The clinical role of circulating free tumour DNA in gastrointestinal malignancy

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Abstract

Circulating cell-free DNA (cfDNA) is DNA released from necrotic or apoptotic cells into the bloodstream(1). Whilst both healthy cells and cancer cells release cfDNA, tumours are associated with higher levels of tumour-derived circulating cell-free DNA (ctDNA) detectable in blood(2). Absolute levels of ctDNA, as well as genetic mutations and epigenetic changes detected in ctDNA show promise as potentially useful biomarkers of tumour biology, progression, and response to therapy(2). Moreover, studies have demonstrated the discriminative accuracy of ctDNA levels for diagnosis of gastrointestinal cancer compared with benign inflammatory diseases(3). Therefore, ctDNA detected in blood offers a minimally invasive, easily repeated "liquid biopsy" of cancer(4, 5), facilitating real-time dynamic analysis of tumour behaviour that could revolutionise both clinical and research practice in oncology(2, 6).

In this review, we provide a critical summary of the evidence for the utility of ctDNA as a diagnostic and prognostic biomarker in gastrointestinal malignancies.

Circulating cell-free DNA (cfDNA) is DNA released from necrotic or apoptotic cells into the bloodstream(1). Whilst both healthy cells and cancer cells release cfDNA, tumours are associated with higher levels of cfDNA detectable in blood (average 180ng/mL, ranging from 0->1000ng/mL) compared with healthy controls (average 30ng/mL, ranging from 0-100ng/mL)(7). Within these ranges there is considerable variability between individuals, in part influenced by underlying the inflammatory state(7). cfDNA derived from tumour cells is termed circulating cell-free tumour DNA, or ctDNA(2). cfDNA is highly fragmented, consisting of short segments (<185-200bp in length)(8, 9) which are generated by cellular apoptosis and long fragments (200bp-21kbp) generated by necrosis, the latter being more prevalent in ctDNA in the presence of cancer(1). ctDNA is therefore distinguishable from cfDNA using this method, as well as the presence of genetic alterations present in tumour cells but not healthy cells. ctDNA is rapidly cleared from serum and plasma and therefore represents a highly dynamic marker of tumour biology(10, 11). However, studies have shown that mutations detected in ctDNA do not correspond perfectly with those identified in primary tumour tissue DNA, particularly for early-stage tumours. Contributing factors to this current limitation in ctDNA detection are that copy numbers of ctDNA are generally very low compared with copy numbers of wild type cfDNA, and limitations in accuracy of current sequencing technologies limit sensitivity for detecting specific mutations in cancer(2, 12). Additionally, tumour cells prone to release of ctDNA may be genetically different to the majority populations detected in the primary tumour due to genetic heterogeneity (2).

There are several ways in which cfDNA provides invaluable genomic data for clinical studies. Absolute levels of ctDNA and genetic point mutations within ctDNA detected using point mutation targeted assays, whole exon genetic sequencing or even whole genome sequencing techniques provide invaluable genomic data. Additionally, epigenetic changes such as hypermethylation of CpG residues can also be determined. These alterations in ctDNA in blood at baseline and over time are potentially useful biomarkers of tumour biology, progression, and response to therapy(2, 6). Whilst evidence shows cfDNA levels are also elevated in inflammation(7, 13) and trauma(14), several studies have demonstrated the discriminative accuracy of ctDNA levels in patients with gastrointestinal cancer compared with patients with benign inflammatory diseases(3, 15-17). Therefore, ctDNA detected in blood offers a "liquid biopsy" of cancer(4), potentially obviating the need for invasive tumour biopsy in some clinical scenarios and facilitating dynamic, repeated evaluation of tumour characteristics(2, 7).

Tumour biopsy is invasive, painful and carries a risk to patients of complications such as bleeding and damage to neighbouring structures. Moreover, tumour biopsies may only sample one area of tumour and miss important biological information due to tumour heterogeneity. Some tumours such as pancreatic cancer are difficult to access and therefore repeated sampling of tumours to monitor for prognostic mutations is not practical. ctDNA therefore has the potential to revolutionise both clinical management and research in oncology by offering patients a rapid, minimally invasive means of monitoring tumour behaviour(5), which through being more acceptable to patients, may also improve adherence to tumour management strategies . The non-invasive,

easily repeatable nature of ctDNA detection also offers considerable benefits for large-scale participation in gastrointestinal cancer research studies.

In this review, we provide a critical summary of the evidence for the utility of ctDNA as a diagnostic and prognostic biomarker in gastrointestinal malignancies.

Methods of quantifying cfDNA levels, gene mutations and gene methylation

Significant advancements have been made in cfDNA detection and quantification methods in recent years and there are several excellent reviews and many papers describing technical aspects of cfDNA quantification and ctDNA mutation detection to which we direct the reader(2, 6, 7, 18-20). Methodology will therefore not be discussed in detail in this review. A brief summary of the process of cfDNA isolation, ctDNA mutation detection and methylation pattern determination is provided in Figure 1.

Briefly, cfDNA is isolated from plasma or serum and analysed qualitatively using older fluorescence-based methods for cfDNA detection, or increasingly by using quantitative, highly sensitive digital polymerase chain reaction (PCR)(7). Allele-specific targeted mutation analysis digital PCR methods allow detection of pre-specified gene mutations to distinguish ctDNA from wild-type cfDNA(21), whilst massively parallel sequencing microfluidic techniques and duplex sequencing coupled with next generation sequencing allow efficient, sensitive sequencing of the entire genome(7, 20). It is possible to detect down to 0.01-0.001% of mutation allele fractions using new methods(20, 22).

Short interspersed nucleic acid elements (ALU) repeats and long interspersed nucleotide elements (LINE1) are non-coding repetitive DNA sequences distributed throughout the genome that are used to calculate the DNA integrity index in cfDNA(7, 23). ALU115 fragments reflect cfDNA of healthy cell origin, whereas ALU247 fragments are more frequently detectable in ctDNA(24, 25). This is an older method for distinguishing ctDNA from wild-type cfDNA, is less sensitive and is not widely used now that digital PCR techniques have improved sensitivity and specificity. Relative telomere length is another technique used infrequently in ctDNA studies, where the length of telomere repeat sequences distinguishes ctDNA from wild-type cfDNA(26).

DNA methylation modifies gene expression, genomic imprinting and chromosome structure and stability(27, 28). Methylation of cytosine residues in dinucleotide CpG sites and is detectable using bisulphite conversion(28, 29) and subsequent methylation-specific digital PCR (MSP) (30). Panels of methylation markers for use in MSP have been developed for gene mutations commonly found in malignancy, akin to what have been developed for gene mutation digital PCR.

DNA has also been isolated from free circulating tumour cells in plasma to allow more specific clonal information about tumour cells independently of normal host cells(2). However, this technique is beyond the scope of this review.

Evidence for the utility of ctDNA detection for the diagnosis and management of gastrointestinal malignancies

A landmark paper by Bettegowda et al(6) evaluated the diagnostic utility of ctDNA in a number of different malignancies in a large sample of 640 patients. Their method used next generation sequencing of tumour tissue DNA to determine target mutations, which were then quantified in plasma ctDNA using RT-PCR(6). Over 75% of patients with advanced stage hepatocellular carcinoma, colorectal cancer, pancreatic cancer, stomach and oesophageal cancer had detectable ctDNA in plasma(6). In 223 patients without metastatic disease, ctDNA was detected in 73% of CRC, 57% of gastro-oesophageal cancers and 48% of pancreatic cancers(6). This paper established the broad potential diagnostic utility of ctDNA across diverse gastrointestinal tumour types.

Colorectal Cancer

CRC is the fourth most common malignancy with a global incidence of 17.2 cases per 100,000. CRC follows a stereotyped progression from premalignant polyp through to dysplasia, carcinoma in site and then carcinoma, associated with gradual accrual of genetic mutations. Stage I and II CRC are usually curable and only metastatic advanced stage IV disease has high mortality. Chromosomal instability with mutations in mismatch repair genes and loss of heterozygosity in the *APC* pathway are the most common mutations associated with adenoma development, with subsequent *KRAS*, *BRAF*, *PIK3CA*, *PTEN*, *TP53*, *BAX* and *TGF-β* mutations facilitating progression to carcinoma(31). Currently, premalignant adenoma and carcinoma screening is performed by colonoscopy, which provides easy access to tumour tissue for genetic biomarker analysis. However, patient preference for blood-

based testing instead of procedural screening tests and the drive for cost-effective CRC screening and diagnosis have led to much research into blood-based biomarkers in CRC.

The greatest volume of evidence for the diagnostic and prognostic utility of ctDNA detection in gastrointestinal malignancy exists for colorectal cancer (CRC) (Table 1).

Diagnosis and Screening in CRC

Levels of both overall cfDNA and tumour-specific ctDNA have been shown in multiple studies to be higher in patients with CRC compared with healthy controls(32-36) and appear to distinguish early-stage tumours from benign lesions with considerable accuracy (36, 37). In one study of 118 CRC patients, 49 with polyps and 26 healthy controls, cfDNA levels had a diagnostic accuracy of 80% for early-stage CRC compared with benign gastrointestinal disease (36).

ALU247 fragment concentration is higher in plasma ctDNA from CRC patients compared with healthy controls (23). A small pilot study reported the combination of LINE1, ALU247, ALU115 and mitochondrial DNA detection in plasma ctDNA had an AUC of 0.8 for CRC diagnosis alone, rising to 0.9 (PPV of 81% and an NPV of 74%) when combined with carcinoma-embryonic antigen (CEA) measurement (38). A recent large study by Hao et al(39) of 205 patients with CRC, 63 with polyps and 110 healthy controls found ALU115 and ALU247/115 ratio in ctDNA were significantly higher in CRC patients and patients with adenomas compared with healthy controls. Both levels fell after curative surgical resection(39).

Kirsten rat sarcoma viral oncogene homolog protein (*KRAS*) mutations are common in CRC (40-50%)(40, 41), occur early in the carcinogenic pathway(42) and have frequent mutation "hotspots" in codons 12 and 13 (43), making *KRAS* an attractive marker for CRC diagnosis. In a study of 58 patients with CRC, Lecomte et al(44) found 78% of patients with CRC had detectable ctDNA; 38% had *KRAS* mutations in tumour specimens, in whom 45% also had these mutations detectable in ctDNA(44). In a further study of 106 patients with metastatic CRC, Thierry et al(45) tested for 7 different common point mutations in the *KRAS* gene in plasma ctDNA and found 96% concordance between *KRAS* mutations in ctDNA and matched tumour specimens(45). Sensitivity of detecting *KRAS* mutations in ctDNA was 92% and specificity was 98%. However, two small studies have demonstrated *KRAS* mutations in ctDNA in 35% (22 of 62) patients with benign colorectal disease(46) and 50% (2 of 4) of patients with longstanding ulcerative pancolitis(47), raising concerns about specificity for CRC diagnosis but showing potential utility as a diagnostic tool for adenoma premalignant lesions. In the latter study, no correlation with mutations in primary tumour DNA was undertaken and no follow up of the patients with pancolitis was performed to determine outcome. This is a critical point as pancolitis is a strong risk factor for CRC, which is often sessile and difficult to diagnose. Moreover, others have found no evidence of *KRAS* mutations in ctDNA from patients with benign gastrointestinal diseases (16).

Mutations in the Adenomatous Polyposis Coli (*APC*) gene are also common in CRC (60-70%), occur early in carcinogenesis and over 80% occur in exon 15(48). *APC* mutations in ctDNA significantly correlated with stage of CRC disease in one

study(49), however mutations in the primary tumour were not evaluated. Tumour Protein 53 (*TP53*) is also commonly mutated in CRC (60-70%)(50, 51), however it is a late event in CRC and there are no hotspot sites for mutation, making it a less useful marker for early diagnosis(1). Studies suggest *TP53* mutation detection rates of less than 15% in ctDNA, however in those with known mutations in tumour the detection rate is approximately 40%(1, 52). One small study reported *BRAF1* mutations in ctDNA and matched primary tumour specimens from CRC patients compared with healthy controls (53). This was confirmed by Thierry et al(45), who found 96% concordance in *BRAF* mutations between ctDNA and tumour specimens. However, *BRAF* mutations do not occur frequently in early CRC and therefore may have limited diagnostic utility.

Abnormal gene methylation patterns are common in CRC and appear to be an early event in carcinogenesis, with good potential as biomarkers for diagnosis and screening(54, 55). Around 25% of CRC tumours have evidence of gene hypermethylation and 40% of these are detectable in ctDNA(1). One small study compared overall ctDNA gene methylation between 24 CRC patients, 10 patients with benign gastrointestinal disease and 56 healthy controls(56). High levels of overall DNA methylation were seen in both CRC and benign colorectal disease(56), demonstrating poor specificity of DNA methylation alone as a marker for CRC diagnosis.

Septin 9 (*SEPT9*) methylation in ctDNA is one of the best-validated biomarkers in colorectal cancer. *SEPT9* methylation is present in over 90% of CRC specimens, and has been well validated in several large, well-designed studies using ctDNA, with a

sensitivity of 68-79.3% and specificity of 84.8-89% for CRC diagnosis compared with healthy controls(57-61). In several large studies, *SEPT9* methylation in ctDNA is evident in 69 to 88% of CRC, 30% of benign adenomas and 8-14% of healthy controls(57, 60, 62). In another study of 92 CRC cases (25 stage I tumours and 67 stage II to IV tumours) and 92 controls, *SEPT9* was positive in 96.4% of left-sided CRC and 94.4% of right-sided CRC(57). By contrast, only 50% of right-sided tumours were detected using standard faecal-occult blood testing (FOBT) methods and 41.7% using CEA. This is clinically significant as right-sided tumours are more likely to be asymptomatic and missed by routine screening methods(57).

A well-designed study by Church et al(63) prospectively assessed the utility of methylated *SEPT9* for CRC screening of a large cohort of 7,941 asymptomatic individuals over 50 years of age using a commercial assay. 53 patients were found to have CRC and methylated *SEPT9* had sensitivity of 48.2% and specificity of 91.5% for overall diagnosis of CRC, with sensitivity values of 35.0%, 63.0%, 46.0% and 77.4% for stage I-IV disease respectively(63). Additionally, they found methylated *SEPT9* only had 11.2% sensitivity for detection of advanced adenomas(63). Others have shown *SEPT9* is infrequently detected in adenomas greater than 1cm in size (20%), and has lower sensitivity (14%) than stool DNA sensitivity (82%) for diagnosis of large adenomas in screening populations(59, 62, 64). Another large study found stool DNA had greater sensitivity and specificity for CRC diagnosis than serum methylated *SEPT9* in ctDNA(64). These studies clearly demonstrate that *SEPT9* alone is not a suitable screening marker for CRC, though it may have additional benefit in combination with other markers.

An interesting study by Ladabaum et al(65) evaluated the cost-effectiveness of *SEPT9* as a screening blood test for colorectal cancer in comparison to current screening strategies, including faecal occult blood testing (FOBT), faecal immunohistochemical testing (FIT), sigmoidoscopy and colonoscopy(65). *SEPT9* was cost-effective in comparison to no screening, but was inferior to other screening strategies. The cost-effectiveness of *SEPT9* and similar ctDNA-based markers would improve if they increased uptake and longitudinal adherence to screening(65).

A large study by Lee et al(17) evaluated methylation patterns in ten genes using matched primary tumour tissue and plasma ctDNA from 243 early-stage CRC cases, 64 patients with colonic adenomas and 276 healthy controls. They found aberrant gene methylation patterns in promoters of *p14* (18%), *p16* (34%), *APC* (27%), Death Associated Protein Kinase (*DAPK*) (34%), Helicase-Like Transcription Factor (*HLTF*) (32%), human MutL Homolog 1 (*hMLH1*) (21%), 0,6-Methylguanine-DNA-Methyltransferase (*MGMT*) (39%), Retinoic Acid Receptor Beta 2 (*RARbeta2*) (24%), Ras-Association Domain Family Member 2A (*RASSF2A*) (58%) and WNT Inhibitory Factor-1 (*Wif-1*) (74%) in CRC patients compared with those with benign disease and healthy controls (17). Whilst the methylation score for each individual gene had a sensitivity of less than 40%, by using a diagnostic cut-off methylation score of 1.6 in a model that included *APC*, *MGMT*, *RASSF2A* and *Wif-1*, the sensitivity of cfDNA methylation detection was 85.6% and specificity was 92.1% for CRC diagnosis, with a positive predictive value of 90.6% and a negative predictive value of 88.8%(17). *Wif-1*, *RASSF2A*, *p16* and *HMLH1* hypermethylation in ctDNA of CRC patients has also been reported by others(44, 66-71). *HMLH1* promoter hypermethylation appears to be an early event in carcinogenesis (66, 67). Others have also identified

hypermethylation of Neuropeptide Y (*NPY*) (sensitivity 97%, specificity 47%), Proencephalin (*PENK*) (95% sensitivity, 61% specificity) and Neurogenin 1(*NEUROG1)* (sensitivity 61%) in ctDNA as potential diagnostic markers of CRC(69, 72).

Leary et al described massively parallel sequencing and personalised analysis of rearranged ends (PARE) in matched tumour and ctDNA specimens to identify somatic structural variants (including gene copy number alterations and rearrangements) (12, 20) for personalised cancer monitoring with greater sensitivity than other methods (0.001% variant detection in ctDNA)(20). Whilst structural variants are rare in healthy cells and almost ubiquitous in cancer, they are highly unique between tumours and their utility is therefore confined to personalised tumour monitoring rather than diagnosis and screening.

Finally, microsatellite instability and loss of heterozygosity is a common feature in CRC and reflects defective DNA repair mechanisms(73). Evidence of microsatellite instability is evident in plasma ctDNA in approximately 35% of CRC patients(1). A small study found 16 of 27 (59%) patients who had CRC tumours with confirmed microsatellite instability also had detectable markers of microsatellite instability in ctDNA(74). However, detection artefacts and high false positive rates for detection in ctDNA are likely to limit its utility as a diagnostic marker of CRC(1).

To summarise, these studies collectively demonstrate utility of both ctDNA levels and gene mutations for the diagnosis of CRC compared with the healthy state. However, non-invasive detection of colonic adenomas to triage the need for colonoscopic

removal using ctDNA would also be desirable, and there is currently less evidence to support the utility of ctDNA techniques for distinguishing benign adenomas from the healthy state. Therefore, this should be an active area of research in future studies in CRC.

Prognosis in CRC

A small prospective study found *KRAS* mutations and *RASSF2A* hypermethylation in ctDNA were associated with reduced disease-free survival at one year in patients with metastatic CRC receiving chemotherapy(71). A larger study of *KRAS* mutations in 58 patients reported two year survival was only 48% in CRC patients with detectable plasma ctDNA, compared with 100% in those without(44). *KRAS* mutations in ctDNA have high diagnostic accuracy for CRC metastases, with sensitivity of 87.2% and specificity of 99.2 (6). A further small case-control study identified *TP53* mutations in cfDNA was associated with advanced clinical stage and liver metastases, but not lymph node metastases, tumour size or vascular invasion(52).

TAC1 and *SEPT9* methylation have prognostic utility in CRC. In a large study of 150 patients who underwent curative resection for CRC, methylated *TAC1* and *SEPT9* in serum ctDNA were independent predictors of tumour recurrence post surgery and cancer-specific mortality and were detectable in blood earlier than CEA levels(75). Additionally, hypermethylated *HMLH1*, *HLTF*, *HPP1* and *APC* promoters in ctDNA are associated with reduced overall survival in CRC in several prospective studies(66, 67, 70, 76). Others have also reported an association between *HLTF* and *HPP1/TPEF* hypermethylation and advanced tumour stage(70, 76). Further large prospective

studies are needed to validate the prognostic utility of gene alterations in ctDNA in CRC.

Post-surgical recurrence

Absolute levels of cfDNA predict survival post surgery in CRC, with lower pre-operative levels associated with greater survival(77, 78). Plasma cfDNA levels rises steadily immediately post surgery, perhaps reflecting inflammatory responses(35, 79). However, a rapid rise by day 3 post-operatively without a subsequent fall in levels is associated with tumour recurrence(34, 35, 78).

KRAS mutations and hypermethylation of p16 in ctDNA have both been associated with tumour recurrence post resection (35, 80). In one study, two-year recurrence-free survival post curative resection was 66% in patients with detectable ctDNA levels compared with 100% in those without detectable ctDNA (44). Importantly, one study showed only 3 of 16 CRC patients with post-operative tumour recurrence and *KRAS* mutation detection in ctDNA had elevated carcino-embryonic antigen (CEA) levels, suggesting combining ctDNA gene mutation and CEA levels may improve sensitivity for post operative recurrence detection.

In a well-designed, small study of 11 CRC patients who had undergone curative resection by Reinert et al(81), large somatic structural variants were identified in primary tissue specimens using next generation sequencing, then confirmed in plasma ctDNA using droplet-digital PCR. They demonstrated ctDNA detection was useful post resection for determining completeness of resection, response to adjuvant

chemotherapy, recurrence post surgery and development of metastases(81). Importantly, using this method allowed early and highly accurate diagnosis of recurrence prior to conventional techniques of tumour recurrence surveillance (both sensitivity and specificity were 100%). For metastatic disease, detection was an average of ten months earlier than conventional follow-up(81).

Taback et al (82) described an interesting technique to improve accuracy of ctDNA as a prognostic marker post resection, by isolating ctDNA from mesenteric venous samples taken during surgical resection, the hypothesis being that ctDNA levels may be higher in mesenteric venous samples due to CRC venous drainage via the mesenteric and portal systems. They confirmed that gene hypermethylation was more commonly detected in mesenteric (11/11) compared with peripheral plasma samples (9/11) (82). This novel technique is straightforward to employ during surgery and requires validation in larger studies.

Chemotherapy response and resistance

One of the most exciting applications of ctDNA is for determining tumour treatment response and resistance to chemotherapy. *KRAS* and *EGFR* mutations conferring resistance to EGFR inhibitors can be detected in ctDNA of patients who are failing EGRF inhibitor therapy(6, 83). *KRAS* and *BRAF* mutations measured in ctDNA prior to therapy and quantification of levels during therapy with cetuximab and irinotecan predict response to therapy(84, 85) and these mutations are detectable prior to radiological evidence of tumour progression (84, 86, 87). Though correlation between *BRAF* mutations in ctDNA and tumour specimens is high, studies have generally

found *BRAF* mutations to be rarely detected in tumour specimens of CRC patients, which limits its clinical utility compared with *KRAS* (36, 85, 88). A recent large study of 503 patients with CRC by Tabernero et al(88) confirmed the utility of detecting *KRAS, BRAF* and *PIK3CA* mutations in ctDNA in CRC patients on regorafenib therapy and demonstrated that mutations present in ctDNA change dynamically during chemotherapy treatment and may differ to those present in baseline, pre-treatment tumour samples.

Similarly, overall cfDNA levels have also been used to assess response to neo-adjuvant chemo radiation in rectal carcinoma. Responders have a significant reduction in plasma cfDNA levels after treatment compared with non-responders (89). DNA integrity index in cfDNA, measured by ALU repeats, is also an independent predictor of response to neo-adjuvant chemo radiation (90).

In summary, there is good evidence from large validation studies that methylated *SEPT9* and *KRAS* mutations in ctDNA are useful and accurate markers for CRC diagnosis, prognosis including detection of metastatic disease, and for rapid detection of post surgical recurrence. *KRAS* mutations additionally predict response to EGFR-based biologic agents. Methylated *RASSF2A*, *HMLH1* and *Wif-1* may also prove useful for diagnosis. Though few studies have specifically evaluated the utility of ctDNA in CRC for distinguishing patients with metastatic disease, pilot data suggest potential utility of ctDNA for metastatic disease detection in CRC. The time is ripe for further studies validating the cost-effectiveness of these markers within current management guidelines. However, available data suggest current test sensitivity is inadequate for their use as sole screening markers in CRC compared with current

screening strategies. Studies validating of combinations of biomarkers with CEA, and further assessment of the impact if blood-based biomarkers on CRC screening uptake and follow up are urgently needed.

Oesophageal Carcinoma

Incidence of oesophageal cancer is 5.9 per 100,000 globally(91) and it has 5-year survival rates of only 17% despite treatment. The majority of oesophageal carcinomas are adenocarcinoma (10%) or squamous cell carcinoma (90%). Barrett's oesophagus, metaplasia induced by chronic reflux esophagitis, represents the premalignant lesion in adenocarcinoma(92). Adenocarcinoma incidence and mortality have been steadily increasing over the last decade. The most common genetic mutations in oesophageal cancer are *TP53*, *CDKN2A*, *SMAD4* and *ARID1A* in adenocarcinoma and *TP53*, *RB1*, *CDKN2A*, *PIK3CA* and *NOTCH1* in squamous cell carcinoma(92). Diagnosis and screening in Barretts oesophagus is by gastroscopy, which has a low complication rate and allows simultaneous treatment options for many lesions as well as ready access to genetic material. However, preference for non-invasive approaches to screening and monitoring and a lack of other biomarkers provides a potential role for ctDNA in oesophageal carcinoma management.

To date, there have only been a handful of small case-control studies evaluating the clinical utility of cfDNA and ctDNA in oesophageal carcinoma. These are summarised in Table 2.

Diagnosis and screening of oesophageal carcinoma

Zhai et al (93) conducted genome-wide sequencing in cfDNA and matched tumour specimens in a small case-control study of 28 patients (8 with oesophageal carcinoma, 10 with Barrett's oesophagitis and 10 healthy controls). They found that ctDNA gene methylation profiles correlated significantly (r=0.92) with methylation profiles of the primary tumour and identified differences in genetic profiles between oesophageal carcinoma, Barrett's oesophagus and healthy controls(93). This requires further exploration in larger cohorts.

Disease prognosis and survival in oesophageal carcinoma

Hypermethylation of the MutS Homolog 2 (*MSH2)* promoter was identified in primary tumour specimens of 101 of 209 patients (48%) with oesophageal squamous cell carcinoma, of which 77 patients (76%) had matching findings in plasma ctDNA. *MSH2* hypermethylation detected in plasma ctDNA predicted reduced disease-free survival post oesophagectomy in one study (94), whilst another reported *APC* hypermethylation in ctDNA was associated with reduced survival in oesophageal adenocarcinoma, but was not commonly detected (95). Both studies were small and had no adjustment for confounding variables.

Current data are insufficient to define the role of ctDNA in oesophageal cancer diagnosis and management and further studies are warranted.

Gastric Cancer

The global incidence of stomach cancer is 12.1 cases per 100,000 (91, 96). Early stage tumours are curable by resection, however locally advanced disease has only 25-25% 5-year survival and most patients with metastatic disease survive less than 12 months despite treatment(96). Common genetic alterations in sporadic gastric cancer include altered methylation pattern of the mismatch repair genes and mutations in *TP53* and *HER2/ ERBB2/ EGFR* pathways, whereas hereditary cancer syndromes including gastric cancer include mutations in *APC, TP53, STK11, CDH1* and *CTNN1A*(96). As these mutations represent therapeutic targets with available treatments, ctDNA could prove useful for identifying patients most likely to respond to systemic targeted therapies or early detection of drug resistance mutations.

Diagnosis of gastric cancer

A summary of current data of blood-based biomarkers in gastric cancer is found in Table 2. cfDNA levels are significantly higher in patients with gastric carcinoma compared with healthy controls (97, 98). Kim et al (99) found that cfDNA levels had a sensitivity of 96.67% and specificity of 94.11% for diagnosis of gastric cancer compared with healthy controls when a cut-off of 90ng/mL was used, however cases were not early stage tumours and sample size was small.

Studies have found significant association between gastric cancer and promoter hypermethylation of the XIAP association factor 1 (*XAF1*), *APC*, *HMLH1* and Tissue Inhibitor of Metalloproteases 3 (*TIMP3*) genes detected in ctDNA (100, 101). *XAF1* plays a tumour suppressor role in carcinogenesis and is frequently down-regulated in gastric carcinoma specimens (100). *XAF1* promoter hypermethylation in ctDNA had

reasonable accuracy for diagnosis of gastric cancer from healthy controls (AUC of 0.9) and levels correlated with shorter overall survival in one study (100). Another study reported 33 of 60 cancer subjects (55%) and 3 of 22 healthy controls (14%) had detectable target gene methylation in serum(101). Promoter methylation was detected for *APC* in 17%, E cadherin in 13%, *HMLH1* in 41% and *TIMP3* in 17% of gastric cancer subjects(101). Furthermore, methylated *APC*, *HMLH1* and *TIMP3* concentrations were significantly associated with stage III and stage IV disease(101), while E cadherin and *APC* combined were associated with shorter overall survival(101). However, of concern in this study was the relatively high detection of methylated target genes in the healthy control group. This may reflect underlying predisposition to gastric cancer and therefore poor specificity of ctDNA for gastric cancer diagnosis(101). In addition, ctDNA findings were not confirmed in primary tumour specimens.

Another study highlighted increased Sex determining region Box 17 (*SOX17*) promoter methylation in gastric carcinoma, with a trend to shorter overall survival. However, this study was small and included no controls (102). *RASSF1A* promoter methylation in ctDNA was also identified in 34% of 47 patients with gastric adenocarcinoma compared with 3% of 30 patients with benign gastric disease and 30 healthy controls ($p<0.001$), suggesting potential utility as a diagnostic marker for gastric carcinoma (68). ctDNA *RASSF1A* methylation correlated strongly with *RASSF1A* methylation in tumour specimens(68).

Multimodular adaptor proteins, or *MINTS*, are members of the X11 family and have key roles in cell membrane function and cellular transport. Methylation of *MINT2*

promoter was detected in ctDNA of 39% of 92 patients with gastric carcinoma, 6% of 48 patients with chronic gastritis and no healthy controls(103). The findings in ctDNA were confirmed in matched primary tumour specimens. *MINT2* methylation was significantly associated with tumour progression, metastatic disease and shorter overall survival, making this a potentially valuable diagnostic and prognostic biomarker that warrants further study.

Post surgery prognosis and recurrence detection in gastric cancer

Levels of cfDNA fall rapidly after surgery for gastric carcinoma, and recurrent levels of detectable ctDNA have been associated with tumour recurrence(99). More specifically, others have demonstrated that detectable *XAF1* methylation in serum ctDNA post resection for gastric carcinoma is associated with tumour recurrence(100).

Gastrointestinal Stromal Tumours (GIST)

Several groups have evaluated ctDNA mutation detection as a diagnostic and prognostic marker for GIST tumours, however studies have been small and require validation. Maier et al(104) found *cKIT* and Platelet-Derived Growth Factor Receptor A (*PDGFRA*) mutations in ctDNA were common in GIST compared to healthy controls. Moreover, mutation concentration in ctDNA was higher in patients with active disease compared with those in clinical remission or who responded to therapy. Rawnaq et al (105) assessed loss of heterozygosity in twelve polymorphic marker regions in 91 patients with recurrent GIST. They found microsatellite instability in

ctDNA in 33% (30 of 92) patients with recurrent disease (105). However, confounding factors such as age and duration of follow up were not considered. Another very small study by Yoo et al(106) identified *cKIT* mutations in exon 17 which predicted response to tyrosine kinase inhibitor-based chemotherapy regimens in GIST patients.

In gastric carcinoma, current evidence suggests *XAF1, HMLH1, RASSF1A, APC* and *TIMP3* methylation are potential diagnostic markers, while *XAF1* is also useful for post surgical recurrence detection. Methylated *MINT2* appears to have both prognostic and diagnostic utility in gastric carcinoma, including detection of metastatic disease. Further studies validating these markers and defining their clinical role are needed.

Pancreatic cancer

Pancreatic cancer accounts for 2% of cancers globally with a stable incidence of 1-10 per 100,000 people(91, 107). It is the eighth most common cause of cancer-related death with 5 year survival rates of only 5% as it is frequently clinically silent until very advanced, when curative therapies are often no longer (108). Pancreatic cancer progresses from premalignant lesions to cancer in similar molecular fashion to CRC. Over 90% have *KRAS* mutations, of which 80% are in exon 12 (109-112). Other common rate-limiting mutations as lesions progress include *CDKN2A*, *TP53* and *SMAD family 4* (*SMAD4*) mutations (107). Currently there is no effective screening tool for premalignant lesions or pancreatic cancer(107). Obtaining pancreatic tissue for diagnosis and genetic biomarkers is difficult as it requires either endoscopic

ultrasound guided biopsy with an associated risk of tumour seeding, therefore there is a potential role for ctDNA to reduce the need for invasive pancreatic biopsy.

The discovery of identical *KRAS* mutations in both pancreatic tumour specimens and ctDNA by Sorenson et al (113) was a pioneer publication in ctDNA research. Their findings have been confirmed by various groups A recent paper by Kinugasa et al(112) in 75 patients with pancreatic cancer demonstrated *KRAS* mutations were detected in 74.7% of tumour specimens and 62.6% of ctDNA samples. Survival was reduced in those with *KRAS* mutations identified in ctDNA, but not in tissue samples(112). Another study of 47 patients with pancreatic cancer and 31 patients with chronic pancreatitis reported 47% detection of *KRAS* mutations in ctDNA in pancreatic cancer patients, compared with 13% in patients with chronic pancreatitis, with a sensitivity of 47% and specificity of 87% for cancer diagnosis(15). However, combining *KRAS* mutation detection in ctDNA with CA19-9 had a sensitivity of 98% and specificity of 77% and negative predictive value of 96%(15). Interestingly, none of the four patients with chronic pancreatitis went on to develop pancreatic cancer in 36 months of follow-up. This study highlights that ctDNA *KRAS* detection may be a useful adjunct to CA199 testing when CA199 results are equivocal(15).

Liggett et al(108) used a microarray methylation detection method for 56 fragments (MetDet56) and found methylation patterns in 8 gene promoter regions could reliably distinguish pancreatic cancer from healthy controls, with sensitivity of 82% and specificity of 78% (108). These genes included Breast Cancer Associated gene 1 (*BRCA1*), Cyclin D2 (*CCND2*), *HMLH1*, Cyclin Dependent Kinase Inhibitor 1C (*CDKN1C*), Progesterone Receptor (PGR)-distal, PGR-proximal, Spleen Tyrosine

Kinase (*SYK*) and Von Hippel-Lindau (*VHL*) (108). A second panel of 14 gene promoter regions could distinguish pancreatic cancer from chronic pancreatitis with sensitivity of 91% and specificity of 91%(108). These genes included *CCND2*, *DAPK1*, Oestrogen Receptor 1 (*ESR1*), *PromA*, *HMLH1*, *MGMT*, Mucous gel forming protein 2 (*MUC2*), Myogenic Differentiation 1 (*MYOD1*), *CDKN2B*, *CDNK1C*, Phosphoglycerate Kinase 1 (*PGK1*), *PGR proximal*, *RARbeta*, Retinoblastoma 1 (*RB1*) and *SYK*(108). *CDKN1C*, *CCND2*, *HMLH1*, *PGR-proximal* and *SYK* were identified in both panels and therefore may provide a simple broad marker panel worthy of validation for pancreatic cancer diagnosis (108). In this study, ctDNA gene mutations were not compared to those present in the primary tumour.

Yi et al(114) reported sensitivity of 81% and specificity of 95% for the combination of methylated Basonuclin 1 (*BNC1*) and ADAM Metallopeptidase with Thrombospondin 1 (*ADAMTS1*) in ctDNA for diagnosing early-stage pancreatic carcinoma, including Pancreatic in-situ Neoplasia (PIN) 3(114). Other small studies have reported abnormal methylation of the *CCDN2*, *VHL*, Thrombospondin 1 (*THBS1*), Suppressor of Cytokine Signalling 1 (*SOCS1*) and Plasminogen Activator and Urokinase (*PLAU*) genes in pancreatic cancer (115).

In summary, *KRAS* mutations in ctDNA appear useful for diagnosis of pancreatic cancer and can distinguish from chronic pancreatitis, which addresses an important clinical diagnostic need. However, detecting combinations of *KRAS* mutations with other biomarkers may improve early stage diagnostic accuracy of ctDNA and validation studies are needed.

Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the sixth most common malignancy worldwide and third most common cause of cancer-related death and has a high mortality despite treatment(91, 116). HCC usually develops within background liver disease, usually in the presence of cirrhosis. Due to the many potential predisposing aetiologies of liver disease, HCC is genetically heterogeneous and involves many molecular carcinogenic pathways. Commonly mutated genes include *CTNNB1*/ *APC*/ *AXIN1*, *TERT*, *CDKN2A*, *PIK3CA*, *TP53* and *ARID1* and ARID*2* (117). To date, this has been a key limitation in the translation of genetic markers in HCC to clinical care. Currently, HCC screening is performed using twice yearly ultrasound and diagnosis does not require tumour tissue biopsy(116). Therefore, ctDNA would provide an alternate means of obtaining genetic information about the tumour in the absence of liver biopsy.

There have been several studies evaluating cfDNA and ctDNA utility in hepatocellular carcinoma (HCC) management. A summary of available ctDNA data in HCC is found in Table 3.

Diagnosis and screening in HCC

Currently available tumour markers for HCC show only moderate sensitivity and specificity for HCC. Several studies show cfDNA levels are significantly higher in patients with HCC compared with chronic liver disease and healthy controls and

reasonable accuracy for distinguishing between HCC and chronic liver disease(118-123), with reported sensitivity of 56.4% to 69.2% and specificity of 93.3% (118, 121) In these studies, attempts to distinguish ctDNA from cfDNA were not made. El-Shazly et al (124) found longer cfDNA fragments were more common in HCC cases compared with healthy controls, with DNA integrity more strongly associated with HCC diagnosis than cfDNA concentration (124). An interesting study by Fu et al (26) found relative telomere length in serum cfDNA was significantly higher in 140 Hepatitis B (HBV)-related HCC cases without cirrhosis compared with 280 HBV infected non-cirrhotic controls and this remained significant on multivariate analysis (26). Large sample size and statistical adjustment for clinical confounders make this a strong study design and further assessment of telomere length in cfDNA in non-cirrhotic HCC should be prioritised.

Whilst gene mutations are very common in HCC, they are highly varied, with most gene alterations reported in less than 30% of tumours and few "hot spots" of frequent mutation. Tumour suppressor *TP53* 249Ser is one exception, a "hotspot" mutation very commonly associated with aflatoxin exposure, HBV infection and HCC. It occurs predominantly in south Asia and sub-Saharan Africa, where exposure to aflatoxin through groundnut consumption is high (122). However, this mutation is very uncommon in Caucasian HCC populations(122, 125). There are several high quality genetic epidemiology studies published by Kirk et al demonstrating a strong association between ctDNA *TP53* mutation Ser249 and hepatitis B (HBV)-related HCC in Gambians and this correlates closely with serum aflatoxin adduct levels (122, 126-128). The largest of these studies detected the mutation in 74 of 186 HCC cases, 15 of 98 patients with HBV-related liver cirrhosis and 12 of 348 HBV infected

controls, with an odds ratio of 20.3 for HCC (122). This study was particularly important for highlighting the potential utility of ctDNA for diagnosis of HCC in resource-poor settings. However, mutations that predispose to HCC and pre-date HCC development are unlikely to be highly specific for HCC diagnosis and may be better suited to HCC risk stratification for the purposes of screening. This study also described confounding adjacent gene mutations that affected the accuracy of *TP53* 249Ser mutation assays, highlighting an important potential cause of reduced diagnostic sensitivity of ctDNA point mutation analysis for HCC diagnosis (122).

There have been few other studies of ctDNA gene mutations in HCC. An Egyptian case-control study reported low levels of TP53 detection and an absence of *CTNNB1* mutations in ctDNA of HCC patients of mixed aetiology, and these findings were confirmed in primary tumour tissue specimens in a smaller subset of patients (119).

Promoter methylation of *RASSF1A* occurs in up to 70% of HCC patients compared with patients with chronic liver disease and healthy controls (6-8% *RASSf1A* hypermethylation) (30, 129-132). Importantly, several groups have found elevations in methylated *RASSF1A* as well as *p15* and *p16*, *APC*, Fragile Histidine Triad (*FHIT*) and *E cadherin* (30, 133) in ctDNA pre-dates HCC diagnosis (30, 132). Overall accuracy of *RASSF1A*, *p15* and *p16* methylation detection in ctDNA for HCC diagnosis was 89% (sensitivity of 84% and specificity of 94%), after adjusting for confounding variables (30).

Hypermethylation of G protein-coupled bile acid receptor GPBAR1 (*TGR5*) in ctDNA was significantly more common in HCC cases (77/160) compared with

chronic hepatitis B infection (12/88) and healthy controls (2/45) in one large study (134). When combined with alpha-fetoprotein, *TGFR5* significantly improved sensitivity for diagnosis of HCC (81.25% for AFP cut-off of 20ng/mL), however, this was at the expense of reduced specificity (38.64%). Interestingly, there was significantly greater methylation in those over 60 years of age, confirming the importance of adjusting for confounding factors such as age in gene methylation studies (134). By contrast, hypomethylation of LINE1 repeats in ctDNA is more common in HCC cases compared with cirrhosis and healthy controls, though diagnostic accuracy has not been assessed(24).

Prognosis in HCC

Several studies have reported significant associations between overall cfDNA levels and tumour differentiation and tumour size (121, 135), as well as a negative association with 3 year disease-free survival(135). In a large study of 87 HCV-related HCC with chronic HCV infection, Tokuhisa et al(120) found high cfDNA levels were an independent predictor of shorter overall survival and distant metastases after hepatectomy on multivariate analysis. However, in another cohort of 96 HCV-related HCC and 99 chronic HCV controls not undergoing surgery, the same group found no association between cfDNA levels and tumour size, stage or overall prognosis (123). Interestingly, they found that cfDNA levels correlated with inflammatory cytokine gene expression (123).

Hypomethylation of LINE1 repeats in ctDNA was an independent predictor of shorter overall survival and associated with HBV infection, large tumour size and advanced

CLIP score in one study(24). High DNA integrity has also been shown to be an independent marker of shorter overall survival, tumour size, TMN stage, vascular and lymphatic invasion and distant metastases (124).

Two markers of microsatellite instability *D8S258* and *D8S264*, in combination with ctDNA concentration, were independent predictors of overall and 3 year disease-free survival in HCC(136). Moreover, D8S258 was independently associated with tumour stage, tumour differentiation and vascular invasion (136).

To summarise, available evidence suggests concentration of overall cfDNA and *TP53* 249Ser mutation in ctDNA are important diagnostic markers of advanced stage HBV and aflatoxin-related HCC in African patients, but not in caucasians. cfDNA levels appear prognostic for both advanced disease stage and metastases, whilst *RASSF1A*, *p15* and *p16* methylation appear promising diagnostic markers for early-stage HCC. It is important to note that few large validation studies have been conducted of ctDNA in HCC patients. Future studies should also include sub-analyses of different aetiologies of HCC.

Considerations for the design of future studies

There are three main limitations common to many studies investigating ctDNA detection in gastrointestinal malignancy. The first is lack of a standardised approach to isolation, detection and quantification of cfDNA levels or gene mutations and epigenetic changes in ctDNA. cfDNA concentration is higher in serum than plasma due to release from cells during coagulation (137-141), however both methods are

widely used and results are not comparable. Furthermore, whilst DNA is relatively robust, studies have demonstrated degradation in sample quality over time and use of historical samples may reduce accuracy for cfDNA quantification and analysis and likewise reduce comparability across studies (139, 142, 143). Studies validating different techniques in parallel are needed.

Secondly, not all studies have determined gene mutations and gene methylation patterns in both ctDNA and matched primary tumour specimens, and few have compared ctDNA sequence to germline sequence in non-tumour cells within the same subject. For diagnosis, genetic variants ideally must only be present in ctDNA, not cfDNA from healthy cells. For prognosis, ctDNA must accurately reflect mutations currently present in the primary tumour. These comparisons are essential to establish credibility of ctDNA as a dynamic marker of tumour..

The third criticism of many published studies in this field is their small sample size, which limits discriminative power to determine the effects of clinical confounding variables. For example, studies have shown DNA methylation is independently influenced by age, smoking, alcohol consumption, gender, toxin exposure, diet (particularly folate intake), physical activity, BMI(144), even socio-economic status(28, 145-149). Background polymorphisms can also affect epigenetic methylation and tumour phenotype and should be accounted for (150). Aetiology of underlying disease may also be a factor influencing the carcinogenesis pathway through altered inflammatory mechanisms. Detailed analysis of these potentially important clinical variables cannot be performed rigorously without sufficient numbers of subjects included. Indeed, development of

strict statistical analysis benchmarks for studies in ctDNA akin to bioinformatical analysis standards developed for GWAS studies would greatly benefit this field.

Future directions for the practical use of ctDNA techniques in the clinic

Collectively, these data support a potential role for ctDNA at the bedside in gastrointestinal malignancy. ctDNA levels and mutation detection has proven utility for diagnosis in gastrointestinal malignancy, including more limited evidence for diagnosis of premalignant lesions for some cancers (such as colonic adenomas and Barrett's' oesophagitis) which could allow a screening blood test to triage the need for more invasive endoscopy to detect and remove premalignant lesions. Prognostic information offered by ctDNA mutation detection could facilitate early detection of metastatic disease and personalise treatment algorithms to maximise outcomes. ctDNA also provides rapid detection of tumour recurrence post curative therapy, with evidence for this in CRC and HCC. Identification of systemic treatment resistance-conferring genetic mutations in ctDNA also has proven utility in CRC. Moreover, development of epigenetic methylation inhibitor therapies means ctDNA gene methylation detection may also become an important biomarker for prognosis and treatment response. Genetic mutations and altered methylation patterns have proven useful for prognosis in CRC, pancreatic cancer and HCC, whereas to date methylation changes have been the main biomarker identified in ctDNA in gastro-oesophageal cancer.

From a practical perspective, there are still limitations to use of ctDNA in the clinic. Cost of whole exome sequencing is currently very high and unlikely to be a cost-

effective approach in the short term. Moreover, limitations in sensitivity for detecting the majority of mutations present in primary tumour tissue in ctDNA still exist, as outlined in many of the studies described, particularly for early-stage disease and well-differentiated tumours with lower metastatic potential such as HCC. However, the rapid pace of genomic technology and associated bioinformatics analysis platform developments, coupled with subsequent reductions in sequencing costs over time, mean it is likely that these barriers will be overcome and targeted whole exon sequencing will become more readily available, with lower detection limits and greater sensitivity and specificity for ctDNA detection. Whole exon sequencing will also expand the clinical utility of ctDNA in malignancies with greater heterogeneity of genetic mutational sequences such as HCC, where individual targeted point mutation assays are unlikely to be helpful. However, for malignancies such as CRC, targeted combinations of point mutation in panels are likely to prove very useful for both diagnosis and prognosis. Targeted mutation assays are also likely to have an important role for following individual mutations located in tumour specimens over time for early detection of recurrence post curative therapies. Whilst in CRC the mutational landscape is well recognised and the future looks bright for translation of ctDNA-based technologies into the clinic, for other malignancies such as HCC and oesophageal cancer more research is still required to identify the best genetic biomarkers for diagnosis and prognosis. Arguably, where ctDNA will prove most clinically useful will be malignancies such as HCC and pancreatic cancer, where tumour tissue is not routinely obtained or is not recommended due to the potential for tumour seeding of biopsy tracts. Finally, how we successfully combine biomarkers spanning genomic, metabolomics and proteomic domains and incorporate them into current gastrointestinal malignancy screening and management guidelines to

maximise early cancer diagnosis and prognosis remains a critically important future challenge for translational researchers. Greater translational data quality and cost-effectiveness analyses will support regulatory changes to allow incorporation of these exciting new technologies at the bedside.

Conclusion

There is a wealth of data supporting the utility of ctDNA for both diagnosis and prognosis in various gastrointestinal malignancies, with particularly strong evidence for diagnosis and prognosis in colorectal cancer, pancreatic cancer and HCC. Monitoring for tumour recurrence post surgery and detection of mutations indicating resistance to chemotherapy are two of the most promising clinical uses of ctDNA detection. Pilot data also support a role for ctDNA in metastatic disease detection in CRC, gastric cancer and HCC. However few studies have specifically evaluated the accuracy of ctDNA techniques for distinguishing metastatic from non-metastatic disease with sufficient power. Further large-scale validation studies of ctDNA biomarkers will help refine their role in the clinical management of gastrointestinal malignancies.

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Figure 1A. Development of cancer and relationship to circulating cell-free tumour DNA levels (ctDNA)

ctDNA levels remain low in the healthy state. ctDNA levels increase with the presence of adenomas and become increasingly elevated with progression of malignancy from carcinoma-in-situ to early-stage cancer. Curative treatment causes a rapid fall in ctDNA levels back to baseline levels. However, ctDNA levels are detectable in recurrent disease and increase rapidly as advanced carcinoma and systemic metastases develop.

Figure 1B. The process of circulating cell-free tumour DNA (ctDNA) procurement, genetic analysis and use in the clinic

A blood sample is taken from the patient in the clinic. Whole blood undergoes centrifugation and the plasma or serum supernatant is then isolated, then applied to cfDNA isolation columns and a multi-step process of elution and precipitation of DNA from plasma occurs.

ctDNA is then quantified using real-time polymerase chain reaction (PCR). Specific gene mutations of interest are detected using allelic imbalance methodology, targeted PCR mutation assay, microarray of whole genome Next Generation Sequencing (NGS).

Hypermethylated CpG dinucleotides can be detected by bisulphite conversion of DNA. Briefly, bisulphite conversion protocols convert unmethylated, but not methylated, cytosine residues to uracil. The modified DNA is then analysed using

either methylation-specific polymerase chain reaction (PCR), whole genome sequencing, or methylation marker microarray panels.

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