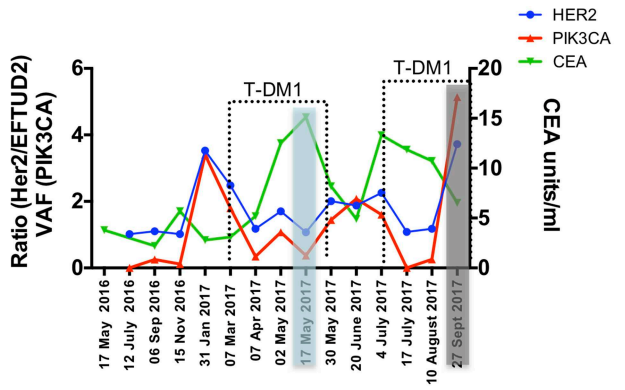

FIGURE 3 | Circulating markers were measured in serial plasma collected from the patient throughout 18 months of clinical management. Data are presented as variant allele frequency (VAF) for the PIK3CA mutation, ratio of number of copies of HER-2 /EFTUD2 and units per ml for CEA. Dashed lines delineate time points of T-DM1 treatment and shaded areas correspond to clinical response (blue) and progression (gray).

generate the next treatment cohort of 11 NOG mice which were treated with the same drugs as above. Tumor size was monitored by electronic caliper measurement and mice were monitored for signs of toxicity (weight loss, bleeding etc) and tumor burden.

Nucleic acid extractions: DNA and RNA were extracted from the patient's tumor and PDX samples as previously reported (31).

ctDNA Assays

Cell free DNA (cfDNA) extraction and development of ddPCR assays were performed as previously reported (32). Briefly, in order to estimate PIK3CA (G106V) AF a pre-amplification of 5–10 ng cfDNA step was incorporated prior to ddPCR reaction using primers listed in **Supplementary Table S1a**. The PCR amplified products were diluted and combined with mutant and wild-type probes for PIK3CA G106V mutation detection (**Supplementary Table S1a**). ddPCR was then performed on Biorad C1000 thermal cycler incubating the plates at 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 55°C for 60 s, followed by 10 min incubation at 98°C and plates were read on a Bio-Rad QX200 droplet reader.

To test HER-2 gene amplification status with ddPCR we used the EFTUD2 gene as a reference since it shows a highly stable copy number ratio with the ERBB2 locus (33). HER-2 amplification was defined as the HER-2/EFTUD2 ratio. For each sample 10–50 ng of cfDNA were partitioned into 20,000 droplets with primers and probes for HER-2 and EFTUD2 genes (**Supplementary Table S1b**). ddPCR was then performed on Biorad C1000 thermal cycler incubating the plates at 95°C for 10 min followed by 40 cycles of 98°C for 15 s, 55°C for 60 s, followed by 10 min incubation at 98°C and plates were read on a Bio-Rad QX200 droplet reader.

DATA AVAILABILITY

Raw data were generated at the Sick Kids Hospital and Jewish General Hospital Molecular Pathology facilities. Derived data supporting the findings of this study are available from the corresponding author upon request.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Jewish General Hospital Ethics board with written informed consent from all subjects. The patients gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Jewish General Hospital Ethics Review Board.

AUTHOR CONTRIBUTIONS

AA-M, MBa, and GB designed the study. SJ and UK collected clinical data. UK collected clinical samples, MBu performed PDX experiments, LC developed ctDNA assays. AA-M supervised and managed the data generation and data analysis. MBa, GB, AA-M, and LC contributed to the analysis and interpretation of the data. AA-M, MBu, MBa, GB, SJ, and LC reviewed all drafts of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00698/full#supplementary-material

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REVIEW
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The Mutational Landscape of Pancreatic and Liver Cancers, as Represented by Circulating Tumor DNA

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The mutational landscapes of pancreatic and liver cancers share many common genetic alterations which drive cancer progression. However, these mutations do not occur in all cases of these diseases, and this tumoral heterogeneity impedes diagnosis, prognosis, and therapeutic development. One minimally invasive method for the evaluation of tumor mutations is the analysis of circulating tumor DNA (ctDNA), released through apoptosis, necrosis, and active secretion by tumor cells into various body fluids. By observing mutations in those genes which promote transformation by controlling the cell cycle and oncogenic signaling pathways, a representation of the mutational profile of the tumor is revealed. The analysis of ctDNA is a promising technique for investigating these two gastrointestinal cancers, as many studies have reported on the accuracy of ctDNA assessment for diagnosis and prognosis using a variety of techniques.

Keywords: circulating tumor DNA (ctDNA), pancreatic ductal adenocarcinoma (PDAC), hepatocellular cancer (HCC), somatic mutations in cancer, tumor heterogeneity

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INTRODUCTION

Both pancreatic and liver cancers show high mortality rates and a poor outcome, in part due to a complex and heterogeneous mutational landscape that hinders diagnosis and prognosis. The detection of this mutational profile has traditionally required tissue biopsy, a highly invasive procedure. Recent developments have indicated the potential of liquid biopsies, such as those which analyse circulating tumor DNA (ctDNA) (1). By understanding the mutational landscape of these tumors, and to what extent the ctDNA mutational landscape reflects this, our understanding of how liquid biopsies can be useful in personalized therapy will be improved.

Pancreatic and liver cancer most commonly present in the forms of pancreatic ductal adenocarcinoma (PDAC) and hepatocellular carcinoma (HCC). PDAC is the fourth most common cause of cancer death and thirteenth most common cancer, with incidence and mortality on the increase. Risk factors include chronic pancreatitis, alcohol abuse and obesity (2). HCC is the second leading cause of cancer death worldwide and the sixth most common cancer, with incidence rates highest in Eastern Asia and sub-Saharan Africa. Risk factors for HCC development include hepatitis infection, alcoholic and non-alcoholic liver disease, cirrhosis, and exposure to aflatoxins (3).

Mutations within the genome drive the progression of both pancreatic and liver cancer. While some mutations are very commonly observed across multiple cancer patients, others are less

frequent, representing heterogeneity within the mutational landscape. Both pancreatic and liver cancers show a high amount of somatic mutations, around 50 per tumor (4, 5). The presence and/or absence of specific mutations can dictate cancer therapy, and hence detection of the mutational profile of a given patient is a necessary step in effective treatment (6).

Tumor heterogeneity, an effect of genome instability, reduces the efficacy of targeted agents in personalized therapy, a therapeutic approach in which treatments are chosen based on the molecular basis of the disease in the individual (7). While markers for both PDAC and HCC exist, these markers have limitations which affect their clinical use. In PDAC, the most commonly used biomarker is elevated levels of carbohydrate antigen 19-9 (CA19-9), though this approach shows low positive predictive value for asymptomatic patients (<0.9%) despite high sensitivity (100%) and specificity (98.5%) (8). The most widely used HCC biomarker is serum alpha-fetoprotein (AFP), though its clinical use is limited by its lack of sensitivity (39–65%) and specificity (76–94%) (9, 10).

In this review, we discuss the mutational landscapes of both pancreatic and liver cancer, and how well they are represented by analysis of ctDNA. We begin by discussing the pathology of PDAC and HCC, and the signaling pathways on which these mutations converge. We then look at what is known about ctDNA and its release, and then discuss the methods used for isolation and analysis of ctDNA. We finally consider the many studies which have detected ctDNA in the analysis of PDAC or HCC, and the extent to which these studies can accurately identify mutations within the disease state (**Figure 1**).

PANCREATIC AND LIVER CANCER PATHOLOGY

PDAC and HCC development are both driven by somatic mutations, meaning they occur within an individual cell after conception and were not present in the previous generation. Driver mutations, which directly promote tumor growth, vary between different cancers, but tend to occur early on in disease development (11).

PDAC occurs in around 90% of pancreatic cancer cases, developing from normal acini, through precursor lesions, to ductal carcinoma. Mutations within pancreatic epithelial cells drive acinar-ductal reprogramming, and then the development of various stages of pancreatic intraepithelial neoplasia (PanIN) and then full PDAC. This development also involves the appearance of environmental characteristics such as desmoplasia, hypoxia, solid and fluid pressure, and autophagy (12).

The development of PDAC, and its connection with the underlying genetic alterations of driver genes, is proposed to follow the multi-hit model. The first hit involves a mutation in the *KRAS* gene and overexpression of the receptor tyrosine kinase ErbB2 (*ERBB2* gene). Surviving cells are then altered by the second hit, in which the tumor suppressor gene *CDKN2A* becomes mutated through promoter methylation, leading to PanIN-1. Thirdly, tumor suppressor genes such as *TP53* and *SMAD4* become inactivated through mutation, leading to PanIN-2/3 and then PDAC (13–15). Loss of heterozygosity (LOH), where deletion of one of the copies of the gene occurs and therefore sensitizes the remaining copy to oncogenic mutations, is also linked to progression of different PanIN stages directly to PDAC. e.g., loss of heterozygosity at 17p, 18q, and 9p promotes PanIN-1 progression to PDAC (16) (**Figure 2A**).

The pathogenesis from healthy liver to HCC, the most common form of liver cancer, can be instigated in multiple ways. Chronic hepatitis B or C virus (HBV/HCV) infection, a diet rich in aflatoxins, or metabolic diseases can promote chronic hepatitis, which progresses to cirrhosis, a state often reached with high alcohol intake. This progression is associated with genetic instability. Cirrhosis precedes HCC in around 90% of patients and contains areas of abnormal hepatocytes known as dysplastic foci (<1 mm). These foci then develop into dysplastic nodules (>1 mm), and further develop into HCC. This progression from cirrhosis to HCC involves the accumulation of genetic and epigenetic alterations. Overexpression of *TERT*, and inactivation of *GSTP1* and *RASSF1A*, leads to the formation of dysplastic nodules following cirrhosis, and then inactivation of *TP53* and *CDKN2A* through mutation promotes HCC development. Wnt signaling pathway mutations (*CTNNB1* and *AXIN1*) occur at a later stage to promote advanced HCC (**Figure 2B**) (17, 18). Aflatoxin exposure can also promote progression to HCC without cirrhosis (19).

The downstream effects of the mutations which drive the progression of PDAC and HCC generally converge onto the pathways surrounding cell cycle regulation and oncogenic signaling (20). Progression through the cell cycle relies on a set of proteins which regulate various checkpoints, where activation must occur or the cell cycle is arrested. The different phases of the cell cycle are controlled by cyclins and the cyclin-dependent kinases (CDKs) they activate. These proteins oscillate in concentration during the phases of the cell cycle (21). Many effectors regulate the activity of these proteins, only allowing progression under certain cellular circumstances. Oncogenic signaling pathways in the cell promote survival and proliferation in response to external cues, such as growth factors or cytokines. Mutations in the effector proteins within these pathways, and those which regulate these effector proteins, are also common and lead to uncontrolled cell division.

CTDNA RELEASE

DNA can be released from multiple cells in different forms. As a highly charged molecule, DNA easily forms complexes with other molecules, and these structures protect the DNA from nuclease action and recognition by the immune system (22). Alternatively, DNA can attach to the external side of the cell membrane. Circulating DNA in healthy patients is generally double stranded and between 500 and 21,000 base pairs in length, whereas ctDNA is much smaller. Furthermore, the double stranded DNA derived from tumors has been shown to be less stable that that from non-tumor cells (23).

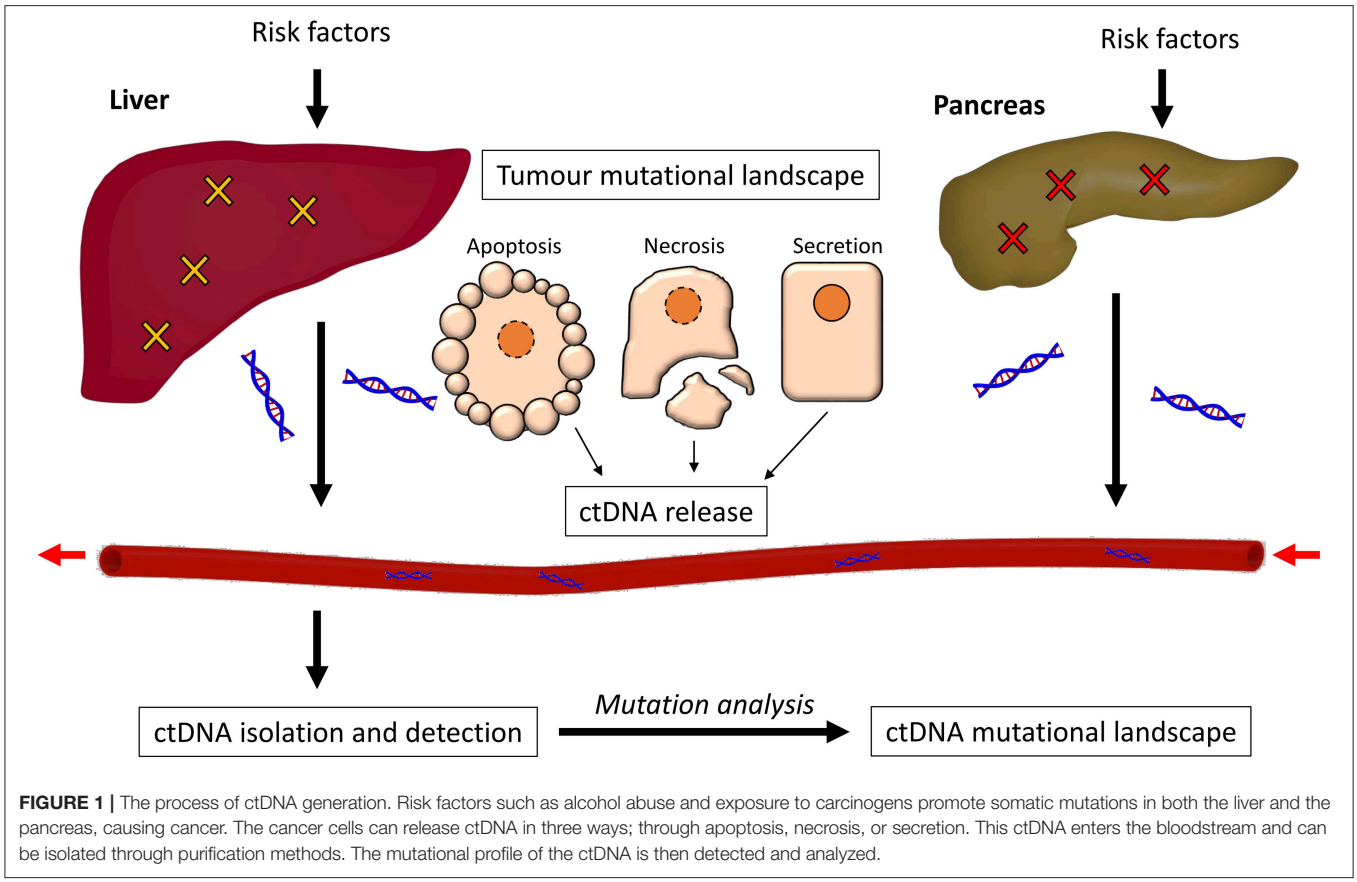

FIGURE 1 | The process of ctDNA generation. Risk factors such as alcohol abuse and exposure to carcinogens promote somatic mutations in both the liver and the pancreas, causing cancer. The cancer cells can release ctDNA in three ways; through apoptosis, necrosis, or secretion. This ctDNA enters the bloodstream and can be isolated through purification methods. The mutational profile of the ctDNA is then detected and analyzed.

DNA is released from cells in processes associated with both health and disease. Living cells can secrete newly synthesized DNA as part of a protein complex, with many proteins implicated in this interaction including Argonaute2 and high density lipoprotein (24). It has been suggested that DNA within cells is regularly replaced to maintain a threshold level of DNA repair activity for maintenance of genome integrity (25). This controlled secretion may be important for ctDNA, as it has been observed that cell free DNA from breast cancer cells is released primarily through active secretion *in vitro* (26).

In contrast, apoptosis leads to the shedding of DNA as cell integrity is lost. Apoptosis involves a stepwise degradation of chromosomes into singular nucleosomal units, around which 146 base pairs of DNA are wrapped. DNA is then packed into apoptotic bodies to be shed, which are rapidly cleared by phagocytes. However, the pathology of cancer often inhibits the clearing ability of phagocytes (25). In necrosis, DNA degradation is much more random, and as such, releases DNA fragments of different sizes, up to 10,000 base pairs, which can be found in blood. Necrosis does not contribute to cell free DNA in healthy patients but does occur in tumor cells (27, 28).

Since there is increased DNA fragmentation in apoptosis compared to necrosis, apoptotic cell free DNA tends to be shorter, around the size of a nucleosomal unit. The size of ctDNA varies, with many fragments around 145–160 base pairs, suggesting apoptosis as an important mechanism. However, many fragments are smaller than 145 base pairs, indicating further degradation in the blood stream (29). Cell free DNA half-life is limited, as the spleen, liver, and kidney promote clearance, with an average half-life for cell-free fetal DNA of 16 min (30).

Concentrations of ctDNA increase with tumor stage and burden, but the overall proportion of cell free DNA that is tumor-derived can also be affected by the release of DNA from non-tumor cells, i.e., following lysis of white blood cells (31). Fragmentation of ctDNA is further increased as the tumor mass increases (32). Tumor location, size, and vascularity all affect the rate at which ctDNA is shed, though a lot of uncertainty still exists on how and why these effects occur, and this can influence the power of liquid biopsy tests (33).

Circulating DNA has a role in intercellular messaging independent of disease. One example of this is the co-operation between B and T lymphocytes in mediating the humoral immune reaction, in which T cell released DNA is suggested to provide the genetic information needed for B cells to synthesize the correct antibody (29). In contrast, genometastasis, the transfer of mutated DNA from one cell to another, is an oncogenic process that involves circulating DNA. For example, ctDNA from colon cancer has been shown to promote the oncogenic transformation of murine embryonic fibroblasts (34).

Other tumor biomarkers are also of interest in the field of liquid biopsy, and also contain genetic information which may shed light on the tumor mutational landscape. Circulating tumor

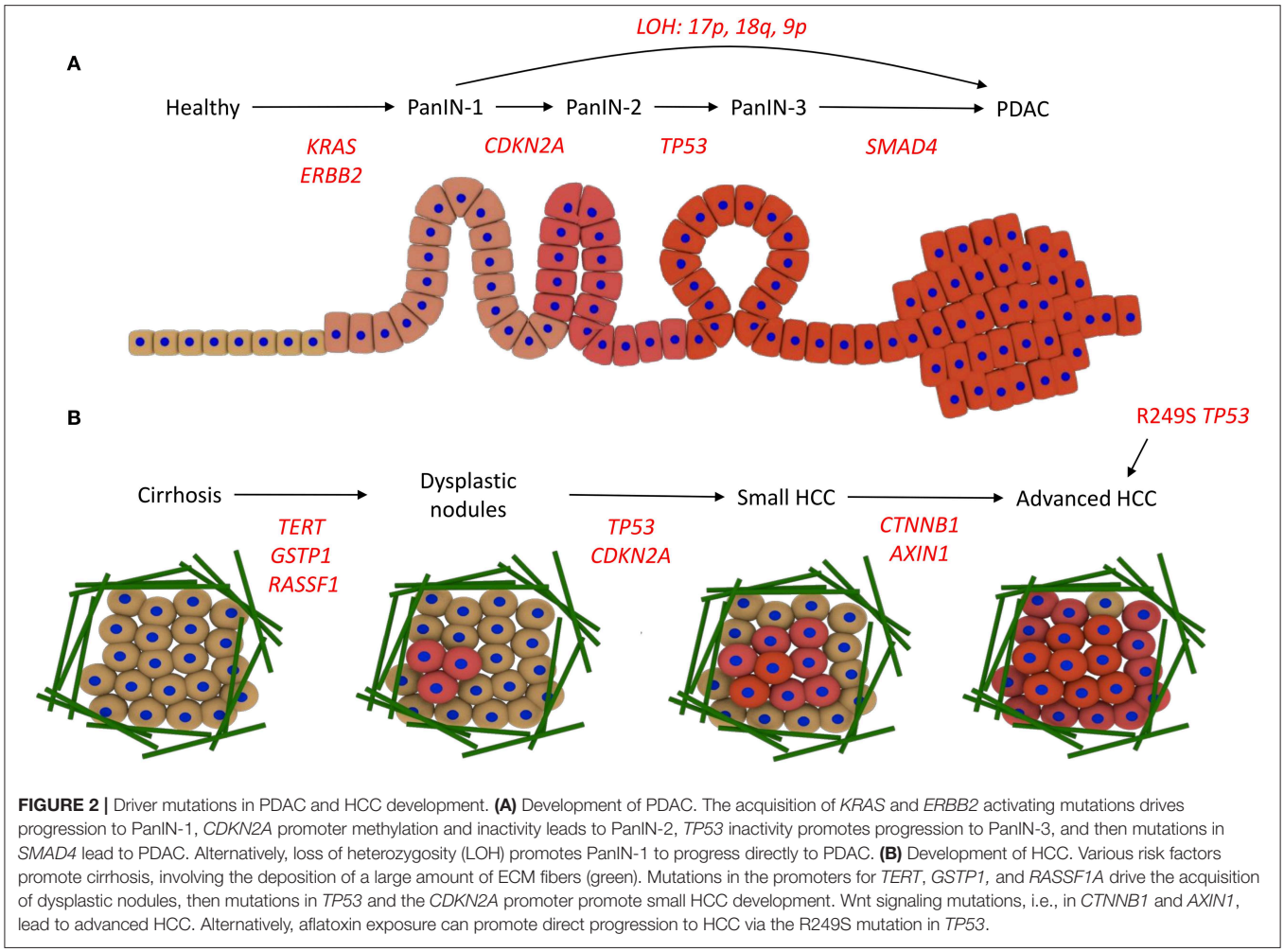

FIGURE 2 | Driver mutations in PDAC and HCC development. **(A)** Development of PDAC. The acquisition of *KRAS* and *ERBB2* activating mutations drives progression to PanIN-1, *CDKN2A* promoter methylation and inactivity leads to PanIN-2, *TP53* inactivity promotes progression to PanIN-3, and then mutations in *SMAD4* lead to PDAC. Alternatively, loss of heterozygosity (LOH) promotes PanIN-1 to progress directly to PDAC. **(B)** Development of HCC. Various risk factors promote cirrhosis, involving the deposition of a large amount of ECM fibers (green). Mutations in the promoters for *TERT*, *GSTP1*, and *RASSF1A* drive the acquisition of dysplastic nodules, then mutations in *TP53* and the *CDKN2A* promoter promote small HCC development. Wnt signaling mutations, i.e., in *CTNNB1* and *AXIN1*, lead to advanced HCC. Alternatively, aflatoxin exposure can promote direct progression to HCC via the R249S mutation in *TP53*.

cells (CTCs) have been suggested as a source of ctDNA, but these are unlikely to contribute much as CTCs are rare within the blood, and ctDNA is often present in the absence of CTCs (35). Exosomes, nanovesicles secreted by cells, contain DNA, but are also not suggested to contribute to ctDNA abundance (36).

CTDNA ISOLATION AND DETECTION

It is important to understand the variety of analysis methods for ctDNA, as appreciating their particular benefits and disadvantages allows a critical approach to the current set of studies and will improve the choices made in future ctDNA research.

ctDNA analysis is able to detect point mutations and copy number variation, but unable to detect larger scale mutations such as chromosomal aberrations (1). The concept of genomic variation as a guiding marker for therapy selection has been previously demonstrated, e.g., the variation in the gene *SLC15A2* as a marker for responsiveness to sorafenib in HCC (37). In addition, ctDNA analysis, for detection of the T790M mutation in the epidermal growth factor receptor (EGFR), has been recently used to guide therapy selection in non-small lung cancer (38).

ctDNA can be isolated from multiple body fluids, but most is most commonly extracted from blood plasma or serum. Pancreatic juice, bile, saliva, urine, and pleural effusion can also be used as a source of ctDNA (36). In one isolation method, guanidinium-thiocyanate-phenol-chloroform is used to separate RNA from DNA under acidic conditions, where DNA remains in the organic phase whereas RNA remains in the aqueous phase (39). Other common DNA isolation methods use kits that involve silica-based columns, polymer-mediated enrichment, or magnetic beads (40). Other pre-analytical variables that should be considered include the choice of body fluid, collection and processing materials, storage conditions and thawing temperatures (41).

Specific Mutation Detection

Amplification of ctDNA requires faithful duplication of the DNA sequence. Quantitative PCR or real-time PCR (qPCR) is used to exponentially amplify a segment of DNA and concurrently quantify levels of DNA. Primers are designed to flank the sequence to be amplified, e.g., a specific exon of a gene, are therefore independent of the presence of a mutation.

TaqMan PCR and SYBR green analysis allow for real-time quantitative analysis of PCR amplification (**Figure 3A**). In

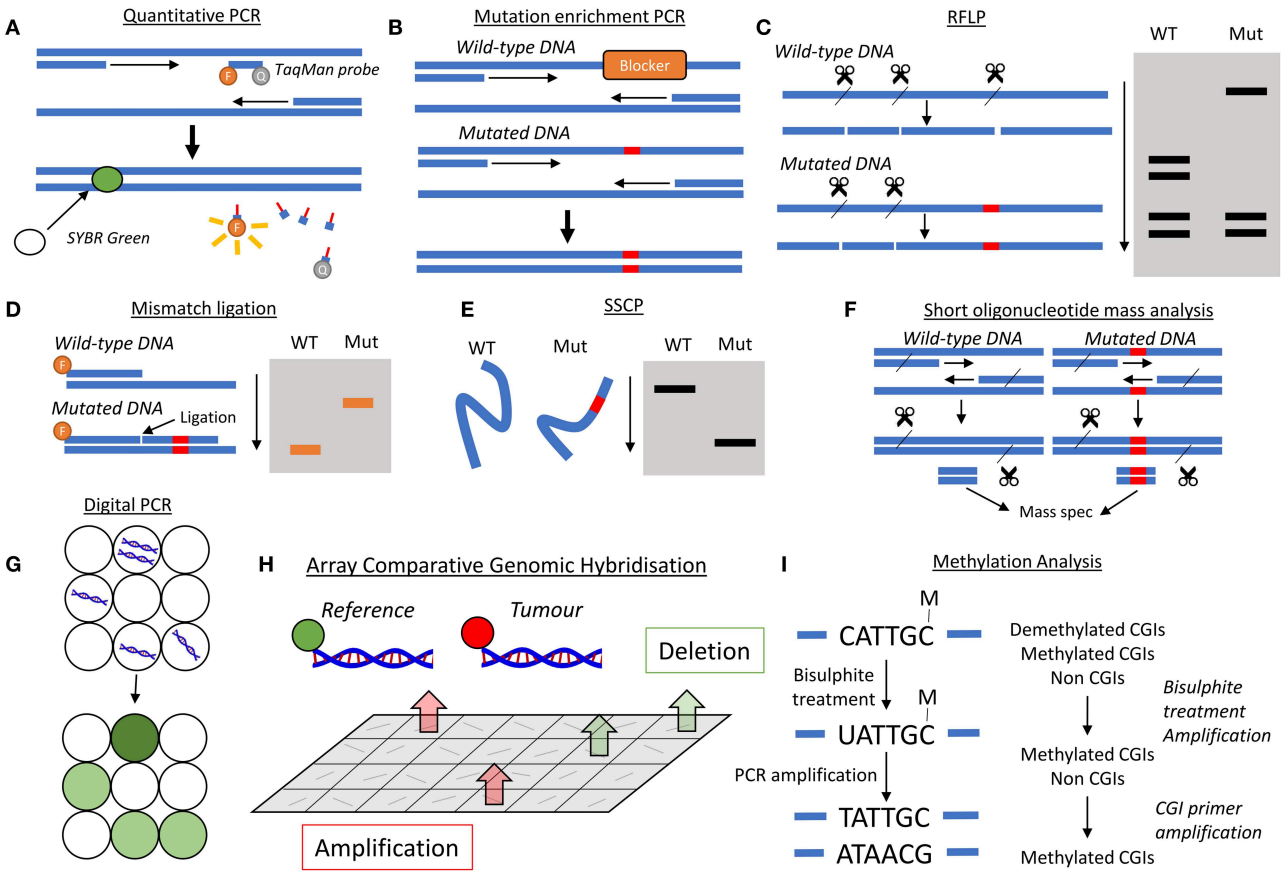

FIGURE 3 | Analysis methods to ctDNA analysis. **(A)** Quantitative PCR, either with a TaqMan probe or a double stranded DNA probe such as SYBR green. TaqMan probes bind to regions of interest, which could be a specific mutation, and nuclease activity during amplification separates the fluorophore and quencher, leading to fluorescence. SYBR green is unspecific for sequence, but binds to double stranded DNA and becomes fluorescence, which can be detected. **(B)** Mutation enrichment PCR. A blocking molecule binds to the wild-type sequence preventing its amplification. Therefore, only mutated DNA is amplified. **(C)** Restriction fragment length polymorphism (RFLP). If mutations alter the short sequences recognized by nucleases, then the fragment profile differs between wild-type and mutated DNA. When run on an electrophoresis gel where fragments are separated by length, the band profile will differ between wild-type and mutated DNA. **(D)** Mismatch ligation. Two probes are used, one attached to a fluorophore and another with a sequence to detect the mutation. By adding a ligase enzyme, longer fragments are generated for mutated DNA, which run differently on an electrophoresis gel. **(E)** Single strand conformation polymorphism. Wild-type (WT) and mutated DNA will form slightly different conformations are single strands, leading to a different movement on an electrophoresis gel. **(F)** Short oligonucleotide mass analysis. A very short fragment, around 7 base pairs, from the gene of interest is generated and subjected to mass spectrometry. **(G)** Digital PCR. The DNA solution is separated into many discrete volumes, containing none or some of the DNA. This DNA is amplified and the proportion of DNA positive volumes is used to quantify ctDNA levels. **(H)** Array comparative genomic hybridization. Reference and tumor DNA are labeled differently and allowed to bind to an array of DNA targets from a library. If deletion has occurred in the tumor, more reference DNA will attach to a particular sequence. If amplification has occurred in the tumor, more tumor DNA will attach to a particular sequence. These changes manifest in different intensities of each label. **(I)** Methylation analysis. Left: bisulfite treatment leads to unmethylated cytosine residues becoming uridine residues. When amplified, these Us pair with adenine residues, so any cytosines present following amplification are those that were methylated. Right: methylated CpG tandem amplification sequencing (MCTA-Seq) isolates methylated CpG islands (CGIs) for analysis of global genome methylation.

TaqMan PCR, a probe is designed to bind a specific sequence of interest and contains both a fluorophore and a quencher probe located near each other, hence no fluorescence is observed. If the sequence of interest is present, the probe binds to that sequence, and then PCR amplification leads to degradation of the probe through 5′ to 3′ exonuclease activity. This separates the fluorophore and quencher, leading to fluorescence. SYBR green analysis is not specific to any DNA sequence but becomes fluorescent upon binding to the minor groove of double stranded DNA, where more binding sites are created with PCR amplification. The SYBR green assay is low cost and easy to use, though can suffer from a lack of specificity. The sensitivity of TaqMan probes are similar to SYBR green, but do show an increased specificity (42).

Amplification of only mutant alleles can be achieved through mutation enrichment PCR (**Figure 3B**). In this method, a blocking segment of DNA is used that only binds to the wild-type version of the gene, and its presence blocks the progression of DNA polymerase. Where a mutation has occurred, this blocking segment does not bind and therefore DNA polymerase is able to amplify this DNA region (43). A further development is PNA-PCR clamping, in which peptide nucleic acids (PNA) are used to bind more strongly to specific sequences of DNA to block PCR amplification. Locked nucleic acids and morpholinos can

also be used for this purpose (44). This specific amplification is often combined with a non-specific quantification method e.g., SYBR green.

Restriction fragment length polymorphism (RFLP) can also be used to detect mutations (**Figure 3C**). The premise behind the analysis technique is that alterations of bases in DNA change the interaction of various nucleases with the DNA. If a nuclease cannot bind, then cleavage at that site does not occur, leading to a difference in the fragment profile following nuclease treatment. Therefore, if a mutation occurs in a nuclease binding site, then the wild-type DNA will be cleaved whereas the mutated DNA will not, leading to polymorphism, i.e., a difference in the length of the variety of restriction fragments (45). Many studies have used RFLP in DNA analysis, including the detection of mutations in the gene *TP53* in HCC (46).

The mismatch ligation assay involves the use of DNA probes that target mutated sequences, as well as labeled probes (**Figure 3D**). Both probes are allowed to attach, where the mutant probe only attaches in the presence of a mutation. A DNA ligase enzyme is added to ligate the two probes into one, which is then removed. The probes are then run on a gel, where movement is dependent on DNA size, and detected. If a mutation is present in the analyzed DNA, then the ligation produces a longer labeled fragment, and therefore the longer probe moves differently within the gel. This method has been used to analyse common mutations in pancreatic cancer (47).

Mutations in pancreatic cancer have also been analyzed through single strand conformation polymorphism (SSCP) (**Figure 3E**) from DNA from pancreatic juice (48). In SSCP analysis, the gene of interest is amplified using PCR, denatured into single strands and then run on an electrophoresis gel. The slight differences in sequence due to mutations affect the conformation of the single strands, altering their movement within the gel (49).

In short oligonucleotide mass analysis (**Figure 3F**), a short region of the genome (as small as 7 base pairs) is amplified by PCR, with the primers engineered to contain an endonuclease site. Following amplification, digestion of the PCR product leaves only the short genomic region, which is subsequently analyzed by electrospray ionization mass spectrometry to determine its sequence (50). This method has been used to assess a specific *TP53* mutation in HCC (51).

Digital PCR is a recent development which increases sensitivity. This process involves separating DNA templates into discrete volumes, such that some contain no DNA template and some contain at least one DNA template (**Figure 3G**). PCR amplification is then performed, so that the volumes with a relevant DNA template will be amplified whereas those without will not be amplified. The number of DNA positive volumes following PCR amplification, often determined with the TaqMan assay, is then used to calculate the DNA concentration. In droplet digital PCR (ddPCR), the discrete volumes are oil droplets within a water-based solution (52). Heterogeneity in the mutation profiles of ctDNA of HCC patients has been demonstrated through this assay (53).

Since, the copy number of genes can be altered by amplification or deletion mutations, methods for analyzing copy number such as Array Comparative Genomic Hybridization (aCGH) have been developed (**Figure 3H**). In this method, tumor DNA is labeled with one fluorophore and reference DNA from a healthy sample is labeled with another fluorophore. These DNA solutions are then mixed and added to an array of DNA targets. If deletion has occurred, then there will be more reference DNA attached to a specific DNA target, and if amplification has occurred, then there will be more tumor DNA attached to a specific DNA target (54). The copy number of various genes in the ctDNA of breast cancer has been analyzed this way (55).

Methylation Detection

Since, many reported mutations in both pancreatic and liver cancer involve aberrant methylation of specific gene promoter regions, detection of these mutations within ctDNA must use specific techniques to maintain a marker of methylation during PCR amplification, most commonly sodium bisulfite treatment (**Figure 3I**). Methylation occurs primarily on the C5 position of cytosine bases within the cytosine-guanine dinucleotide (CpG). The product, 5-methylcytosine, is unaffected by treatment with sodium bisulfite, whereas unmethylated cytosine residues are converted into uracil. PCR amplification converts uracil bases into thymine bases, and therefore when the PCR product is sequenced, any cytosine residues present are those that were methylated in the original DNA (56). One interesting technique is known as "methylation on beads," which combines DNA extraction, bisulfite conversion and PCR in one tube using silica superparamagnetic beads, and has been used to analyse the promoter region of the *CDKN2A* gene in lung cancer (57).

Global methylation is not suited for bisulfite analysis as large amounts of DNA would be needed to represent the whole genome. Shotgun massively parallel bisulfite sequencing has been developed, a sequencing platform with high throughput and has been used to assess global hypomethylation in HCC (58). Another analysis technique for methylation across the genome is methylated CpG tandem amplification and sequencing (MCTA-Seq) (**Figure 3I**). This method looks at the methylation state of the 7-mer CGCGCGG, also known as a CpG island, which is common in the genome. In the 1st step, following bisulfite treatment, unmethylated sequences are eliminated as they are amplified less than methylated sequences by a specific primer. Methylated sequences, but those that are not CpG islands, are then eliminated by a CpG island specific primer. The product, containing only methylated CpG islands, is then amplified for quantification purposes (59).

Sequencing Analysis

For larger scale analyses, next-generation sequencing (NGS) is increasingly used. NGS involves the sequencing of millions of short fragments of DNA in parallel, and multiple platforms have been developed for this high throughput analysis technique (60). The detailed mechanisms of the wide variety of NGS platforms is outside the scope of this review but have been well-reviewed elsewhere (61, 62). The main difference between NGS analysis of DNA directly from cells and ctDNA analysis is the lack of a ctDNA fragmentation step in the preparation of a DNA library,

as ctDNA is already present in small fragments. NGS methods can also be used for analysis of copy number in ctDNA (63).

One NGS platform that has been recently used for analysis of mutations in ctDNA from HCC patients is Guardant360. This platform uses a panel of oncogenes and tumor suppressor genes and analyses each ctDNA sample set for single nucleotide variants, copy number amplification and other fusion and insertion mutations. The assay reports the type of mutation, if present, for each gene for each patient (64).

Other NGS assays using gene panels, currently in development for other cancer types, may also show promise for either pancreatic or liver cancer in the future. For example, the Cobas® EGFR Mutation Test v2 is used for non-small-cell lung carcinoma since EGFR, a KRAS-activating receptor, is often mutated in this particular cancer (65). Another NGS platform, the PlasmaSELECT-R64 assay, evaluates a panel of 64 genes and has been directly compared with the Guardant360 assay on samples from patients with metastatic prostate cancer. In this study, the genomic alterations observed varied greatly depending on the assay used despite an overlap of genes tested, and these inconsistencies mean that the effectiveness of personalized medicine could vary depending on the NGS platform used (66).

CTDNA MUTATIONAL LANDSCAPE AND DETECTION

PDAC and HCC have characteristic mutational landscapes, where some genes are hotspots for driver mutations which facilitate disease progression. Though these two gastrointestinal diseases share many common genetic alterations, some are specific to each pathology. For example, both pathologies commonly show mutations in the genes *TP53* and *CDKN2A*. In addition to these shared alterations, PDAC frequently exhibits mutations in *KRAS,* as well as *ERBB2* and *SMAD4,* whereas HCC is often characterized with mutations in the *TERT* promoter, *CTNNB1* and *AXIN-1*.

Only a subset of the mutations present in the tumor mutational landscapes of PDAC and HCC have been detected by studies which have analyzed ctDNA in the body fluids of cancer patients. The metrics commonly used in ctDNA analysis are absolute values for ctDNA abundance (either specific to a target gene or overall ctDNA levels) or the percentage of patients in a cohort with a particular mutation. For diagnosis purposes, a mutation must be highly sensitive, in that its detection indicates the presence of the disease, but also highly specific, in that lack of its detection indicates the absence of the disease. Prognosis involves relating mutations or ctDNA abundance to clinical metrics such as overall survival or time to relapse.

Here we describe what is currently known about the mutational landscapes of PDAC and HCC, and how well recent studies have been able to represent this landscape through analysis of ctDNA.

Cell Cycle

TP53

One role of each cell cycle checkpoint is to ensure that there is no DNA damage before cell cycle progression occurs. If arrest at these checkpoints is not properly controlled by the multitude of signaling proteins involved in their regulation, then cancer can develop (67). One key DNA damage response protein is p53, coded for by the gene *TP53* located on chromosome 17. p53, a commonly mutated tumor suppressor, is activated by DNA damage, leading to transcriptional upregulation of its target genes to halt the cell cycle. For example, by promoting the expression of p21, which inhibits multiple cyclins and their CDKS, p53 inhibits progression through both the G1/S and G2/M checkpoints (**Figure 4**) (68).

Mutations in *TP53* are present in HCC and exist in 35–50% of patients (69, 70). The most common missense mutation is R249S and is linked to exposure to the mycotoxin aflatoxin B_1, which can promote both cirrhosis-dependent and independent progression to HCC (**Figure 2B**). Furthermore, the HBx protein, expressed from insertion of the hepatitis B virus into the genome in HCC, has been shown to inhibit the activity of the wild-type p53 protein (71). The ctDNA analysis of mutations in the *TP53* gene for HCC diagnosis has mostly analyzed populations with a high dietary exposure to aflatoxin B_1. The R249S mutation has been detected at a higher level in ctDNA from HCC patients than in healthy controls in a variety of studies, suggesting its diagnostic potential, and has been also been shown to be associated with worse survival than wild-type *TP53* (72).

In a 2000 study in The Gambia, the R249S mutation was detected in 36% of HCC patients but in only 6% of healthy controls (73). In Nigeria, the same approach showed a smaller detection rate of 8% in HCC and 0% in healthy controls (74). Another African study, which analyzed data from a variety of tribal groups, detected this mutation in 18% of their cohort of 158 black southern Africans (75). In contrast, an analysis of patients in Egypt detected this *TP53* mutation in only 1.3% of HCC cases and 1.4% of healthy cases, though higher levels (17%) were detected in chronic liver disease cases (46).

As well as Africa, Asian regions have also been studied as places where aflatoxin β_1-mediated mutation of *TP53* occurs. In the Qidong region of China, the R249S mutation was detected in HCC cases in 2003 at a sensitivity of 44% and specificity of 93% (76). A study in Thailand in 2005 found the mutation R249S in 26% of HCC cases but only 15% of healthy cases (51). These studies indicate that the detection of the R249S mutation shows promise but may only be highly specific for HCC in certain regions.

Quantitative analysis of the plasma concentration of R249S *TP53* has also been performed for HCC and healthy cases. A 2005 study from The Gambia determined that the median concentration of R249S *TP53* in HCC cases (2,800 copies/mL) was higher than that of cirrhotic or healthy cases (both 500 copies/mL). HCC diagnosis was significantly associated with >10,000 copies of R249S *TP53* per mL (77).

TP53 is inactivated in 20–76% of pancreatic cancers, primarily through a mutation in one allele along with loss of the other allele. Many of these mutations occur in the DNA binding domain of p53 (78, 79). Mutations in *TP53* cannot initiate pancreatic cancer (13) and tend to appear in later stage PanINs (**Figure 2A**). Only a few studies have looked at ctDNA *TP53* mutations for the analysis of PDAC, despite mutations occurring abundantly in the tumor. One study in 2017 used NGS to identify a range

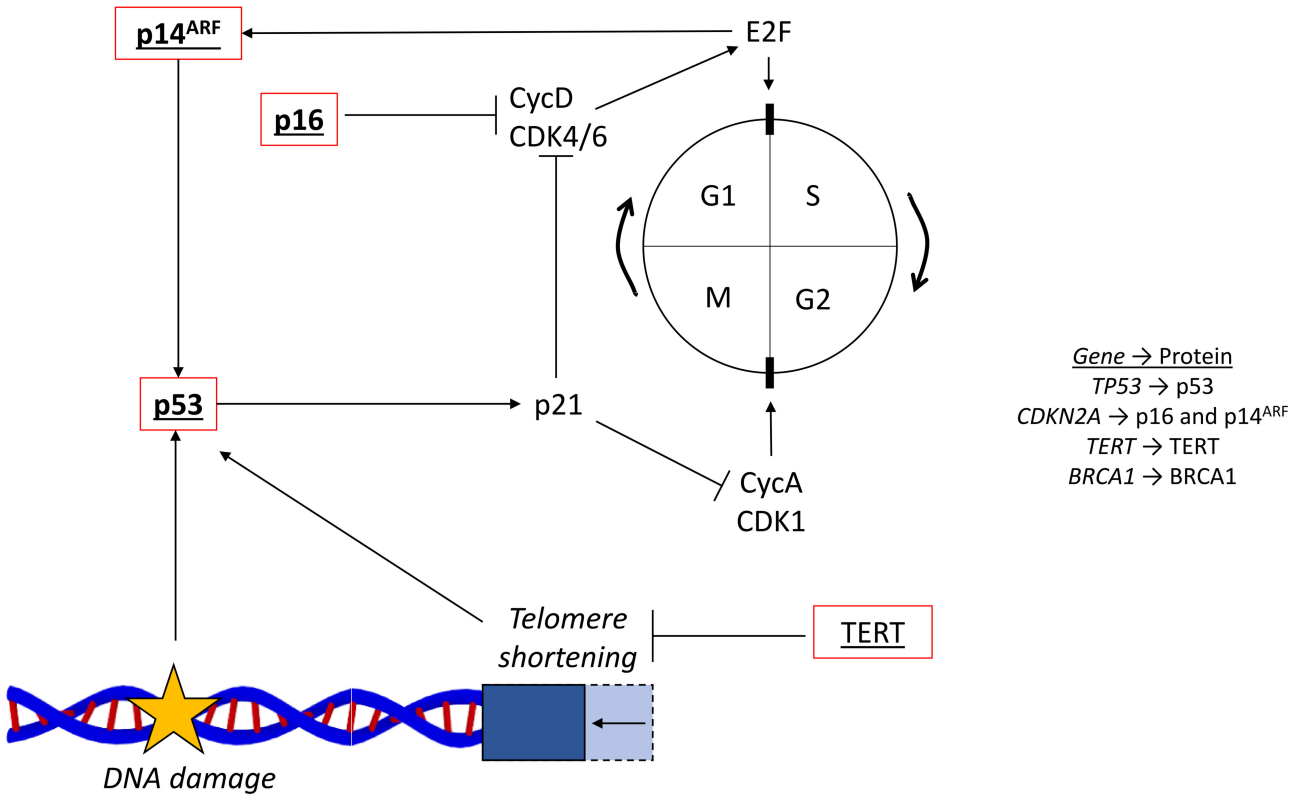

FIGURE 4 | Cell cycle and common mutations. The cell cycle (. . . G1 → S → G2 → M → G1. . .) is regulated by cyclins and their associated cyclin-dependent kinases. The G1/S transition is controlled by CycD and CDK4/6, which activates E2F. The G2/M transition is controlled by CycA and CDK1. The tumor suppressor p53 responds to DNA damage and activates p21, which inhibits cell cycle progression via the cyclins. Telomere shortening, reversed by the activity of TERT, also activates p53. The two *CDKN2A* coded proteins, p16 and p14ARF, are also involved in the regulation surrounding E2F, CycD, and CDK4/6. Red outline, commonly downregulated in tumors; bold, commonly mutated in PDAC; underlined, commonly mutated in HCC; bold and underlined, commonly mutated in both PDAC and HCC; CycD, cyclin D; CDK4/6, cyclin dependent kinase 4/6; CycA, cyclin A; CDK1, cycling dependent kinase 1; TERT, telomerase reverse-transcriptase; NFE2L2, nuclear factor erythroid 2-related factor 2.

of *TP53* mutations from ctDNA in pancreatic juice with high specificity. In 59% of PDAC patients, *TP53* showed some sort of mutation, whereas no control cases exhibited any alterations (80). The aflatoxin β_1-mediated mutation of R249S has additionally been detected in ctDNA from pancreatic cancer patients in Iran in a 2013 study, with an 11% incidence in pancreatic cancer but only 3.5% of healthy cases (81). Furthermore, the *TP53* mutations, I251M and R175G, have been detected in the ctDNA of individual pancreatic cancer patients pre-surgery, and the mutations G293E, M340Cfs*5, S362Afs*8, and T211I have been detected in individual patients who developed metastasis after resection of the primary tumor (82).

CDKN2A

Another tumor suppressor gene that is commonly mutated, *CDKN2A*, encodes two other cell cycle regulatory proteins, p16 and $p14^{ARF}$. *CDKN2A* is situated on the short arm of chromosome 9, with p16 and $p14^{ARF}$ generated from different reading frames. Inactivation of the *CDKN2A* promoter by hypermethylation is a common occurrence in both HCC and PDAC (78). p16 has a key role in regulation of the G1/S checkpoint in the cell cycle, and $p14^{ARF}$ is involved in activating p53 (**Figure 4**) (83).

From an analysis of 71 HCCs, it has been shown that 66% of HCC cases exhibit inactivation of p16 and 15% exhibit inactivation of $p14^{ARF}$ (69). Most commonly, the *CDKN2A* promoter is methylated, leading to inactivation, and this hypermethylation has been observed on average in 58% of HCC cases (84). While promoter methylation is the dominant form of mutation, missense and nonsense mutation of *CDKN2A* have also been seen in liver cancers, including H75Y (85) and R58* (86). Additionally, 7% of HCC cases show homozygous deletion of the *CDKN2A* gene (69).

CDKN2A alterations have been detected in the ctDNA of HCC patients in many studies. A 2003 study detected methylation of the *CDKN2A* promoter in the ctDNA of 47% of HCC patients where promoter methylation had been observed in the tumor (87). Other studies by the same group detected *CDKN2A* promoter methylation in around 80% of HCC patients where methylation was present in the tumor. ctDNA methylation was not detected in any patients where none was present in the tumor in both studies (87–89). Furthermore, promoter

methylation was observed in the plasma of liver cancer patients pre-surgery at a rate of 31% and the median amount of methylation of the *p16* genes analyzed was 12-fold lower following surgery (87). NGS methods on ctDNA from HCC patients have also detected the presence of the *CDKN2A* mutant R80* (64).

In pancreatic cancers, *CDKN2A* is inactivated in ∼40% of cases by deletion of both alleles (78), with loss associated with worse survival probability (90). Inactivation occurs in a further 40% by deletion of one allele and a mutation within the remaining allele (91). Furthermore, 15% of pancreatic cancers show hypermethylation of the promoter sequence for *CDKN2A* (78, 92). ctDNA analysis of *CDKN2A* for pancreatic cancer is limited, though one study identified mutations from DNA in pancreatic juice at an incidence of 6% in PDAC and 0% in control cases (80).

TERT

Telomeres are nucleoprotein structures, located at the tip of each chromosome, which protect chromosome ends from fusion, recombination and degradation. Telomeres shorten with every cell cycle, ∼50–150 base pairs per cycle, and when they reach a critically short length, promote cell cycle arrest by activating p53 (**Figure 4**) (93). The gene *TERT* encodes telomerase reverse-transcriptase (TERT), which extends telomeres. Increased activation of TERT therefore promotes the lengthening of telomeres and increases cell growth (94). Upregulation of *TERT* is common in HCC, most commonly through activating mutations within its promoter region (95).

The *TERT* promoter is the most frequently mutated site in HCC, with ∼60% of cases exhibiting alterations, most frequently at the positions 124 and 146 base pairs upstream of the ATG start site. Both sites involve a mutation of a guanine to an adenine, and additionally, position 124 has been shown to mutate a guanine to a thymine (95). This creates a binding site for transcription factors of the ETS family which promote TERT expression (96). A further 10–15% of TERT reactivation occurs through insertion of hepatitis B virus into its promoter, and 5% is due to *TERT* amplification (5).

Despite *TERT* promoter mutations driving the initial progression of HCC (**Figure 2B**), and being highly abundant in liver cancers, their detection within the ctDNA landscape has been limited. The specific mutations in the promoter region for TERT that enhance ETS binding, as seen in HCC biopsies, have yet to be detected through ctDNA analysis. Some studies have used *TERT* DNA as an amplification locus for the quantification of overall levels of ctDNA instead of analyzing mutations in the promoter. One study used real-time PCR to show that the abundance of *TERT* DNA in HCC patients was higher than that of HBV patients and healthy controls, though *TERT* abundance was not associated with tumor size or stage (97). Another study observed that *TERT* levels were significantly associated with reduced overall survival, having analyzed concentrations of *TERT* DNA in patients with HCC, cirrhosis and chronic hepatitis (98).

A study of multiple cancers has revealed that PDAC and pancreatic acinar carcinoma do not show *TERT* mutations (99).

Oncogenic Signaling

KRAS

Located on chromosome 12, the *KRAS* gene codes for the 21 kDa GTPase KRAS and is mutated in over 90% of pancreatic cancers (78). If constitutively activated by mutation, KRAS promotes oncogenic signaling through multiple signaling pathways. In its wild-type form, KRAS is activated by cell surface receptors such as the EGFR, leading to activation of the MAP kinase cascade to promote cell proliferation, metabolism and transcription of target genes. KRAS is activated by guanine nucleotide exchange factors (GEFs) which exchange bound GDP for GTP, and is then deactivated either by GTPase activating proteins (GAPs) or through its intrinsic GTPase activity (**Figure 5**) (100).

Ninety eight percentage of *KRAS* mutations affect the glycine residue at position 12, with missense mutations swapping glycine for aspartate, valine, or arginine. This alteration blocks the intrinsic GTPase activity of KRAS and makes the molecule insensitive to GAPs, leading to constitutive activation (100, 101). A few mutations (overall < 2%) also occur at positions 13, 61, 117, and 146 (79). Mutations occur in around 30% of early neoplasms, increasing to around 95% of advanced carcinomas (102, 103). Mouse models use the mutation $KRAS^{G12D}$ to initiate PDAC development and have been used to demonstrate that this mutation in one of the earliest events in PanIN initiation in humans (**Figure 2A**) (101).

As the most commonly mutated gene in pancreatic cancer, detection of mutated *KRAS* in ctDNA is a highly studied area. *KRAS* mutations have been detected in plasma or serum DNA at a range of incidence rates (from 33 to 94%) (47, 80, 104–107).

A 94% sensitivity was seen in a 2017 study on 189 patients with unresectable PDAC using mutation enrichment PCR, following by NGS, to identify G12 mutations. The most common mutation amongst these was G12D (41%). This study also showed that concentrations of *KRAS* ctDNA were increased in stage IV PDAC patients compared with stage III, and these high values were significantly associated with shorter overall survival (107).

The lowest sensitivity observed, 33%, was seen from plasma DNA samples from PDAC patients in China using PCR. Though a low sensitivity was observed, the presence of mutations significantly reflected clinical parameters, including tumor stage and the presence of liver metastasis. The survival time for patients was also significantly negatively associated with the presence of *KRAS* mutations (105). A similarly low sensitivity of 35% was observed using a sensitive mutation specific mismatch ligation assay on plasma DNA from pancreatic cancer patients (47).

Further delineation of the presence of *KRAS* mutations in different stages of pancreatic cancer has been performed. Mutations have been shown to be more abundant in patients with metastatic disease (90%) than local disease (43%) (106), and the use of *KRAS* mutations to differentiate between pancreatic cancer and chronic pancreatitis has been demonstrated with ctDNA analysis at a sensitivity of 47% and specificity of 87% (104).

KRAS mutations, as detected with ctDNA, have also been associated with poor survival in pancreatic cancer. Using ddPCR and amplification of mutant DNA with TaqMan probes for various *KRAS* G12 mutations, ctDNA abundance in PDAC patients has been significantly associated with reduced overall

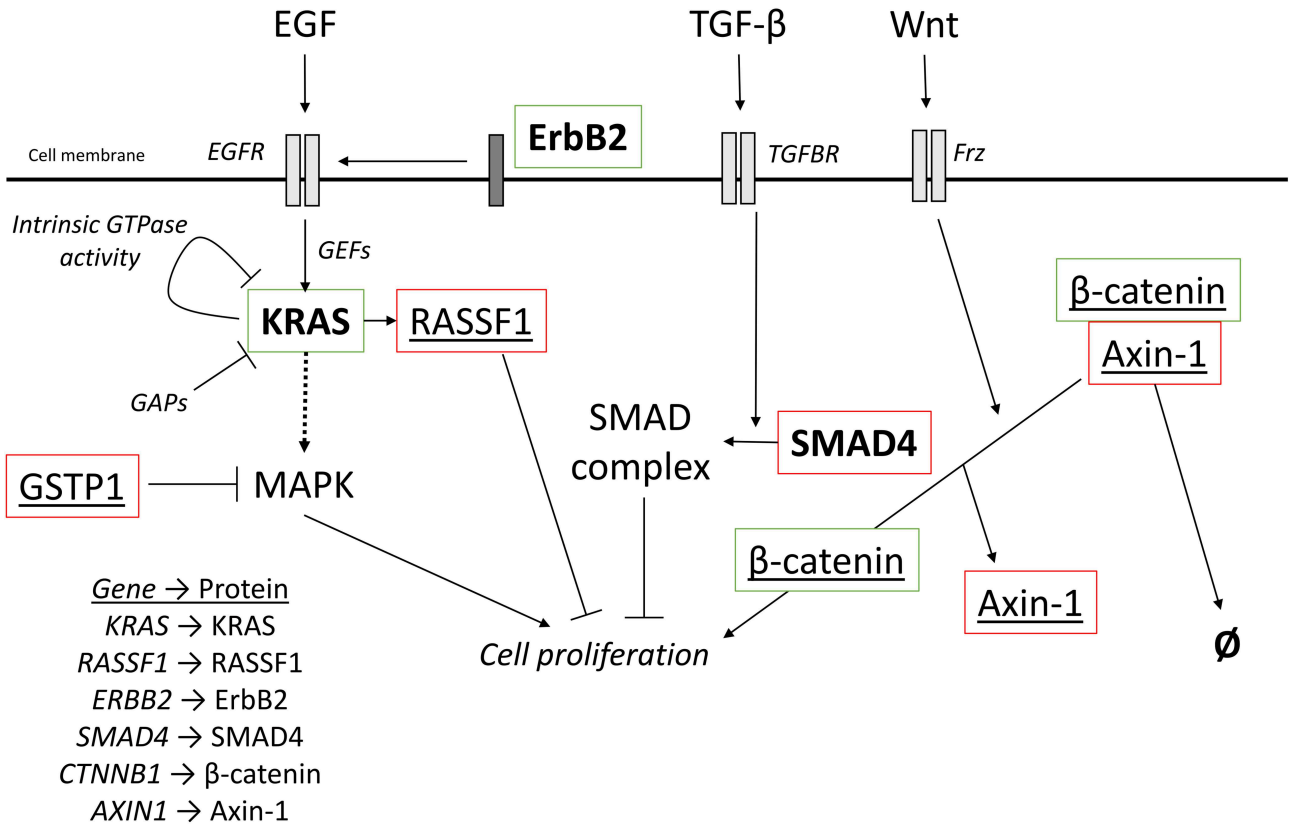

FIGURE 5 | Oncogenic signaling pathways with driver mutations for PDAC and HCC. External signaling molecules, such as EGF, TGF-β, and Wnt, promote intracellular signaling through their respective receptors, EGFR, TGFBR, and Frz. EGFR activation, facilitated by ErbB2, leads to activation of KRAS. KRAS is inactivated by either intrinsic GTPase activity or GAPs. Cell proliferation can be promoted by the MAPK cascade is activated by KRAS but can also be inhibited by the redox regulator GSTP1. KRAS can also activate RASSF1A, which inhibits cell proliferation. TGF-β signaling promotes the incorporation of SMAD4 into a heterotrimeric complex with inhibits cell proliferation. Wnt signaling activated the Frz receptor, which promotes the decoupling of Axin-1 from β-catenin. In the complex, β-catenin is targeted for degradation, but when not complexed, β-catenin promotes cell proliferation. Red outline, commonly downregulated in tumors; green outline, commonly upregulated in tumors; bold, commonly mutated in PDAC; underlined, commonly mutated in HCC; bold and underlined, commonly mutated in both PDAC and HCC. EGF, epidermal growth factor; EGFR, EGF receptor; TGF-β, transforming growth factor β; TGFBR, TGF-β receptor; Frz, Frizzled; GEFs, guanine nucleotide exchange factors; GAPs, GTPase activating proteins.

survival (108). Another study used a PNA clamp specific for the wild-type *KRAS* sequence to perform mutation enrichment PCR for patients about to undergo a chemotherapy regime for PDAC. Pre-therapy mutant *KRAS* ctDNA abundance was significantly associated with reduced progression-free and overall survival (109).

RASSF1A

KRAS mutations have been suggested to not contribute to the pathogenesis of HCC (110). However, RASSF1, a downstream target of Ras family members (**Figure 5**) and an often mutated tumor suppressor protein, is associated with liver cancer (111). Hypermethylation of the *RASSF1* promoter, leading to downregulation of expression, occurs in HCC at a rate of 93% (112). The RASSF1 protein is also a negative regulator of the Hippo pathway, which promotes cell growth (113). Methylation of the *RASSF1* promoter has been seen in pancreatic cancer and is present in 35% of tumors. However, the phenotypic result of this is a variation in the expression of different isoforms of RASSF1 and is not associated with prognosis (114).

RASSF1A has garnered attention for ctDNA analysis and *RASSF1A* promoter methylation has been observed in the analysis of serum DNA in HCC. As part of a longitudinal study, hypermethylation was observed as present up to 9 years before the clinical diagnosis of HCC. Out of the HCC cases, 70% showed *RASSF1* promoter hypermethylation in ctDNA (115). Hypermethylation of the *RASSF1A* promoter has been associated with HCC size of >4 cm (112). Additionally, poorer disease-free survival has been associated with hypermethylation, and this increase was observed in ctDNA longitudinally for patients who carry the hepatitis B virus from enrolment to HCC diagnosis (116).

ERBB2

Various cell membrane receptors promote activation of Ras family members, and one of these receptors which is commonly mutated is the erythroblastic oncogene B2, known as ErbB2

(**Figure 5**). The gene for ErbB2 (*ERBB2)* is located on chromosome 17 and is sometimes referred to as *HER2* (117). ErbB2 expression has been shown to be very low in healthy pancreatic ducts but high incidence has been observed in various ductal malignancies (118).

The *ERBB2* gene is frequently overexpressed in PDAC, and this is associated with a worse prognosis. ErbB2 additionally modulates the resistance of pancreatic cancer cells to the chemotherapeutic gemcitabine (119). Protein overexpression of ErbB2 has been seen at a variety of incidence rates, ranging from 7 (120) to 61% (121). Amplification of the *ERBB2* gene has also been observed at incidence rates from 2 (120) to 24% (122). Furthermore, missense mutations have also been observed in pancreatic cancer, including R103Q, V8421I, and E717D (123).

Despite mutations in the gene *ERBB2* being highly associated with the early stages of PDAC, its mutational status within the ctDNA landscape is less well-considered. Using NGS to detect mutations, and then ddPCR for further analysis, mutations in *ERBB2* (either in exon 17 or 27) have been detected in the ctDNA of 20% of pancreatic cancer patients. Exon 17 mutations were additionally associated with significantly reduced overall survival (124). Amplification of the *ERBB2* gene in pancreatic cancer has also been observed by ctDNA analysis (125).

In HCC, *ERBB2* is rarely altered. One missense mutation (H878Y) has been observed in liver cancer at an incidence of 11% (126). An ErbB effector, ERRFI1, has been reported as being mutated in 5% of HCC cases (70, 127). A literature review which reviewed mutations in a multitude of cancer types did not report any studies where *ERBB2* was mutated in liver cancer ctDNA (128). However, genomic alterations in *ERBB2* are found at a rate of 25% in extrahepatic cholangiocarcinoma, but not in intrahepatic cholangiocarcinoma (129). Cholangiocarcinoma (CC) is a common type of liver cancer which begins in the bile ducts which connect the liver to the gallbladder, and cases are classified as intra- or extrahepatic depending on which part of the biliary system they arise in Massarweh and El-Serag (130). Future ctDNA testing for *ERBB2* may therefore be useful for some CC cases, as well as those rarer cases where patients have the mixed malignancy where both HCC and CC are present.

SMAD4

Another oncogenic signaling pathway is the TGF-β pathway, and involves the effector protein SMAD4, which is often inactivated through mutation in PDAC. With its gene located on the long arm of chromosome 18, SMAD4 promotes inhibition of epithelial cell growth (131). Extracellular transforming growth factor beta (TGF-β) promotes the formation of SMAD complexes, with SMAD4 a subunit of a heterotrimer which promotes expression of tumor suppressor genes (**Figure 5**) (132). *SMAD4* is inactivated in 35% of pancreatic cancers by homozygous deletion (78), where loss is a negative prognostic indicator and associated with poor survival (133).

As a low abundance mutation, detection of *SMAD4* mutations has generally proceeded through NGS approaches. Using digital NGS to efficiently sequence low-abundance mutations, *SMAD4* mutations, either frameshift or missense, have been identified from DNA in pancreatic juice at an incidence of 15% in PDAC and 0% in control cases (80). Using targeted resequencing to focus on specific genes for amplification and analysis, NGS has also been used to demonstrate that *SMAD4* mutations were present in the ctDNA of only 5% of PDAC patients (134).

Wnt Signaling (CTNNB1 and AXIN1)

The Wnt signaling pathway also transduces extracellular signals which affect cell development and is closely associated with cancer. Canonical Wnt signaling involves an AXIN-containing protein complex that promotes the degradation of the signaling effector β-catenin, coded for by the gene *CTNNB1*. Upon activation of the pathway by extracellular Wnt ligand, this complex is disrupted and β-catenin translocates to the nucleus to regulate gene expression (**Figure 5**).

Activating mutations of *CTNNB1* occur at a rate of 11–37% in HCC (5). Large in-frame deletions in exon 3, and missense mutations between residues 32 and 37 lead to high levels of β-catenin activation, as they prevent the binding of β-Trcp which would promote ubiquitination and degradation. Other mutations, such as those involving Ser45, lead to weak activation of β-catenin as they block a phosphorylation site that promotes degradation. Ser45 mutations only lead to development of benign tumors, but selective duplication of this mutated allele and production of double the dose of mutated β-catenin is suggested to promote progression to a malignant tumor (135).

Mutations in *CTNNB1,* with the nucleotide changes A121G and T133C, have been detected in 13% of the ctDNA of HCC patients using ddPCR with primers specific for certain mutations (53). With NGS, *CTNNB1* mutations leading to the amino acid changes of S29T, S33C, H36P, and G34V were detected in 29% of HCC patients (64). A previous study published by the same group that year analyzed ctDNA in a further 26 patients with HCC using NGS and demonstrated that 31% of HCC patients showed *CTNNB1* mutations. These were missense mutations leading to the amino acid changes D32N, S45P, S45F, S37F, T41A, as well as S33C, H36P, and G34V which were observed in the group's later study (136).

Axin-1 is a protein involved in the protein complex that regulates β-catenin and is coded for by the *AXIN1* gene. In a study involving 100 HCC cases, *AXIN1* mutations were observed at a rate of 6%, including nonsense and frameshift mutations. These mutations are predicted to truncate Axin-1 to remove the β-catenin binding site, and therefore Axin-1 is no longer able to facilitate β-catenin degradation (137). However, *AXIN1* mutations have not been detected in ctDNA from HCC patients (136).

GSTP1

One protein that can regulate kinases within oncogenic signaling pathways is glutathione S-transferase π (GSTP1). The main role of GSTP1 is to detoxify the cytoplasm by conjugating with xenobiotics and maintaining redox homeostasis. If GSTP1 expression is reduced, carcinogen detoxification is diminished and therefore genome instability is promoted. GTSP1 has the additional role of negatively regulating kinases that act as effectors which promote cell proliferation (**Figure 5**) e.g., MAPK (138) and c-Jun (139).

Downregulation of expression from *GSTP1* occurs in HCC through methylation of its promoter region in around 53% of HCC cases (140), with methylation of certain regions occurring more often than others, and more specifically in HCC. For example, methylation in one promoter region has been shown in 77% of HCC cases and no healthy cases, and methylation in another region of the promoter has been shown in 80% of HCC cases, but also in 100% of healthy cases (141). High levels of methylation of the specific region (5' of−48) of the *GSTP1* promoter are more abundant in HCC (37%), compared to other liver conditions including hepatitis, cirrhosis, as well as healthy control (all 0%), and only 15% of HCC cases show no methylation (141). The *GSTP1* promoter region has also been shown to be methylated in 23% of PDAC patients but in 0% of healthy patients (142).

Methylation of the promoter region of *GSTP1* has been analyzed in ctDNA samples. Using bisulfite treatment to maintain a marker for methylated cytosine residues, 50% of HCC patients from China have been observed exhibiting *GSTP1* promoter hypermethylation on ctDNA extracted from serum. However, this 50% incidence rate was also observed for patients with liver cirrhosis, suggesting a lack of specificity in ctDNA analysis (143).

In addition to analysis of promoter methylation, *GSTP1* has also proved beneficial in ctDNA analysis as an amplification locus for overall assessment of ctDNA levels. ctDNA has been shown to be significantly higher in HCV-HCC (141 ng/mL) patients than those in HCV carriers without HCC (34 ng/mL) and control patients (46 ng/mL) (144). The same group performed another study the next year with more HCV-induced HCC patients, demonstrating similar results for HCV-HCC (116 ng/mL) and HCV carriers (34 ng/mL). This increased ctDNA level was significantly associated with worse survival (145).

Global Hypomethylation

The overall level of DNA methylation, in addition to specific oncogenic methylations on promoter regions, can also be a marker for cancer. Global DNA hypomethylation promotes genomic instability, and the methylation status of LINE-1 is often used as a marker for global DNA methylation. LINE-1 is a transposable element, i.e., a DNA sequence that moves and duplicates within the genome and makes up ∼17% of the genome. Its hypomethylation, representative of global hypomethylation, is associated with a poor prognosis in many cancers (146). LINE-1 methylation levels have been shown to be decreased in HCC cases (146, 147), as well as pancreatic cancer cases (148), compared to healthy controls.

The methylation status of LINE-1 from ctDNA of HCC patients has been analyzed, showing that the percentage of unmethylated LINE-1 was significantly higher for HCC compared with healthy controls. Furthermore, LINE-1 hypomethylation could be correlated significantly with advanced tumor stages, indicating that LINE-1 hypomethylation is a significant and independent prognostic factor for overall survival (149).

Gene Panels

Liver cancer is associated with many types of mutations at a moderate abundance, compared to the domination of the pancreatic cancer landscape by *KRAS*. ctDNA analysis methods have also used gene panels in order to improve sensitivity and specificity in both HCC and PDAC.

By looking at specific mutation hotspots in the genes *CTNNB1*, *TP53,* and the *TERT* promoter, a study identified mutations in 20% of patients. In addition, by quantifying total cell free DNA with a double stranded DNA stain similar to SYBR green, it was shown that total cell free DNA amount was not correlated with mutation status. Despite the limited promise of this study, a significant correlation between detectable mutation status and survival probability was observed (150). The same genes have been used in a panel in a ddPCR assay of ctDNA from HCC patients, with mutation detection at a higher incidence (56%) in this case (53). Furthermore, a deep sequencing technique for ctDNA, which amplified and analyzed 46 coding and non-coding genes, detected mutations in 63% of HCC patients (151).

Since aberrant methylation is a key part of the HCC mutational landscape, methylation marker panels have also been used for ctDNA analysis. In one study, a methylation marker panel was identified using a learning set of patients, identifying methylation in genes such as *NOTCH3* and *PPFIA1*. It was then tested on a different set of patients and showed a higher combined diagnosis score for HCC than healthy controls or liver disease. This panel was also a significant predictor of overall survival (152). Another study used a set of 4 methylation markers (*RGS10*, *ST8SIA6*, *RUNX2*, *VIM*) to detect HCC, with 94% sensitivity and 89% specificity (59). It should be noted that the methylation markers used in these studies are not methylation events which driver disease progression, such as *TERT* promoter methylation.

Panels of markers that analyse copy number variation have also been used for HCC detection. Using NGS to detect a panel of size alterations (e.g., gain in chromosomal region 1q and loss in chromosomal region 13q), HCC has been correctly identified in 84% of patients, with 100% specificity against cirrhosis and chronic hepatitis (63).

Though most combinatorial studies have analyzed HCC, some studies have evaluated ctDNA from PDAC with gene panels. One study analyzed the concentrations of mutated DNA for a multiple gene panels to test their diagnostic potential for PDAC identification. These panels were *KRAS* alone, *TP53* alone, *TP53* in combination with *SMAD4*, or a full panel of 9 genes including *KRAS*, *TP53*, *SMAD4* as well as other genes such as *CDKN2A*. For PDAC vs. control, the highest sensitivity (85.3%) was achieved by the 9 gene panel, whereas when comparing PDAC vs. intraductal papillary mucinous neoplasm (IPMN), *TP53* + *SMAD4* showed the highest sensitivity (64.7%). The *TP53* + *SMAD4* combination, as well as *TP53* alone, showed 100% specificity for PDAC vs. control, with the 9 gene panel less specific at 83.4%. *KRAS* was also highly specific at 91.7%. For PDAC vs. IPMN, the 9 gene panel was the most specific (85.7%) (80). This study highlights well that increasing the number of genes analyzed does not necessarily improve detection as both sensitivity and specificity must be considered.

CONCLUSION AND FUTURE INSIGHTS

ctDNA analysis is emerging as a sensitive and specific method for analyzing the mutational landscape of patients with HCC and/or PDAC. Over the years, a multitude of ctDNA studies have identified the presence, or indeed absence, of mutations associated with either of these gastrointestinal diseases. ctDNA research has progressed by improving and updating detection and analysis techniques, and by understanding how to apply results from ctDNA analysis in a clinical setting.

A key goal of ctDNA research is for analysis of ctDNA from an individual patient to accurately represent the mutational landscape of that patient, and therefore be useful in diagnosing any malignancies such as PDAC or HCC, and dictating clinical practice (1). PDAC and HCC share many common driver mutations, e.g., alterations within the genes *TP53* and *CDKN2A*, but other frequent mutations only occur in one of these pathologies, e.g., *KRAS* mutations are abundant in PDAC but rare in HCC. Models for the mutational development of PDAC and HCC are well-established (**Figure 2**) and indicate how specific mutations drive steps along the pathway from health to disease, though heterogeneity in mutational profiles has limited current understanding.

ctDNA mutational analysis represents the end of a long process that begins with the occurrence of somatic mutations within tumors cells. Inevitably, the rate at which specific mutations are then detected in ctDNA often differs from that detected in tumor biopsies. For example, mutations in *TP53* in PDAC are observed between 55 and 75% from tumor biopsies, but the range of incidence rates for ctDNA is from 11 to 59%. In this case, part of the reason could be that the ctDNA analysis only looks at the specific mutation R249S and does not consider others. However, the G12D mutation in *KRAS* in PDAC has been shown in tumors at an incidence of 98%, yet ctDNA analysis has detected this specific mutation at lower rates (33–94%). Additionally, it must be noted that many mutations often detected within the liver or pancreas have either been detected at a low incidence or not detected at all in ctDNA (**Figure 6**). For example, *TERT* promoter mutations are highly prevalent in HCC but have yet to be specifically detected in ctDNA from HCC patients, though have been used as part of a gene panel for ctDNA analysis (150).

The variance between the tumor and ctDNA mutational landscape is likely to be primarily generated from the variety of isolation and detection techniques and tumor heterogeneity, but other factors may also be involved. ctDNA only makes up part of the cell free DNA present within the body, and as such, non-mutated DNA fragments are also included in the analysis which dilutes ctDNA. Since ctDNA is often released through apoptosis, it can be speculated that the cells that more readily undergo apoptosis could be those where the driver mutation is not present, leading to less mutated DNA in the circulating population. Furthermore, it has been demonstrated that different DNA sequences exist at different concentrations within plasma, and it has been suggested that sequence may affect the rate of DNA cleavage within the blood (153). This may underlie why some genes are more readily detectable than others.

One key aim of ctDNA is to determine the genotype of the patient, which can be used to dictate therapy choices. For example the *BCR-ABL* oncogene, present in various leukaemias, can be targeted with the specific agent imatinib (154). Other genotypes are associated with a predicted lack of response to therapy e.g., mutant *KRAS* in colorectal cancer is associated with a lack of response to the therapeutic cetuximab (155). Some of the studies we highlight here link mutational status in ctDNA to survival metrics (e.g., 98, 107, 116, 124). Correlations between mutations, as detected by ctDNA analysis, and therapy response to specific agents are required for combining the field of ctDNA mutational analysis with clinical prognosis and therapy choices.

The diagnostic and/or prognostic potential of a particular mutation requires high sensitivity and specificity, and therefore high accuracy. Many of the driver mutations that have been so far detected through ctDNA show limited accuracy, though others show more promise in reaching the goal of 100% accuracy. The hypothetical perfect analysis technique would be able to detect a mutational change or changes present in all cases of the disease (i.e., 100% sensitive) and in no cases without the disease (i.e., 100% specific). An improved understanding of the molecular biology that drives disease initiation will be informative for identifying all possible mechanisms for the disease. For example, while *KRAS* mutations are highly prevalent in early PDAC development and seen as driver mutations, they are not present in 100% of cases (156). An understanding of which mutations drive these *KRAS*-independent developments may converge on a pathway, or set of pathways, from which all known disease progressions develop from. A full ctDNA analysis of the genes encoding these proteins may show a perfect sensitivity and specificity.

Sensitivity and specificity are highly dependent on many pre-analytical parameters, e.g., plasma separation and method of ctDNA isolation, and optimization of the assay may require extra steps within the workflow (65). However, perfect sensitivity and specificity may not necessarily be required for therapy choices based on mutation status. If known ctDNA mutations are significantly associated with various survival metrics, including post-therapy survival, then therapeutic decisions could be made. A patient may still have the disease state, and further monitoring may be required, but if the patient has a form of the disease that does not present with ctDNA mutations, their survival prospects may be better and hence therapy would not be needed. A highly sensitive, but perhaps less specific, assay could be used for longitudinal monitoring purposes, to ensure that patients are not characterized as healthy when they have the disease.

Both HCC and PDAC develop through multiple stages, and these stages are associated with specific mutations e.g., *KRAS* as an early mutation that occurs in PanIN, and *TERT* promoter methylation that occurs following liver cirrhosis. As such, more studies are needed which analyse ctDNA during different parts of disease progression. One good example of ctDNA monitoring is a study which analyzed hypermethylation of the *GSTP1* promoter hypermethylation in healthy individuals and patients with cirrhosis and HCC (141). Since there is sometimes a discrepancy between tumor mutation status and ctDNA mutation status, more studies that specifically analyse ctDNA mutations at different tumor progression stages will improve

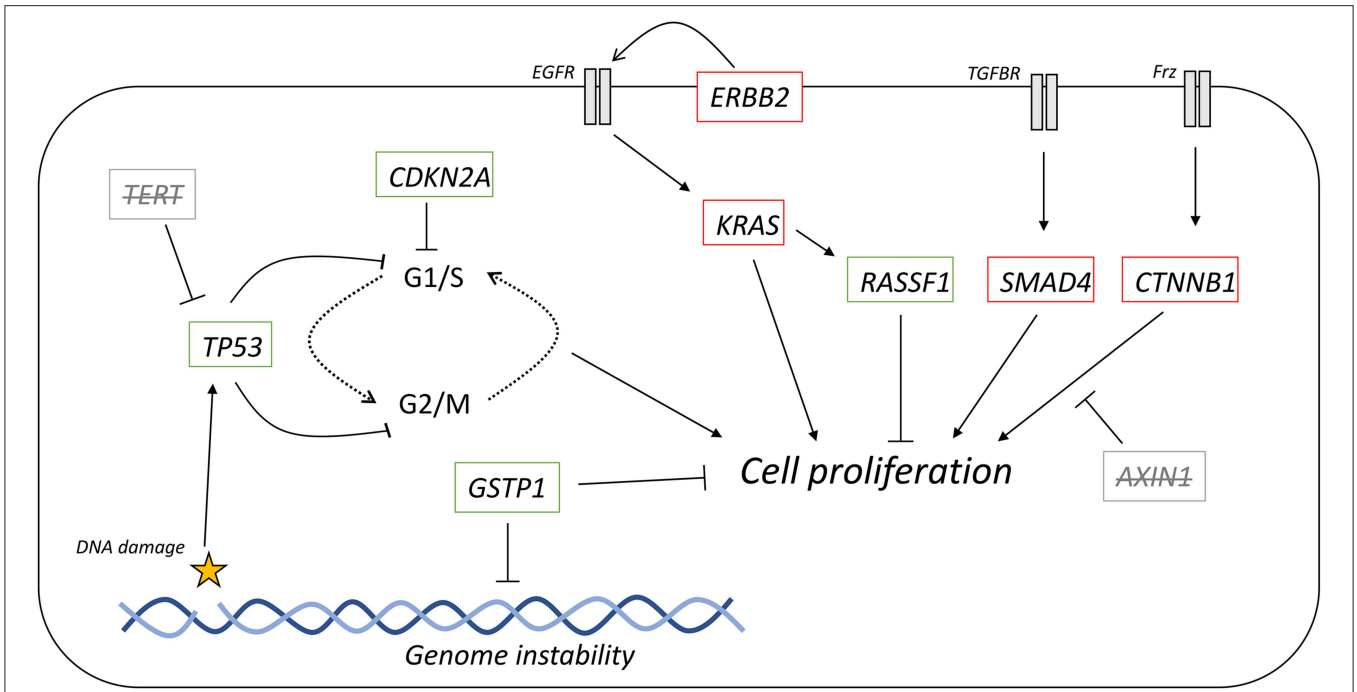

FIGURE 6 | Representation of the tumor mutational landscape by ctDNA analysis. Commonly mutated genes in HCC and PDAC, and their role in cell signaling. Those genes with strikethrough have none or limited results in studies detecting these mutations. Red outline, commonly downregulated in tumors; green outline, commonly upregulated in tumors.

our understanding of the circulating mutational landscape, a landscape which is clinically available.

One feature of diagnosis rarely considered within studies that analyse ctDNA is the differentiation between different diseases. For example, many studies report high specificity for HCC compared to healthy and other liver disease states, but do not compare HCC vs. diseases of other organs, such as PDAC. *KRAS* is seen as a high accuracy marker for PDAC, though has also been detected in the ctDNA of colorectal cancer patients (157). Similarly, *TERT* promoter mutations, seen as key driver mutations in HCC, are present in multiple other cancers including bladder and skin cancer (158). Gene panels, which assess multiple genes, may be able to differentiate cancers from each other, if further detail on the cancer mutational landscape, as present in ctDNA, could be found. Studies that compare and analyse ctDNA from a cohort of patients exhibiting various diseases may lead to identification of gene panels that show high specificity. These efforts would likely be affected by intertumor heterogeneity amongst patients. Furthermore, choice of bodily fluid, e.g., pancreatic juice instead of blood, may allow the specific identification of particular cancers.

In conclusion, the ctDNA mutational landscape differs from the tumor mutational landscape, and research must be undertaken to evaluate the mechanisms behind this discrepancy. Future studies should also, if possible, report on how ctDNA mutation detection is related to survival metrics and/or therapy response. With this information, ctDNA analysis may become an indispensable tool in analyzing, and basing therapeutic decisions on, the mutational status of tumors in individual patients, and further progression in the field of personalized therapy.

AUTHOR CONTRIBUTIONS

AR and ARH wrote the manuscript.

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Telomere Length of Circulating Cell-Free DNA and Gastric Cancer in a Chinese Population at High-Risk

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Background: Telomeres have long been found to be involved in cancer development, while little was known about the dynamic changes of telomere length in carcinogenesis process.

Methods: The present study longitudinally investigated telomere alterations of cell-free DNA (cfDNA) in 86 gastric cancer (GC) subjects recruited through a 16-year prospective cohort with 2–4 serums collected before each GC-diagnosis from baseline and three follow-up time-points (a total of 276 samples). As the control, 86 individual-matched cancer-free subjects were enrolled with 276 serums from the matched calendar year.

Results: In the 73 pairs of baseline serums from GC and control subjects, shortened telomeres showed increased subsequent GC risk [odds ratio (OR) = 9.17, 95% CI: 2.72–31.25 for 1 unit shortening]. In each baseline gastric lesion category, higher risks of GC progression were also found with shortened cfDNA telomeres; ORs per 1 unit shortening were 6.99 (95% CI: 1.63–30.30) for mild gastric lesions, 6.06 (95% CI: 1.89–19.61) for intestinal metaplasia and 15.63 (95% CI: 1.91–125.00) for dysplasia. With all measurements from baseline and follow-up time-points, shortened telomeres also showed significant association with GC risk (OR = 7.37, 95% CI: 2.06–26.32 for 1 unit shortening). In temporal trend analysis, shortened telomeres were found in GC subjects compared to corresponding controls more than 3 years ahead of GC-diagnosis (most $P < 0.05$), while no significant difference was found between two groups within 3 years approaching to GC-diagnosis.

Conclusion: Our findings suggest that telomere shortening may be associated with gastric carcinogenesis, which supports further etiological study and potential biomarker for risk stratification.

Keywords: cell-free DNA, prospective cohort, gastric cancer, serum, telomere length

INTRODUCTION

Telomeres are tandem repeats of TTAGGG nucleotides at the ends of eukaryotic chromosomes. They have long been shown to maintain chromosome integrity and genomic stability (1, 2). Shortened telomeres can induce cellular senescence or apoptosis (3). Persistent cell division bypassing apoptosis may cause genomic instability and tumorigenesis as a result of shortened telomere in chromosome (4, 5). A recent prospective multi-center cohort study reported that intestinal metaplasia (IM) subjects with shortened telomeres in gastric mucosa were associated with subsequent progression to dysplasia (DYS) or gastric cancer (GC) (6), suggesting that telomere shortening may be involved in the process of gastric carcinogenesis.

The application of tissue biomarkers is restricted for invasive collection and high cost in prevention and clinical practices, especially in a large population setting. Consequently, an increasing number of studies have evaluated telomere length of peripheral blood lymphocytes DNA and GC risk, but reported inconsistent results (7–9). The single time-point measurement in most previous studies has been a major limitation, which made it difficult to investigate the longitudinal alterations of telomere length affected by aging, environmental exposures or carcinogenesis processes.

Circulating cell-free DNA (cfDNA) is becoming a promising target in early detection, therapy response monitor and prognosis evaluation of cancer (10). Serum cfDNA may be released in inflammatory, infection or carcinogenesis processes from a wide spectrum of cells, including necrotic and apoptotic cells, active blood cells and circulating tumor cells. Therefore, studies suggested that the alterations of cfDNA may be more sensitive than peripheral blood leukocyte DNA to reflect the overall organism status for their multiple origins (11–13).

In 1994, we initiated an intervention trial for GC prevention in Linqu County, a rural area in northeast China, which has one of the highest GC mortality and precancerous gastric lesions rates in the world (14, 15). Our previous follow-up study and intervention trial in this area found that *H. pylori* infection, cigarette smoking, and low level of dietary Vitamin C may contribute to the development of GC (16). With multiple serum samples collected from baseline and three subsequent follow-up clinical examinations, this prospective cohort study provided us a unique opportunity to explore the temporal trend and dynamic attrition of cfDNA telomere length during the long-term process of gastric carcinogenesis.

METHODS

Study Subjects

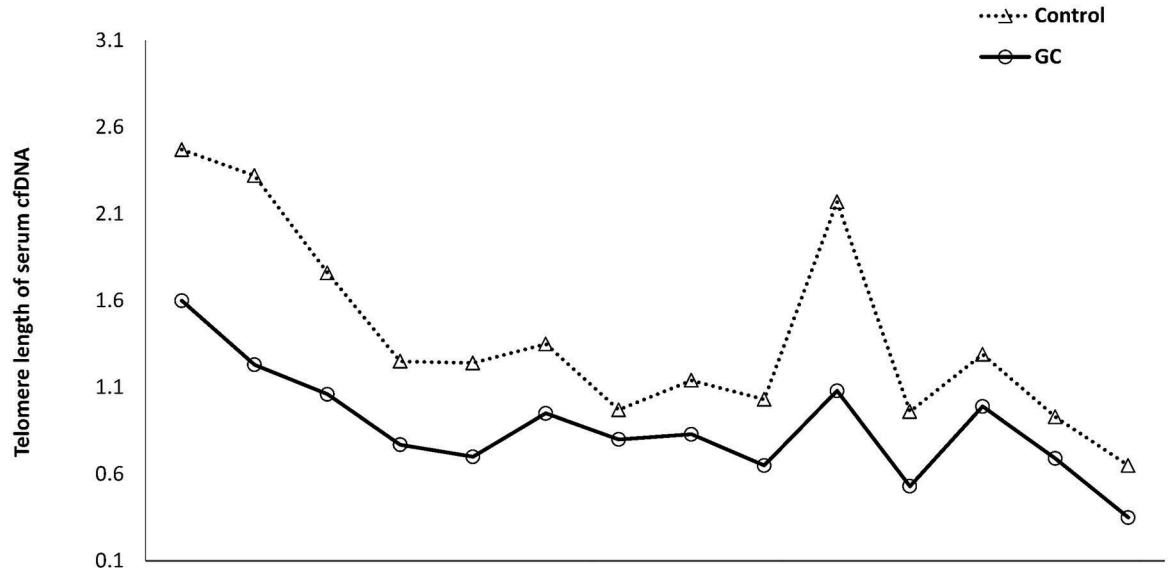
The details of this intervention trial were described elsewhere (14, 15). Briefly, a total of 3,411 residents aged 35–64 years from 13 randomly selected villages in Linqu County were enrolled in an initial screening program with endoscopic examinations and blood sample collections in 1994. Then, 3,365 eligible subjects were assigned randomly to receive three interventions or corresponding placebos for GC prevention in 1995, including anti-*H. pylori* treatment for 2 weeks, vitamins or garlic supplementations for 7.3 years. To monitor the serum levels of micronutrients after interventions, blood samples were collected from trial participants in 1996 and 1997, respectively. In 1999, an endoscopic screening was performed to follow up the effects of interventions on gastric lesion progression with blood sample collections at the same time. The incidences of cancers were followed from 1995 until 2010, with 106 GC patients identified.

For the present study, 86 GC cases were enrolled with 2 to 4 serum samples before each GC-diagnosis from baseline and three follow-up time-points, respectively. Among these GC cases, 79 (91.9%) were pathologically confirmed as 75 (87.2%) intestinal and 4 (4.7%) diffuse type. The locations of the GC were identified in 82 (95.3%) cases, with 54 (62.8%) in angulus or antrum, 20 (23.3%) in body, 6 (6.9%) in cardia, and 2 (2.3%) in pylorus of stomach. A total of 79 (91.9%) GCs had records of lymph node and distant metastasis when initially diagnosed, including 39 (45.3%) with lymph node or distant metastasis, 40 (46.5%) without any kind of metastasis. In all the 86 GC cases, 36 (41.9%) cases had serum samples from all the 4 time-points, 32 (37.2%) cases had samples from 3 time-points, and 18 (20.9%) cases had samples from 2 time-points. A total of 276 pre-diagnostic serum samples were selected, including 73 from baseline, 73 from 1996, 74 from 1997, and 56 from 1999. For each pre-diagnostic sample, the number of years before GC-diagnosis was identified by the time interval between the dates of sample collection and clinical diagnosis, ranging from 1 to 16 years (**Figure 1**). Since only a small number of samples were collected at 15 or 16 years before GC-diagnosis ($n = 1$ and 5, respectively), they were combined into the group of $\geq$14 years before GC-diagnosis.

For each GC case, one control without any types of cancer during the follow-up period was randomly selected from the cohort participants, matched by gender, age ($< \pm 5$ years) and calendar year of serum sample collection. Finally, a total of 276 serum samples from 86 controls were available for the current study. This study was approved by the Institutional Review Board of Peking University Cancer Hospital.

Telomere Length Measurement

CfDNA was extracted from 150 μL serum sample using QIAamp DNA Blood Mini kit (Qiagen, CA) according to the manufacturer's protocol and eluted in 40 μL of elution buffer. The telomere length of each DNA sample was determined by quantitative real-time polymerase chain reaction (qRT-PCR) according to a protocol by Cawthon (17). This method measures relative telomere length by determining the ratio of telomere repeat copy number to single copy gene 36B4 in individual samples relative to a standard pooled DNA with a 7500 FAST real-time PCR system (Applied Biosystems, CA). Briefly, the PCR reaction (20 μL) for the telomere or 36B4 amplification consisted of 2X SYBR Green Master Mix (Thermo Scientific), 250 nmol/L each telomere or 36B4 specific primers, and 4 μL purified cfDNA sample. The thermal cycling conditions for both telomere and 36B4 were 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 59 s with signal collection. The primer sequences were as follows: forward telomere primer (Tel-1), 5′-CGGTTTGTTTGGGTTTGGGTT



Years before GC-diagnosis		≥ 14	13	12	11	10	9	8	7	6	5	4	3	2	1
N (Pairs in different years)		14	12	16	17	18	30	14	30	24	21	24	24	22	10
Median of telomere length	Control	2.47	2.32	1.76	1.25	1.24	1.35	0.97	1.14	1.03	2.17	0.96	1.29	0.93	0.65
	GC	1.60	1.23	1.06	0.77	0.70	0.95	0.80	0.83	0.65	1.08	0.53	0.99	0.69	0.35
	P	<0.001	<0.001	<0.001	0.022	0.163	0.002	0.519	0.007	0.017	0.011	0.043	0.148	0.235	0.115

FIGURE 1 | The temporal trends of cfDNA telomere length in GC and control groups. The X axis presents the years before GC-diagnosis of pre-diagnostic samples from GC subjects. The number of sample pairs was shown in each group, and the *P*-values were calculated by linear regression with adjusting age variable between GC and control groups.

TGGGTTTGGGTTTGGGTT-3′; reverse telomere primer (Tel-2), 5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTAC CCT-3′; forward human 36B4 primer, 5′-CAGCAAGTGGGA AGGTGTAATCC-3′; reverse human 36B4 primer, 5′-CCCATT CTATCATCAACGGGTACAA-3′.

For quality control and calibration of PCR efficiency, the same standard DNA extracted from pooled serum samples were included on each plate with all samples assayed in duplicate. Melting curve analysis was performed after every reaction to verify specificity and identity of the PCR products. The standard DNA was diluted using 3-fold increment per dilution to produce a seven-point standard curve, ranging from 0.05 to 40 ng/μL template DNA. The standard curves for amplifications of telomere and 36B4 were constructed every 10 plates before the detection of samples, with all the R^2 values were ≥0.99. The samples with low concentration of cfDNA (Ct value of 36B4 >34) were deleted for out of the linearity of the standard curve. The intra-assay coefficient of variation for telomere/36B4 ratio was <8.0%. The average coefficients of variation were 3.1% for telomere reaction and 2% for 36B4 reaction, respectively. When the coefficient of variation was higher than 15%, the measurement was repeated. The paired samples were analyzed on the same plate by one technician with group information blind.

H. pylori Antibody Assay

As described previously, *H. pylori* antibody assays were used to determine infection status in 1994, 1996, 1997, and 1999 (18). In brief, serum levels of anti-*H. pylori* IgG and IgA were measured separately in duplicate with enzyme-linked immunosorbent assay procedures. An individual was defined to be positive for *H. pylori* infection if the mean optical density for either IgG or IgA was 1.0. Quality control samples were assayed at Vanderbilt University, Nashville, TN.

Statistical Analysis

For the baseline data analysis, the distributions of general characteristics between GC subjects and controls were compared by the chi-square tests for categorical variables and paired *t*-test for age. Telomere lengths were compared between subjects who progressed to GC and their paired controls by Wilcoxon rank-sum test. The odds ratio (OR) and corresponding 95% confidence interval (CI) for the association between baseline

telomere length and GC risk were calculated by conditional logistic regression adjusting for age, *H. pylori* infection and gastric lesion status. Within different categories of baseline gastric lesion, the associations between baseline telomere length and GC risk were assessed by unconditional logistic regression adjusting for age and *H. pylori* infection.

Using multiple measurements of samples from all collection time-points, linear mixed models were applied to examine the associations between cfDNA telomere length and potential influencing factors by the regression coefficients and 95%CIs. The association between cfDNA telomere length in multiple time-points and the risk of GC was further evaluated by generalized linear mixed model. The changes of telomere length before and after interventions were compared between active and placebo groups by Wilcoxon rank-sum test. To compare the temporal trends of cfDNA telomere length, all the pre-diagnostic samples were sub-grouped according to the years before GC-diagnosis and compared with their corresponding controls by linear regression adjusting age. The annual attrition rate of telomere length in a specific time span for each subject was calculated by dividing the telomere length difference between two consecutively collected serum samples by interval years and baseline telomere length. Therefore, we had 3 average annual telomere attrition rates for subjects with 4 serum samples, and 2 average annual telomere attrition rates for subjects with 3 samples. Differences of average annual attrition rates between GC and control groups were compared by Wilcoxon rank-sum tests.

All statistical analyses were performed using the SAS software version 9.2 (SAS Institute, Cary, NC). All statistical tests were two-sided, and $P < 0.05$ was considered as statistically significant.

RESULTS

Selected Characteristics of the Subjects in GC and Control Groups

The baseline characteristics and the intervention assignment of the subjects in GC and control groups were shown in **Table 1**. Compared with controls, GC group had a significantly higher proportion of *H. pylori* infection (81.4 vs. 58.1%, $P < 0.001$), and more advanced gastric lesions such as IM or DYS (46.5, 40.7 vs. 31.4, 13.9%, $P < 0.001$). No significant differences were observed between GC cases and controls in age, sex, smoking, alcohol intake and any of the three interventions.

Relationships Between Epidemiologic Parameters and Telomere Length of cfDNA

With a total of 276 pairs of serum samples collected at baseline or three follow-up time-points from the 86 pairs of GC and control subjects, we firstly evaluated the associations between telomere length of cfDNA and age or other potential influencing factors (**Table 2**). Linear mixed model found that aging was a significant risk factor for telomere shortening (regression coefficient $= -0.009$, $P < 0.001$ per 1 year older), after adjusting with other factors in multivariate model. No significant associations were found between cfDNA telomere length in serum and sex, smoking, alcohol drinking or *H. pylori* infection status (all $P > 0.05$).

Effects of Interventions on Telomere Length of cfDNA

To evaluate the effects of the three interventions on serum cfDNA telomere length, we calculated the change of telomere length in 86 *H. pylori*-positive subjects with serum samples from 1994 and 1996 for the comparison between anti-*H. pylori* and placebo treatments, and in 104 subjects with serum samples from 1994 and 1999 for the comparison between long-term supplementation of garlic or vitamin and placebo treatments. No significant differences in change of telomere length were found between any active treatments and corresponding placebo groups, with *P*-values as 0.587 for anti-*H. pylori* treatment, 0.363 for garlic supplementation and 0.457 for vitamin supplementation (**Table 3**).

The Associations Between Baseline cfDNA Telomere Length and Risks of GC or Precancerous Lesions

Among 86 pairs of GC and control subjects, 73 pairs possessed serum samples at baseline in 1994. The baseline cfDNA telomere length median was shorter in those who progressed to GC during the follow-up period than in controls, $P < 0.001$. Shortened cfDNA telomere length at baseline was associated with increased subsequent GC risk (OR = 9.17, 95%CI: 2.72, 31.25, $P < 0.001$, for 1 unit shortening), after adjusting with age, *H. pylori* infection and gastric lesions (**Table 4**).

In addition to the risk of GC, telomere lengths were also analyzed among different baseline gastric lesions. Statistical difference was found in telomere length medians (interquartile range, IQR) among various gastric lesion groups [mild gastric lesions (no more than chronic atrophy gastritis): 2.12 (IQR, 1.35–2.40); IM: 1.55 (IQR, 1.17–2.16); DYS: 1.71 (IQR, 1.22–1.99), $P = 0.038$]. While after adjusting with age and *H. pylori* infection, unconditional logistic regression showed no significant associations between shortened cfDNA telomere and IM (OR = 1.56, 95%CI: 0.88–2.75 for 1 unit shorting) or DYS (OR = 1.62, 95%CI: 0.86–3.07 for 1 unit shorting) with mild gastric lesions group as reference.

The risks of subsequent GC progression were further investigated in different categories of baseline gastric lesion. Telomere length medians were significantly shorter in GC than in control group for each baseline lesion category. Significantly higher risks of GC progression were also found by multivariate analysis with shortened cfDNA telomere length; ORs per 1 unit shortening were 6.99 (95% CI: 1.63–30.30, $P = 0.009$) for mild gastric lesions, 6.06 (95% CI: 1.89, 19.61, $P = 0.002$) for IM and 15.63 (95% CI: 1.91, 125.00, $P = 0.010$) for DYS (**Table 3**).

TABLE 1 | Selected characteristics of subjects in GC and control groups.

Variables	Total ($n = 172$)	GC ($n = 86$)	Control ($n = 86$)	*P*
Baseline characteristics				
Age (years, mean ± SD)	50.8 ± 9.6	51.9 ± 9.7	49.8 ± 9.5	>0.999[a]
Sex (%)				>0.999[b]
Female	42 (24.4)	21 (24.4)	21 (24.4)	
Male	130 (75.6)	65 (75.6)	65 (75.6)	
Smoking (%)				0.519[b]
Never	58 (33.7)	27 (31.4)	31 (36.1)	
Ever	114 (66.3)	59 (68.6)	55 (63.9)	
Drinking (%)				>0.999[b]
Never	60 (34.9)	30 (34.9)	30 (34.9)	
Ever	112 (65.1)	56 (65.1)	56 (65.1)	
H. pylori infection (%)				<0.001[b]
Negative	52 (30.2)	16 (18.6)	36 (41.9)	
Positive	120 (69.8)	70 (81.4)	50 (58.1)	
Gastric lesions (%)				<0.001[b]
Normal/SG/CAG	57 (33.1)	10 (11.6)	47 (54.7)	
IM	67 (39.0)	40 (46.5)	27 (31.4)	
DYS	47 (27.3)	35 (40.7)	12 (13.9)	
Missing	1 (0.6)	1(1.2)	–	
Intervention treatments assigned in 1995				
Anti-*H. pylori* treatment[c]				0.339[b]
No	59 (49.2)	37 (52.9)	22 (44.0)	
Yes	61 (50.8)	33 (47.1)	28 (56.0)	
Garlic supplement				0.360[b]
No	88 (51.2)	47 (54.7)	41 (47.7)	
Yes	84 (48.8)	39 (45.3)	45 (52.3)	
Vitamin supplement				>0.999[b]
No	90 (52.3)	45 (52.3)	45 (52.3)	
Yes	82 (47.7)	41 (47.7)	41 (47.7)	

[a] *Equivalence t-test.*
[b] *Pearson's Chi-square test without missing values.*
[c] *Anti-H. pylori treatment and corresponding placebo was only assigned to 120 H. pylori positive subjects.*
CAG, chronic atrophic gastritis; DYS, dysplasia; GC, gastric cancer; H. pylori, Helicobacter pylori; IM, intestinal metaplasia; SD, standard deviation; SG, superficial gastritis.

The Association Between cfDNA Telomere Length and the Risk of Subsequent GC Based on All Serums From Four Collection Time-Points

Considering the significant association between baseline cfDNA telomere length and GC risk, we further evaluated the association using 276 pairs of serum samples from all four collection time-points by generalized linear mixed model. After adjusting with age, smoking, drinking, *H. pylori* status, baseline gastric lesions and calendar year of serum collection, shortened cfDNA telomere length was significantly associated with higher risk of GC progression (OR = 7.37, 95%CI: 2.06, 26.32, $P = 0.002$, for 1 unit shortening).

Temporal Trend of cfDNA Telomere Length in GC Development

To investigate the temporal trend of cfDNA telomere length in the process of gastric carcinogenesis, 276 pairs of serum samples from GC subjects and corresponding matching controls were assigned into sub-groups according to the interval years between sample collection and clinical diagnosis of GC (**Figure 1**). Compared to their corresponding controls, GC subjects showed shorter age-adjusted telomere lengths for most interval year sub-groups from 4 to ≥14 years ($P < 0.05$, except for the sub-group of 8 or 10 years). From 1 to 3 years before GC-diagnosis, no significant differences were found between progress-to-GC group and matching control group (*P*-values as 0.148, 0.235, and 0.115, respectively).

TABLE 2 | Relationships between epidemiologic parameters and telomere length of cfDNA from baseline and follow-up time-points.

Variables	Unadjusted model[a]		Adjusted model[b]	
	Regression coefficient (95%CI)	*P*	Regression coefficient (95%CI)	*P*
Age	−0.009 (−0.012, −0.006)	<0.001	−0.009 (−0.012, −0.005)	<0.001
Sex				
(Female=0, Male=1)	−0.056 (−0.132, 0.020)	0.150	−0.050 (−0.169, 0.069)	0.409
Smoking				
(Never=0, Ever=1)	−0.079 (−0.147, −0.011)	0.024	−0.040 (−0.147, 0.066)	0.455
Drinking				
(Never=0, Ever=1)	−0.008 (−0.076, 0.061)	0.824	0.042 (−0.049, 0.133)	0.366
H. pylori infection				
(Negative=0, Positive=1)	−0.025 (−0.096, 0.046)	0.490	−0.044 (−0.114, 0.027)	0.223

[a] *Univariate linear mixed model.*
[b] *Multivariate linear mixed model adjusting for age, sex, smoking, alcohol intake, and H. pylori infection status.*
CI, confidence interval; H. pylori, Helicobacter pylori.

TABLE 3 | Changes of cfDNA telomere length after intervention.

Intervention treatments assigned in 1995	Total		
	n	Changes of telomere length median (interquartile range)	*P*[e]
Anti-*H. pylori* treatment[a,c]			0.587
No	41	0.13 (-0.34, 0.69)	
Yes	45	0.42 (-0.12, 0.83)	
Garlic supplement[b,d]			0.363
No	54	1.23 (0.75, 1.67)	
Yes	50	1.07 (0.71, 1.47)	
Vitamin supplement[b,d]			0.457
No	56	1.21 (0.71, 1.71)	
Yes	48	1.11 (0.86, 1.36)	

[a] *For anti-H. pylori treatment, the change of telomere length was defined as the difference of serum cfDNA telomere length between 1994 and 1996.*
[b] *For garlic supplement and vitamin supplement, the change of telomere length was defined as the difference of serum cfDNA telomere length between 1994 and 1999.*
[c] *A total of 86 subjects who received anti-H. pylori or placebo treatment in 1995 and possessed serum samples both from 1994 and 1996 were analyzed.*
[d] *A total of 104 subjects who received supplementation of garlic or vitamin or corresponding placebos and possessed serum samples both from 1994 and 1999 were analyzed.*
[e] *Wilcoxon rank-sum test.*

The Association Between Annual Telomere Length Attrition Rates and the Risk of GC

The medians of annual attrition rate were 0.165/year (IQR, −0.001 to 0.277/year) in GC group and 0.157/year (IQR, 0.004–0.251/year) in control group, respectively. No significant difference was found between the two groups, $P = 0.656$. We further divided the interval years between serum collection and GC-diagnosis into five categories (≥14 years, 11–13 years, 7–10 years, 4–6 years, and 1–3 years). The annual attrition rate medians of telomere length in GC and control groups showed no significant difference in each category (**Figure 2**).

DISCUSSION

Based on a long-term follow-up study in a high-risk population of GC, we found that subjects with shortened cfDNA telomere lengths were associated with a higher risk of GC progression. Moreover, shortened cfDNA telomere length can be detected more than 3 years ahead of GC diagnosis. To our best knowledge, this is the first population-based prospective study to dynamically explore telomere length of serum cfDNA in the process of GC carcinogenesis.

Although associations between telomere length and cancer risk or prognosis have been extensively investigated in tumor tissues in the past decades (19–21), telomere in peripheral blood leukocytes is attracting more attentions for its non-invasion and accessibility in the recent studies (22). However, inconsistent results were found in most of the case-control studies with positive (longer telomere with higher cancer risk), negative (shorter telomere with higher cancer risk), or even U-shape associations (9, 22). A previous longitudinal study based on American Normative Aging Cohort suggested longer blood leukocyte DNA telomere only occurred at 3–4 years ahead of cancer incidence (23). While, the main cancer types in this cohort were prostate and skin tumors, which may limit the extension

TABLE 4 | Associations between telomere length of cfDNA and GC risks.

	GC		Control			
	n	**cfDNA telomere length median (interquartile range)**	***n***	**cfDNA telomere length median (interquartile range)**	**OR (95%CI)**	***P***
Total subjects	73	1.37 (1.08–1.74)	73	2.17 (1.76–2.51)	9.17[b] (2.72–31.25)	<0.001[b]
Baseline gastric lesions[a]						
Mild gastric lesions	10	1.36 (1.08–1.86)	39	2.18 (1.76–2.48)	6.99[c] (1.63, 30.30)	0.009[c]
IM	35	1.26 (1.08–1.74)	23	2.16 (1.60–2.79)	6.06[c] (1.89, 19.61)	0.002[c]
DYS	27	1.52 (1.09–1.87)	11	2.19 (1.81–2.65)	15.63[c] (1.91, 125.00)	0.010[c]

[a] *One subjects was excluded for missing pathology diagnosis at baseline.*

[b] *Conditional logistic regression analysis for the OR per one unit decrease of telomere length, adjusting for age, H. pylori infection and gastric lesions.*

[c] *Unconditional logistic regression analysis for the OR per one unit decrease of telomere length, adjusting for age, H. pylori infection. cfDNA, cell-free DNA; CI, confidence interval; DYS, dysplasia; GC, gastric cancer; IM, intestinal metaplasia; OR, odds ratio.*

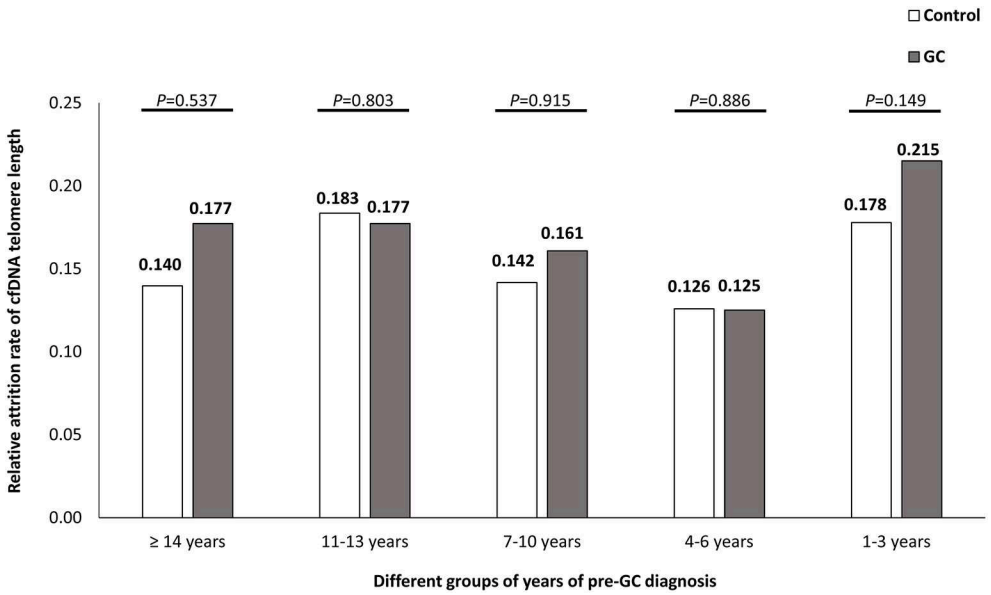


FIGURE 2 | The temporal trends of relative annual telomere attrition rates in GC and control groups. The years before GC-diagnosis of pre-diagnostic samples from GC subjects were classified into five groups as ≥14 years, 11–13 years, 7–10 years, 4–6 years, and 1–3 years, respectively. The annual attrition rates between GC and control groups were compared by Wilcoxon rank-sum tests.

of the result to GC. Our study demonstrated no significant association between the telomere length of blood leukocyte DNA and GC risk (data not shown). The divergence of these studies suggested that the homogeneous hematopoietic blood leukocyte DNA telomere length may not be a sensitive cancer predictor, and alternations are needed, such as cfDNA from serum.

The origins of cfDNA are not totally clear until now. Increasing evidences suggested that it might be passively released by apoptotic and necrotic cells as DNA fragments (24) or actively excreted from distant tumor cells for signal transmission (25). The higher amount of cfDNA was reported from serum compared to plasma (26). Our study found increased concentration of cfDNA in serum within 5 years ahead of clinical diagnosis of GC (data not shown). These results suggested the less degraded serum cfDNA as a better biomarker to synthetically reflect the alterations from infection, inflammation, and carcinogenesis in the whole body. Although the fragmented status of cfDNA may limit the accuracy of quantitative PCR, our well-designed study with matched serum pairs from the same follow-up time-point can relatively show the telomere length difference between GC cases and controls. The significantly shortened cfDNA telomere in the pre-diagnostic serums of GC subjects from baseline and different follow-up time-points further confirmed it as an early event in gastric carcinogenesis.

Precancerous gastric lesions were previously reported to be associated with shorter telomere (6). A recent prospective

genomic and epigenetic profiling study revealed that telomeres were initially reduced in high risk IMs (who developed to GC), but subsequently restored during GC progression (27) for the activation of telomere maintenance mechanisms (4, 28). In the current study, we also found shortened baseline cfDNA telomeres especially in IM subjects, while the telomeres were restored in DYS. When stratified by baseline lesion categories, the higher risk of GC progression for subjects with shortened telomere in each category further confirmed that aberrant alteration of cfDNA telomere may be associated with gastric carcinogenesis from early stage of precancerous lesions. While further prospective validation in larger number of precancerous gastric lesion subjects is still needed.

Although shorter telomeres in high risk IMs were reported previously (27), only 6 IMs progressing to DYS or GC in that cohort limited the generalization of the results. Our prospective study dynamically explored cfDNA telomere length changes in 86 GCs identified in 16-year follow-up period and found significantly shortened telomeres from 4 years to more than 14 years ahead of GC-diagnosis. Our results may reflect the comprehensive effects during the long-term carcinogenesis process, such as more severe inflammation and precancerous lesions in those who progressed to GC subsequently. In addition, genetic variations regulating individual telomere length may also be involved in the susceptibility of GC more than 10 years before clinical diagnosis in the present study (29). Although no serums were detected after GC-diagnosis in our study, non-significant differences between two groups from 1 to 3 years ahead of GC diagnosis may also support the restoration of telomere after cancer incidence.

With 2–4 serum samples collected for each subject in long-term follow-up period, our study had a unique opportunity to calculate the average annual telomere attrition rates. For the first time, we described the alteration of telomere attrition rates from 1 to more than 14 years before GC diagnosis, although no significant difference was found between GC and control groups.

The mechanism of telomere shortening has been well-explained by incomplete synthesis of chromosomal ends with cell division (30), which can be modified by physiological and pathological factors, such as aging (31), inflammation (32), and carcinogenesis (4). Consistent with previous studies (33, 34), our study found shorter cfDNA telomeres in older subjects and continuous telomere shortening in temporal trend analysis in control group, which may be caused by aging. Although infection of *H. pylori*, an important risk factor of GC, was reported to be associated with shorter telomere length in gastric mucosa (35), we did not found a remarkable relationship between *H. pylori* infection status and cfDNA telomere length at baseline. Based on the large intervention trial, our study is further capable of analyzing the effects of anti-*H. pylori* treatment and supplementation of garlic or vitamin on cfDNA telomere length. With the similar distributions of the three intervention arms between GC and control groups, no effective influence on telomere length alteration was found 1 year after 14-day anti-*H. pylori* treatment and after 4-year supplementation of garlic or vitamin, although further long-term effects still need longer follow-up with more bio-sample collection in the future.

The major strength of our study lies in the prospective design, which enabled us to estimate temporal associations between dynamic telomere alterations and GC risk with multiple pre-diagnostic serum samples from GC subjects and corresponding controls based on the same cohort. A limitation of our study is the relative small number of GC subjects. Only 86 GC cases were enrolled with the multiple time point pre-diagnostic serum samples. Individual matched controls were also strictly selected from the same 16-year prospective cohort in Linqu County. Multicenter confirmation with larger sample size is still needed for the extrapolation of the present results. In addition, the mechanism underlying the significant association between cfDNA telomere length and GC risk still require future studies.

In conclusion, our population-based study provided evidence for the first time that aberrant alterations of cfDNA telomere length may happen early in the process of GC development. The dynamic observation of telomere shortening may provide us clues for further etiological study on gastric carcinogenesis and may serve as a potential non-invasive marker for high-risk population screening and monitoring.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Institutional Review Board of Peking University Cancer Hospital with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Board of Peking University Cancer Hospital.

AUTHOR CONTRIBUTIONS

W-CY and K-FP: study design and manuscript proofing. D-JD: experimental design. LZ, J-LM, TZ, Z-XL, W-DL, and W-QL: data collection. YS and YZ: experimental operation, data analyses, and manuscript writing. All authors: results interpretations.

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