Circulating Tumor DNA in Gastrointestinal Cancers

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Gastrointestinal (GI) cancers appear as major health burdens worldwide with high incidences and mortality rates. For these cancers, stage at diagnosis remains the most important prognostic factor for clinical outcome. However, the emergence of simple and reproducible biomarkers is needed for the management of these diseases along their evolution.

circulating tumor DNA | gastrointestinal cancers | personalized medicine | biomarker

1. Introduction

Gastrointestinal (GI) cancers appear as major health burdens worldwide with high incidences and mortality rates. For these cancers, stage at diagnosis remains the most important prognostic factor for clinical outcome. However, the emergence of simple and reproducible biomarkers is needed for the management of these diseases along their evolution. Circulating cell-free DNA (cfDNA) can be detected in plasma, urine, and other bodily fluids for everyone, and is increased in inflammatory diseases, infections and cancers [1][2]. For patients with cancer, a fraction of this cfDNA, called circulating tumor DNA (ctDNA), contains tumor-specific molecular alterations [3][4]. Detection of ctDNA is challenging: First, for the majority of patients, quantities remain very low. Moreover, ctDNA is diluted within total cfDNA and its identification can be difficult. New approaches aretherefore in development to overcome this sensitivity challenge. Depending on cancers subtypes, specific molecular alterations can attest for the presence of ctDNA, which is a promising non-invasive biomarker in the era of personalized medicine. In this review, we tried to resume the molecular aspects of ctDNA and in what extent this biomarker can help clinicians in the detection, screening, diagnosis, prognosis, monitoring and personalization of treatment in patients with gastrointestinal cancers.

2. Early Cancer Detection through Circulating Tumor DNA and Molecular Profile Determination

The GI cancer diagnosis is currently based on a histological assessment and therefore requires tissue sample collected by surgical resection, endoscopic ultrasound, or biopsy of primitive tumor or accessible metastasis. Several studies assessed the interest of ctDNA as screening tool for early tumor stage. However, further studies are still required to prove the clinical utility of ctDNA in early diagnosis as stipulated by American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) in a recent report [5]. Some findings

demonstrated that asymptomatic cancers could be detected years before conventional diagnosis through non-invasive blood tests. In a recent longitudinal study, analysis of ctDNA methylation was performed on plasma samples from 605 asymptomatic individuals. Among them, 191 later developed stomach, esophageal, colorectal, lung or liver cancer within four years of blood draw. This method was able to detect cancer in 95% of asymptomatic individuals who were later diagnosed [6]. However, future longitudinal studies are required to confirm these results. The main risk of this early screening would be over-diagnosis through false-positive results or through the detection of circulating genomic variants from cells that have taken the first step toward transformation but were never meant to become clinically important [5].

Tissue biopsy is usually only performed at diagnosis and can sometimes be hard to obtain. For these reasons, several studies have also evaluated in different GI cancers whether plasma molecular alterations can be detected with ctDNA and are correlated with tissue biopsies.

2.1. Colorectal Cancer

Tumor tissue is routinely used to search for *KRAS* or *NRAS* gene mutations that occur in around 55% of metastatic CRC (mCRC) and predict a lack of response to the EGFR-targeted monoclonal antibodies, such as cetuximab and panitumumab [7][8]. In the same context, BRAF mutation is another alteration known as a poor prognostic factor that can be targeted by a doublet-therapy combining an anti-BRAF kinase inhibitor (encorafenib) and anti-EGFR monoclonal antibody (cetuximab) [9][10][11].

In the context of mCRC, the quantitative PCR (Intplex qPCR) on ctDNA was described by Thierry et al. as a valuable detection method with a high rate of specificity and sensitivity, especially for *BRAF* V600E and *KRAS* mutations, in a prospective study on 106 patients with mCRC [12]. The digital droplet PCR (ddPCR) has also been validated by other group for detection of *KRAS* mutations in mCRC [13]. More recently, in a large prospective multicenter study. Another method consists in using the NGS-BEPER-method (22 genes), and two specific methylated biomarkers (*WIF1* and *NPY*) as a second-step test for NGS-negative specimens. Bachet et al. used this technique to evaluate the concordance of *RAS* mutations between plasma and tissue among 406 chemotherapy-naive patients with mCRC with detectable ctDNA (n = 329/412). By comparing the results of RAS status in ctDNA and in matched tumor tissus, they founded an accuracy of 83% with NGS alone versus 93% with NGS plus methylated biomarkers [14]. Supplementary studies also suggested a good concordance rate between mutations observed in tumor biopsy and those identified on ctDNA [12][13][14][15][16][17][18].

2.2. Pancreatic Cancer

The *KRAS* gene mutations occur in more than 90% of pancreatic cancer (PC), and appears therefore as the best candidate to assess the presence of ctDNA in this tumor [19][20][21][22]. However, the ctDNA detection rate in metastatic PC varies widely from 40% to 80% and could therefore explain some discordance between tumor and plasma mutation assessment [23][24][25][26]. It could explain the results of the recent meta-analysis of Luchini et al. including 14 studies involving 369 patients, that reported a concordance rate of only 32% between ctDNA and

tissue based on large NGS multi-gene mutation panels [27]. The overall pooled sensitivity and specificity of the mutational analysis on liquid biopsy compared to tumor tissue were 70% and 86% respectively. However, when focusing on studies analyzing *KRAS* mutations only, the sensitivity slightly decreased but the specificity increased and were 65% and 91%, respectively [27].

Indeed, apart from *KRAS* mutations for PC screening, adding NGS-based panel for other mutations such as *SMAD4, CDKN2A, ROS1, BRAF* and *TP53* could lead to higher levels of ctDNA detection [28][29][30]. More recently, methylation of promoter of *ADAMST1* and *BNC1* genes were also described as potential tool to assess the presence of ctDNA in PC [31].

However, the use of highly sensitive detection methods of ctDNA might lead to false diagnosis of PC. Indeed, *KRAS* mutations can be detected in plasma in some non-cancerous diseases such as chronic pancreatitis. In a pilot study from Rashid et al., 21.8% of patients with chronic pancreatitis were tested positive for *KRAS* mutations in plasma [32]. Among these 64 patients, none developed a PC, with a mean follow-up duration (by clinic and by positron emission tomography or endoscopic ultrasound) of 2.5 years [32].

Quantitative ctDNA assessment, or combining biomarkers and methylation detection may improve the specificity of ctDNA detection and therefore help to discriminate benign from malignant pancreatic diseases, even at early tumor stages [33][34][35][36].

2.3. Esophageal and Gastric Cancer

In gastric cancer (GC), despite a low-frequency of genomic alterations [37][38], routine tissue-based NGS showed that at least 37% of patients harbor somatic mutations (*TP53, KRAS*) or gene amplification, such as *HER2, MET, EGFR,* and *FGFR2* [39][40][41][42]. Some retrospective studies evaluated the feasibility of ctDNA detection by NGS among GC patients. In a recent study including 55 patients with GC tested by NGS, Kato et al. showed that 31 had concordant mutations between tumor tissue and ctDNA with levels ranged from 61.3% (for *TP53* mutation) to 87.1% (for *KRAS* mutation) [43]. In their meta-analysis, Gao et al., reported that ctDNA detection might be a specific, but still a low sensitive test in GC patients [44]. More recently, the analysis of a large cohort of 1630 patients with GC revealed that ctDNA-NGS genomic landscape was similar but not identical to tissue-NGS [45]. This could reflect the molecular heterogeneity, with some targetable molecular alterations identified at higher frequency via ctDNA-NGS compared with previous matched primary tissue-NGS samples [45].

Despite increasing use of genomic alterations to detect ctDNA in GC, the most investigated technique to prove the presence of ctDNA is detection of hypermethylation of gene promoters which might result in an inappropriate silencing of tumor suppressor genes [44][46]. The promoter methylation of *APC* and *RASSF1A* in cfDNA was described as frequent epigenetic events in patients with early operable GC [47]. Aberrant methylation of other genes such as *PCDH10, SOX17, TIMP3, MINT2* and *WAF1* also showed promising results in GC [46].

In esophageal squamous cell carcinoma (ESCC), preliminary studies suggested the feasibility of ctDNA detection [48]. Luo et al. used exome or targeted sequencing to detect somatic mutations in 11 patients with ESCC and

compared ctDNA from pre- and post-surgery plasma [48]. They compared plasma somatic mutations that were also identified in matched tumors and founded that mutant allelic franction (MAF) decreased after surgery [48].

2.4. Hepatocellular Carcinoma

The analysis of the mutational landscape of hepatocellular carcinoma (HCC) over 3000 samples in the Catalog of Somatic Mutation in Cancer showed that the most frequent tumor mutations were *TP53* (27%), *TERT* (25%) and *CTNNB1* (18%) [49][50][51]. Using targeted methods to detect these three genes mutations in plasma, ctDNA presence was proven from 20% to 55% of patients with HCC across different studies [50][51][52][53][54]. In one prospective study including 27 patients with proven ctDNA, only 22% of them (6/27) also had matched mutants in tumor tissues, underlying the heterogeneity of HCC [50]. Therefore, single specific molecular alterations do not seem to be sensitive or specific enough to be used as a diagnostic tool in HCC. Moreover, some molecular alterations could be unspecific for HCC, such as *TERT* mutations that were present in plasma for 9% of patients with cirrhosis and without evidence of HCC on imaging [52].

When using NGS techniques with panel of frequently altered genes in HCC, ctDNA detection rate reached 63% in a prospective cohort of 30 patients, with two thirds of patients with stage A according to the Barcelona Clinic Liver Cancer score (BCLC A). In this study, the concordance rate between plasma and tissue biopsy was 81% [55].

Despite the utility of gene point mutations, DNA methylation seems to be more broadly informative in HCC. In a recent study, a combination of five aberrant methylation biomarkers was able to distinguish HCC samples from control cirrhotic and not cirrhotic tissue samples, with a specificity of 95% [56].

Some single aberrant methylation genes have shown high concordance rates between plasma and tissue in HCC [57][58][59]. Among patients with hypermethylation of *CDKN2A*, which is described in up to 73% of HCC patients, Wong et al., reported a concordance rate of 81% between plasma and tissue biopsy with a specificity of 100% among control patients [57]. Hypermethylation of *RASSF1A* promoter could also to be a candidate and was found in up to 90% of HCC tissues [60][61][62][63][64]. However, it seems to be also detected in patients with non-malignant liver tumor, such as liver cirrhosis, chronic hepatitis B or in healthy controls, with a lower rate (13%, 4%, and 4%, respectively) [64]. Other single hypermethylated candidates, such as *SEPT9, VIM, FBLN1, TFPI2, TGR5, MT1M, MT1G, APC, SPINT2, SFRP1, GSTP1*, or hypomethylated candidates such as *LINE-1* showed promising results for HCC screening [61][65][66][67][68][69][70].

More recently, whole methylome analysis allowed discovering novel methylated DNA markers in HCC. Creation of a new panel with 6 methylated biomarkers (*HOXA1, EMX1, AK055957, ECE1, PFKP* and *CLEC11A*) was able to detect 75% of BLCL 0 and 93% of BCLC B HCC patients meeting Milan criteria and was superior to AFP [56].

2.5. Other GI Cancers

Molecular landscape of cholangiocarcinoma (CC) has been widely studied in the past few years trying to detect therapeutic targets [71][72][73]. The cholangiocarcinoma (CC) is usually separated between intrahepatic CC (IHCC)

and extra hepatic CC (EHCC). Some mutations such as *KRAS, BRAF* or *TP53* are more frequent in EHCC but remain rare, whereas others, such as *FGFR1-3* fusions and *IDH1/2* mutations are preferentially detected in IHCC and occur in around 15–20% of tumors [71][72][73]. A recent study including 24 CC patients has reported a concordance rate of 74% between mutations in tumor tissue and ctDNA. When stratifying on tumor localization, concordance rate was 92% for IHCC, but only 55% for EHCC [74].

3. Circulating Tumor DNA to Monitor Treatment Response and Detect Acquired Resistance

The non-invasive nature of ctDNA allows for repeated testing and molecular assessment of tumor during treatment. This dynamic assessment is a clear advantage over traditional tissue biopsy. In the advanced tumor stage, baseline ctDNA could be more helpful to capture the molecular spatial and temporal heterogeneity of the disease which is a particularly important biological issue, at diagnosis or later because of clonal evolution and selection [15]. Differences in molecular characteristics have been described between primary tumor and metastases, especially in metachronous lesions [16].

Moreover, the monitoring of ctDNA may also anticipate the evaluation of treatment efficacy by detecting emergent actionable molecular alterations implicated in therapeutic resistance to ongoing treatment.

3.1. Colorectal Cancer

In mCRC, longitudinal quantification of ctDNA appears to be correlated with tumor evolution in several studies [75][76]. By sequencing a panel of 15 genes with frequent somatic variant in CRC tissue sample at diagnosis of 53 patients with mCRC, Tie et al. evaluated ctDNA as disease monitoring. They reported that a level of reduction in ctDNA concentration during first cycle of chemotherapy was significantly associated with the objective radiologic response rate at 8–10 weeks and with a trend for a better PFS. [75]. Similarly, Garlan et al. showed that early changes of the ctDNA concentration could predict the efficacy of first- or second-line chemotherapy in a prospective cohort of 82 mCRC. They used ctDNA monitoring between the first and second or/and third cycle of chemotherapy to define a composite marker that allowed to separate patients in two groups of “bad” or “good” ctDNA responder. This marker was based on the “normalization” of the ctDNA concentration (thresholds of 0.1 ng/mL) and the slope of ctDNA concentration decrease. The group of better ctDNA responders demonstrated a significantly better tumor response rate, PFS and OS [76]. The changes of ctDNA concentration during treatment therefore appear as a relevant early tool to assess treatment efficacy and this biomarker should be evaluated in larger prospective series.

Furthermore, ctDNA can also be used to track clonal evolution. It has been suggested that CRC presumably contains resistant mutant clones before treatment that emerge under therapeutic pressure [77]. The acquisition of resistance can be accompanied by the emergence of *RAS* pathway mutations that could allow to anticipate radiologic progression [78][79]. Several studies have already described emergence of mutations detected by ctDNA under anti-EGFR treatment up to 5–10 months before imaging diagnostic [78][79][80]. By monitoring ctDNA,

Siravegna et al. also showed in a subset of patients, that the proportion of ctDNA, based on the detection of *KRAS* mutations, dynamically varied depending on the presence or the absence of anti-EGFR treatment. These possible dynamic clonal evolutions induced by therapeutic pressure justified to re-challenge anti-EGFR based treatment after a withdrawal period in mCRC. [80][81]. Some retrospective analyses of the phase 2 CRICKET and E-Rechallenge studies suggested that ctDNA could guide this re-challenge therapy because only patients without RAS or BRAF circulating mutations detected plasma at the time of re-challenge might achieve clinical benefit from the retreatment with anti-EGFRs [82][81][83].

In squamous cell carcinomas of the anal canal (SCCA), Human papillomavirus (HPV) is found in 90% [84]. Therefore HPV DNA appears as the best candidate to assess the presence of ctDNA in SCCA and can be detected in plasma by ddPCR with sensitivity up to 93% in HPV positive-cancers [85]. In a recent study enrolling 8 SCCA patients, ddPCR demonstrated 100% of specificity for the detection of HVP ctDNA [86].

3.2. Pancreatic Cancer

In advanced PC, some regimens such as FOLFIRINOX (5-fluorouracil, leucovorin, oxaliplatin, and irinotecan) and gemcitabine plus nab-paclitaxel are effective but are not devoided of toxicities [87][88][89][90]. The monitoring of *KRAS* mutation through ctDNA has been performed in several studies and suggested that its detection could predict radiological progression, but some results were however discordant [91][92][93]. The clearance of *KRAS* ctDNA during treatment predicted better PFS than remaining positive ctDNA [93], and increasing levels of *KRAS* ctDNA were also associated with worse PFS and OS [94][95]. Finally, the decline slope of ctDNA concentration based on mutation of *KRAS* was associated with OS in another study [96]. Apart from *KRAS* mutations, evolution of other mutations in plasma, such as *TP53, SMAD4, CDKN2A, KRAS, APC, ATM, FBXW7* and others could also be used to reliably reflect response to therapy [29][30].

Unlike other GI cancers, there is currently no targetable molecular alteration for all patients with advanced PC in clinical routine. However, some new treatment could be promising in PC, such as PARP inhibitors in case of germline *BRCA1/2* mutations [97]. Moreover, like in other tumors, checkpoints inhibitors seem to be efficient in advanced PC with microsatellite instability [98][99][100]. Molecular alterations could be detected in ctDNA in PC [92] and therefore maybe screen patients for targeted therapies in the future. In this context, Bachet et al. recently confirmed from the data of a randomized phase II trial that the ctDNA could be a predictive biomarker of l-asparaginase encapsulated in erythrocytes (eryaspase) efficacy in advanced PC [101].

3.3. Esophageal and Gastric Cancer

In patients with advanced gastroesophageal adenocarcinoma, the addition of trastuzumab to chemotherapy was associated with improvement of clinical outcomes for tumors with a high level of HER2 expression (IHC3+ or IHC2+ and FISH+) [102]. Some studies have already described the potential for ctDNA to detect *HER2* amplification by ddPCR with high concordance with classic immunohistochemistry and fluorescent in situ hybridization on tissue samples [103][104]. However, in the recent cohort of Maron et al. seven patients with advanced disease were tested

for *HER2* amplification in both primary and metastatic tumor, and in ctDNA. Among them, only 2 patients (28%) were concordant for *HER2* amplification detection in the three samples, underlying possible missed detection of *HER2* amplification by NGS and then the risk of missed opportunities to use anti-HER2 therapies [45]. Despite its lack of sensitivity, ctDNA could however be used in combination with tissue NGS to define a group of extremely sensitive *HER2* amplified patients when treated with trastuzumab [45].

Moreover, some authors already suggested that ctDNA could also be used to monitor response to therapy in GC. In a recent study, tumor responses to lapatinib plus capecitabine were closely related with changes of the level of amplification of *HER2* detected in plasma through serial ctDNA sequencing [105]. In the study of Maron et al. dynamic measurements of ctDNA before and during treatment showed that a decrease superior to 50% in MAF was correlated with better OS [45]. The detection of therapeutic resistance to treatment in advanced GC could also be improved by ctDNA. In the cohort of Maron et al. some anti-HER2 therapy acquired resistance mechanisms were detected using ctDNA [45].

3.4. Hepatocellular Carcinoma

In advanced HCC, ctDNA could be used to monitor tumor burden under therapy. A diagnostic prediction model with 10 selected methylation markers through ctDNA was recently developed by Xu et al. and correlated with tumor burden, treatment response, and disease stage [106].

In a study using whole exome sequencing to evaluate ctDNA among HCC patients who underwent surgery, in patients with positive ctDNA after surgery, the levels of serum ctDNA increased with disease progression and responded to the additional treatments [107].

The somatic MAF of ctDNA could also reflect clinical dynamics as demonstrated in one patient with advanced HCC undergoing trans-arterial chemoembolization in whom increasing level of 8 somatic mutations in plasma was detected before imaging diagnosis and the increase of standard biomarker AFP [108].

3.5. Other GI Cancers

In CC, until past years, chemotherapy was the only validated treatment for advanced disease [73][109]. Recently, some targeted therapies emerged in the therapeutic arsenal. Ivosidenib, a first-in-class oral IDH1 inhibitor, has demonstrated an improvement of PFS over placebo in advanced CC with *IDH1* mutations in the phase III ClarIDHy study [110]. In another phase II study (NCT-02150967), BGJ398, an orally bioavailable, selective pan-FGFR kinase inhibitor demonstrated clinical activity against chemotherapy-refractory CC with *FGFR2* fusions [111]. Lastly, the phase II study FIGHT-202 also supported the efficiency of pemigatinib, an oral inhibitor of FGFR1, 2, and 3 in previously treated patients with cholangiocarcinoma with *FGFR2* fusions or rearrangements [112]. Therefore, the interest in monitoring ctDNA in CC is increasing. Goyal et al. already monitored 9 patients with *FGFR2* fusions and detected de novo point mutations that conferred resistance to BGJ298 in all patients (n = 3) who underwent progression [113]. Ettrich et al. recently demonstrated that 63% of treatment naïve patients with advanced CC had changes in their mutational profile during chemotherapy. They evaluated and identified a set of 76 potential

progression driver genes among a large-scale panel sequencing of 710 cancer-related genes [74]. These data suggest that ctDNA could be used to track disease progression.

In GIST, one main application of ctDNA seems to be monitoring response to therapy and tracking therapeutic resistance to tyrosine kinase inhibitors (TKI) [114][115][116][117][118]. Indeed, despite the revolution in GIST management through the contribution of first line TKI such as imatinib targeting *KIT* or *PDGFRA* molecular drivers, the majority of GIST will progress with the acquisition of secondary *KIT* or *PDGFRA* mutations. In this context, second and third line TKI have been used in some refractory GIST patients [119][120][121][122]. Maier et al. first described a dynamic change in MAF in plasma of advanced GIST under treatment. A decrease or a disappearance of ctDNA occurred in patients responding to TKIs [123]. In other studies, the usefulness of ctDNA for the identification of TKI resistance mutations and their prognostic utility was demonstrated [117][118]. In a phase II study patients with secondary *KIT* mutations had significantly worse OS than those with no detectable secondary mutations [117]. ctDNA can also be used to detect resistance mutations in other gene than *KIT*, as demonstrated in a prospective study that collected 30 plasma samples from 22 patients with metastatic GIST [124]. Monitoring ctDNA using NGS patients with GIST under TKI treatment detected primary but also secondary mutations emerging in patients who had a progressive disease whereas only primary mutations were detected in patients with stable disease. These resistance mutations in ctDNA could represent early biomarkers for treatment response [116][125].

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