Cell-free DNA as a liquid biopsy for early detection of gastric cancer (Review)

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Abstract. Gastric cancer (GC) is one of the most common malignant tumors with poor prognosis worldwide, mainly due to the lack of suitable modalities for population-based screening and early detection of this disease. Therefore, novel and less invasive tests with improved clinical utility are urgently required. The remarkable advances in genomics and proteomics, along with emerging new technologies for highly sensitive detection of genetic alterations, have shown the potential to map the genomic makeup of a tumor in liquid biopsies, in order to assist with early detection and clinical management. The present review summarize the current status in the identification and development of cell-free DNA (cfDNA)-based biomarkers in GC, and also discusses their potential utility and the technical challenges in developing practical cfDNA-based liquid biopsy for early detection of GC.

Contents

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1. Introduction

Despite significant progress being made in the prevention and treatment of gastric cancer (GC) in the past decades, GC is still one of the most concerning malignancies as the majority of patients are diagnosed at an advanced stage of disease. Globally, GC ranked fifth for cancer incidence and third for cancer deaths, accounting for 1.3 million estimated incident cases and 819,000 estimated deaths in 2015 (1). Geographically, GC is more prevalent in developing countries, with the majority of cases and deaths occurring in Eastern Asia, including China, Japan and Korea, followed by Central Europe, Eastern Europe and South America (2). Etiologically, GC is a multifactorial disease attributed to both host and environmental factors. Proposed risk factors for GC include *Helicobacter pylori* (*H. pylori*) infection, smoking, alcohol, obesity, salt intake, atrophic gastritis (AG), intestinal metaplasia (IM) and family history of GC. The current therapy for GC includes surgery, chemotherapy, radiotherapy, VEGFR (ramucirumab) and targeted therapy against HER2 (trastuzmab) (3). The overall outcome of GC is largely associated with the stage of the disease at diagnosis. For early GC limited to the mucosa and submucosa, the 5-year survival rate is >90% (4,5). However, due to the lack of distinguishable symptoms at early stages and effective mass screening programs worldwide, the majority of GC cases are typically detected at stage IIIA-IV, with an estimated 5-year survival rate of <30% and a median survival of 12 months (4,5).

To date, four GC screening methodologies have been implemented in clinical settings: *H. pylori* serology, serum pepsinogen (PG) testing, indirect upper gastrointestinal series (UGIS) and endoscopy. Since the 1960s, population-based GC screening programs in several high-prevalence nations such as Japan and Korea have achieved significantly improved survival and cure rates using the above methods. These programs demonstrate the effectiveness of mass screening for GC (6-8). However, each of these screening tools has its limitations. For instance, *H. pylori* serology is unable to detect premalignant lesions, such as longstanding AG and IM. Therefore, *H. pylori* serology alone is not useful as a screening test for GC (9). The combination of upper endoscopy with pathological biopsy examination is the primary screening technique in the

majority of these programs and the gold standard for confirmation of diagnosis (10). In general, endoscopy is superior to UGIS in sensitivity and cost-effectiveness for detecting early GC (11-13). However, endoscopy is an invasive technique that has infrequent but serious complications, and its utility depends largely on the skill of the endoscopist (14). Therefore, the use of endoscopy in mass-screening programs in low-prevalence and low-income countries is impractical and likely to be associated with low participation rates. Currently, the only non-invasive test for GC detection in the clinical setting is the PG assay (15). Changes in serum PG levels reflect the function of the gastric mucosa. Decreased PGI levels and PGI/PGII ratio are indicators of atrophic changes in the gastric corpus. PG tests can detect gastric mucosal atrophy with a sensitivity of 66.7-84.6% and a specificity of 73.5-87.1% (16,17). However, PG assay's sensitivity for GC detection ranges from 36.8 to 62.3% (18,19), which is too low to be acceptable for population-based screening. Therefore, new assays with improved sensitivity, specificity and cost-effectiveness are needed.

Recent advances in genetic testing, such as next-generation sequencing (NGS) and digital PCR, and bioinformatics have accelerated the research on liquid biopsy greatly, which have high potential to change the clinical management of patients with cancer. Meanwhile, considerable efforts have been made to identify novel, early-stage GC biomarkers with potential utility in clinical liquid biopsy testing. Such biomarkers include cell-free DNA (cfDNA), cell-free RNA (cfRNA), proteins, autoantibodies, circulating tumor cells (CTCs), cancer-derived extracellular vesicles (EVs) and metabolites. A number of comprehensive reviews were recently published on CTCs, proteomics, cfRNA biomarkers, exosomes and EVs in GC (20,21). The current review provides an overview of the recent advances in the early detection of GC using liquid biopsy, with a focus on cfDNA, and their origin and mechanism of release into the bloodstream, as well as their potential utility in clinical practice (Fig. 1).

2. Quantification of circulating cfDNA

cfDNA is circulating extracellular DNA existing in the blood serum or plasma, synovial fluid, cerebrospinal fluid and other body fluids. Physiological events producing cfDNA include cellular apoptosis, secretion, micrometastasis and necroptosis (22). In patients with cancer, the plasma cfDNA levels are 2-3-fold higher than those in normal healthy groups (23), suggesting its potential as a complementary biomarker for cancer detection, as well as an indicator of prognosis and therapeutic response. As shown in Table I, a prospective study quantified the cfDNA levels in serum samples from patients with benign or malignant gastrointestinal tract disease using a radioimmunoassay, and it showed that the cfDNA levels in patients with malignant diseases were significantly higher than those of patients with benign diseases (24). Consistently, Sai *et al* (25) reported that increased plasma total cfDNA in patients with GC could be detected compared with the undetectable levels of healthy controls (Table I). The authors measured the short and long forms of β-actin in plasma samples from patients with GC and healthy controls by quantitative PCR (qPCR), and found that the cfDNA concentration in patients with GC was significantly higher. These studies suggest the potential use of serum cfDNA concentration assay to detect GC.

There are various methods for quantitative cfDNA detection, but their efficiency is limited by sample preparation and assay procedures. To address this issue, Park *et al* (26) developed an Alu-qPCR assay for measuring cfDNA concentrations, which demonstrated better sensitivity and reproducibility compares with other technologies, based on Ultraviolet-visible (UV-Vis) spectrophotometry or the PicoGreen fluorophore. Applying this assay, the cfDNA levels were compared between patients with GC and those of age-matched healthy controls, and found that the mean levels of plasma cfDNA were higher in the GC group than in the control group (Table I). To understand the dynamics of cfDNA levels pre- and post-surgery, Kim *et al* (27) measured the plasma cfDNA levels of patients with GC and healthy controls by qPCR. The samples of the patients with GC were collected before and 24 h after surgery. The results showed that average cfDNA levels were increased in patients with GC compared with those of healthy controls, and there was a positive dose-dependent association with more advanced cancer staging. Meanwhile, the levels of cfDNA in the 24-h-post-surgery group decreased significantly, thus supporting the utility of using cfDNA to monitor disease severity and therapeutic efficacy.

Recently, Qian *et al* (28) investigated cfDNA levels in serum samples from 124 patients with GC, 64 patients with benign gastric disease (BGD; namely gastric adenoma) and 92 healthy controls using the Alu-qPCR assay. The results showed that cfDNA levels were significantly higher in patients with GC compared with those with BGD or in healthy controls ($P<0.05$). Further statistical analysis showed that serum cfDNA levels in the GC group were significantly associated with advanced staging (III-IV) and tumor size (>5 cm), but not with sex, age or tumor location. cfDNA was also more sensitive in the detection of stage-I GC than conventional tumor biomarkers, including carcinoembryonic antigen (CEA), carbohydrate antigen (CA)-19-9, CA50 and CA72-4, suggesting that cfDNA assays may be able to replace current protein tumor biomarkers for cancer detection.

Since the measurement of cfDNA levels does not require any prior knowledge of genetic alterations in tumor tissue, this test could be highly useful in non-invasive assays for early GC detection. However, the application of cfDNA quantification alone for early cancer diagnosis is limited by several obstacles: i) Circulating cfDNA is unstable and its kinetics has not yet been well defined, which may affect assay robustness and standardization; ii) cfDNA levels cannot differentiate cancer type or tissue of origin; iii) cfDNA testing is relatively nonspecific, as numerous patients with non-cancer conditions, such as inflammatory disease, infections and cardiovascular disease, and even healthy individuals after exercise, show elevated cfDNA levels (29,30). Therefore, it is expected that the combined detection of cfDNA levels with other markers may achieve improved clinical performance.

3. Detection of genetic alteration of circulating tumor DNA (ctDNA)

Genetic alterations such as mutations, rearrangement and amplification of driver genes result in tumorigenesis. Recent

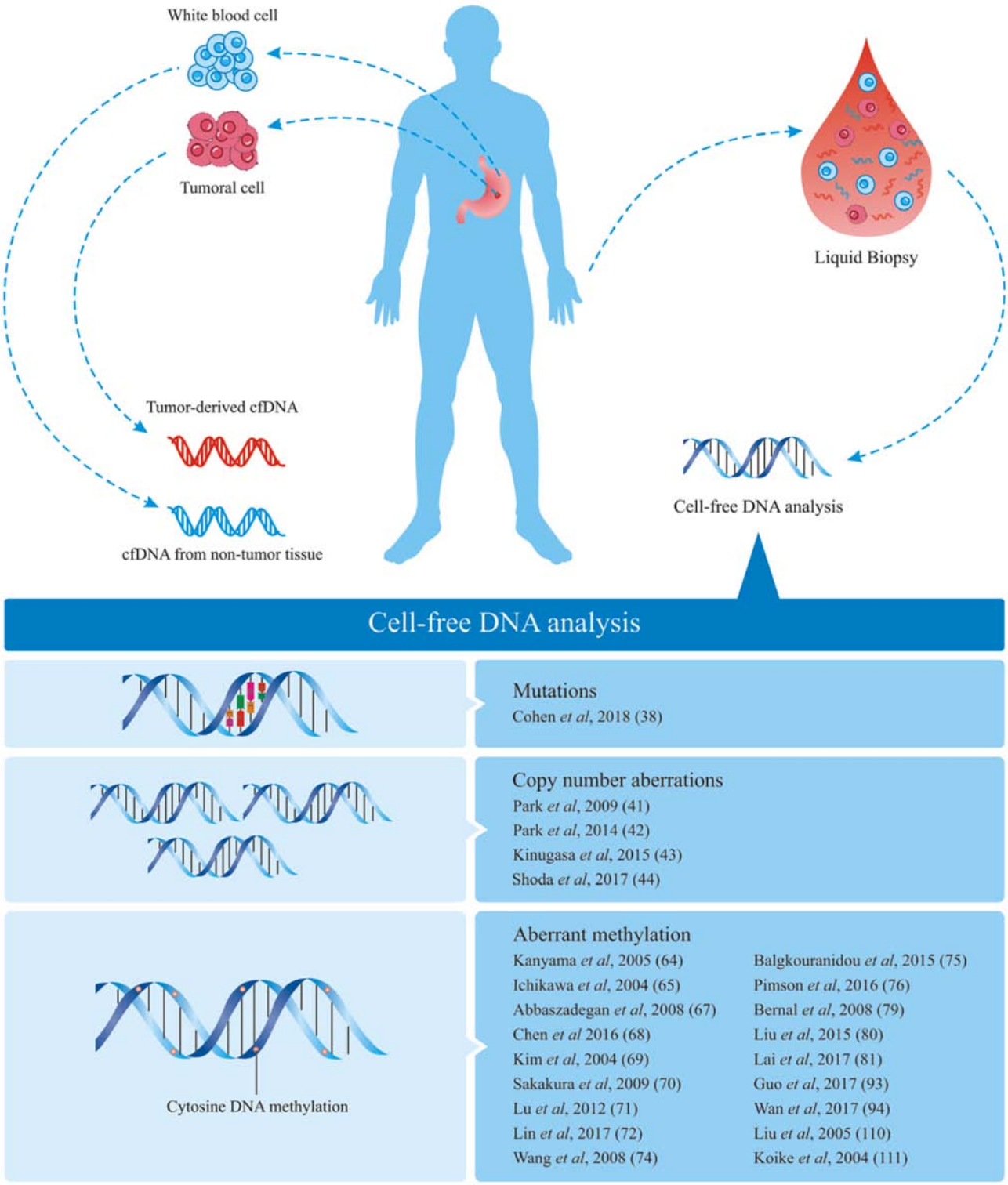

Figure 1. Overview of cfDNA in the early detection of GC through liquid biopsy. Circulating cfDNA provides a variety of clinically informative components. GC-associated changes can be detected through the analysis of cfDNA, including i) mutations; ii) copy number variants of genes; and iii) aberrations in DNA methylation. cfDNA, cell-free DNA; GC, gastric cancer.

advancements in NGS techniques have identified various nucleotide mutations associated with GC (Table II). The top mutated genes showing higher mutation frequency were TP53, TTN, MUC16, CDH1, KMT2C and MLH1 (31-33). In 1977, Leon *et al* (34) reported that numerous patients with cancer had elevated circulating cfDNA, and found that this DNA was tumor derived. Since, scientists focused their attention on the content of ctDNA. Some of the aberrant DNA shed into the blood by cancer cells, such as *EGFR* and *KRAS* mutations, can be potential biomarkers. Previous studies have demonstrated the detectability of mutant DNA released from tumor tissues. In fact, ctDNA analysis has emerged as an additional diagnostic tool to guide clinical management of certain cancer types including lung cancer and colon cancer (35,36). However,

Table I. cfDNA as biomarker for detection of gastric cancer.

				Diagnostic value		
cfDNA	Biomarker candidate	Sample type	Method	Sensitivity	Specificity	(Refs.)
cfDNA	cfDNA levels	Serum	Radioimmuno assay	Mean concentration of 412 and 118 ng/ml respectively (P<0.01)		(24)
		Plasma	qPCR	By 102 bp β-actin assay, GC=5.71 ng/ml, HC=3.20 ng/ml (P=0.03) By 253 bp β-actin assay, GC=0.470 ng/ml, HC=0.212 ng/ml (P<0.0001)		(25)
		Plasma	Alu81-qPCR	75% (41/54)	63% (37/59)	(26)
		Plasma	Measurement of cfDNA concentration	96.67% (29/30)	94.11% (32/34)	(27)

cfDNA, cell-free DNA; qPCR, quantitative PCR; bp, base pair.

the use of ctDNA for early cancer detection is complicated by the generally low abundance of ctDNA in early-stage cancer and the technical challenges in its detection.

Applying the droplet digital PCR (ddPCR) assay, which is currently the most sensitive method, Bettegowda *et al* (37) evaluated ctDNA in 640 plasma samples from patients with various cancer types and stages. ctDNA was detected in >75% of patients with several advanced cancer types, including melanoma, and pancreatic, breast, hepatocellular, ovarian, colorectal, bladder, gastroesophageal, and head and neck cancer. In patients with localized tumors, ctDNA was detectable only in a subset of patients with gastroesophageal cancer (57%), colorectal cancer (CRC) (73%), pancreatic cancer (48%) and breast adenocarcinoma (50%). When *KRAS* mutations were tested for in ctDNA in an additional panel of 206 patients with metastatic CRC, the sensitivity and specificity of detection were 87.2 and 99.2%, respectively. These results indicate that the detectability of ctDNA is affected by cancer type and stage.

Recently, Cohen *et al* (38) developed a blood-based test (CancerSEEK) and investigated its utility in the early detection of eight common cancer types, such as ovarian, liver, stomach, pancreatic, esophageal, colorectal, lung and breast cancer. The above blood test couples targeted parallel sequencing of ctDNA with eight known protein biomarkers using a panel of 61 amplicons in 16 genes, including *TP53*, *KRAS*, *PIK3CA*, *CTNNB1* and *APC*. The authors applied this assay to 1,005 patients with clinically detected stage I-III cancer, and the method yielded 70% of median positivity for all eight cancer types tested and >99% specificity for healthy controls. Using this assay, the authors were also able to track the cancer origin to two possible sites in ~80% of patients. As shown in Table III, GC samples were included in this study, and mutations of a number of driver genes were detected. This study suggests the divergent utility of a single test in detecting multiple early-stage cancer types.

In addition to mutations, the copy number variants of numerous genes such as *c-MYC* and *HER2* were also identified in GC by genome-wide profiling (39,40). Applying a qPCR assay, Park *et al* (41) measured the *MYC*/*GAPDH* ratio in plasma samples from patients with GC and cancer-free individuals. This showed that the mean ratio of *MYC*/*GAPDH* in plasma was significantly increased in GC compared with that in healthy controls (P<0.001). Another study compared the plasma levels of *HER2* and *MYC* genes in patients with GC, gastric adenoma, gastritis or no disease with those in matched tissue samples by reverse transcription-qPCR (42). The results indicated that the *HER2*/*HBB* and *MYC*/*HBB* ratios in tissue and plasma from patients with GC were significantly increased compared with those in gastritis tissue and cancer-free individuals. Similarly, Kinugasa *et al* (43) detected increased *HER2* in ctDNA from serum samples in patients with GC by ddPCR. Recently, Shoda *et al* (44) investigated the *HER2* gene levels in plasma samples of patients with GC and healthy controls using ddPCR. The results showed that the preoperative plasma *HER2* ratio (normalized with an internal control) correlated with *HER2*-positivity status in the tumor (P<0.001) (Table III). Of note, although the increased levels of circulating *HER2* and *c-MYC* genes were detectable in GC, their potential utility in early diagnosis would be limited by their low positivity rates in patients with GC.

4. Measurement of cfDNA methylation

DNA modifications such as 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) could serve as ideal biomarkers for cancer diagnosis. The 5-mC remodeling of DNA has been reported to be involved in cancer initiation, progression and therapeutic response (45). Except for 5-mC, a previous study by Li *et al* (46) showed that 5-hmC from circulating cfDNA was highly predictive of colorectal and gastric cancer, and was superior to conventional biomarkers

Table II. Type and frequency of mutations implicated in gastric cancer.

No. of patients	Method	Top mutated genes	Mutation frequency (%)	(Refs.)
74	WES	TP53	48	(31)
		TTN	37	
		MUC16	15	
		ABCA13	12	
		SYNE1	12	
		DCHS2	11	
		HMCN1	11	
		OBSCN	11	
		ROBO1	11	
63	WES	TP53	48	(32)
		TTN	37	
		MUC16	14	
		ABCA13	13	
		DCHS2	13	
		DNAH11	11	
		HMCN1	11	
		LAMA1	11	
		PCLO	11	
		ROBO1	11	
		SYNE1	11	
43	WES	TP53	41	(33)
		CDH1	26	
		KMT2C	24	
		MLH1	18	
		SMAD4	15	
		GNAS	15	
		CDKN2A	12	
		RPL5	12	
		TAF1	12	
		SETD2	12	
		PTEN	12	

WES, whole-exome sequencing; TP53, tumor protein p53; TTN, Titin; mucin 16, cell surface associated; ABCA13, ATP binding cassette subfamily A member 13; DCHS2, dachsous cadherin-related 2; SYNE1, spectrin repeat containing nuclear envelope protein 1; HMCN1, hemicentin 1; OBSCN, obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF; ROBO1, roundabout guidance receptor 1; LAMA1, laminin subunit alpha 1; PCLO, piccolo presynaptic cytomatrix protein; CDH1, cadherin 1; KMT2C, lysine methyltransferase 2C; MLH1, mutL homolog 1; SMAD4, SMAD family member 4; GNAS, GNAS complex locus; CDKN2A, cyclin dependent kinase inhibitor 2A; RPL5, ribosomal protein L5; TAF1, TATA-box binding protein associated factor 1; SETD2, SET domain containing 2, histone lysine methyltransferase; PTEN, phosphatase and tensin homolog.

and comparable to 5-hmC biomarkers from tissue biopsies. Hypermethylation of promoter CpG islands in tumor suppressor genes plays a crucial role in carcinogenesis (47-49). Eyvazi *et al* (50) verified the promoter methylation of EphA5, HS3ST2 and CDH11 genes in patients with GC using paraffin-embedded tissue sections. Recently, cfDNA in blood plasma has become a promising cancer biomarker for early diagnosis (51). The abnormal methylation of a large number of genes has been demonstrated to have utility for non-invasive detection of cancer in plasma or serum samples (52-54). Among them, *Septin 9* gene methylation, detectable as hypermethylated *Septin 9* DNA fragments in blood plasma, is a front-runner for the clinical screening of CRC (55). *Septin 9*, a member of the Septin family, was originally identified in myeloid neoplasia (56). It functions as a tumor suppressor gene in multiple cancer types (57). Consequently, several assay kits have been developed to detect methylated *Septin 9*. Epi proColon, the first commercial methylated *Septin 9* assay, has been approved by the USA Food and Drug Administration (FDA) for average-risk patients over the age of 50 years, and has also been approved in Europe and China (58). Meta-analysis of existing clinical data showed that the assay's sensitivity and specificity were 71 and 92%, respectively, demonstrating its reliability for CRC detection (59,60). In 2016, a blood test that

Table III. ctDNA as biomarker for detection of gastric cancer.

Type	Biomarker candidate	Sample type	Method	Diagnostic value, % (n)		(Refs.)
				Sensitivity	Specificity	
ctDNA	Gene mutations (TP53, KRAS, PIK3CA, CTNNB1, APC, PPP2R1A	Plasma	NGS	72 (49/68)	99 (805/812)	(38)
	MYC gene copy number	Plasma	qPCR	75.4 (43/57)	76.9 (60/79)	(41)
	(MYC/GAPDH ratio and MYC/HBB ratios)	Plasma	qPCR	72.8 (59/81) for MYC>2.725	77.7 (80/103)	(42)
	HER2 and MYC gene copy number (HER2/HBB and MYC/HBB ratios)	Plasma	qPCR	69.1 (56/81) for both HER2>2.0 and MYC>2.725	92.2 (95/103)	
	HER2 gene copy number	Plasma	qPCR	87.7 (71/81) for HER2>2.0	64.1 (66/103)	
		Tissue and serum	ddPCR	29.2 (7/24)		(43)
		Plasma	qPCR	For discovery: 53.9 (7/13) For validation: 66.7 (2/3)	For discovery: 96.7 (29/30) For validation: 100 (22/22)	(44)

TP53, tumor protein p53; KRAS, kirsten rat sarcoma viral oncogene; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; CTNNB1, catenin beta-1gene; APC, adenomatous polyposis coli gene; PPP2R1A, protein phosphatase 2 regulatory subunit α; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HBB, hemoglobin beta; HER2, human epidermal growth factor receptor 2; NGS, Next generation sequencing; ddPCR, digital droplet PCR.

detects circulating methylated *Septin 9* DNA was approved by the FDA to provide an alternative screening modality (61), thus paving the way for the development of a new generation of liquid biopsy tests for early cancer detection.

Besides *Septin 9*, there is increasing evidence demonstrating that many other hypermethylated genes, including *Reprimo*, *Rassf1A*, *CDH1*, *CDKN2A*, *MLH1*, *RUNX3*, *APC* and *p16*, can be differentially detected in plasma or serum samples of patients with GC (Table IV). Among them, p16 gene hypermethylation in GC has been extensively studied. p16 is a cell-cycle regulator that arrests cells in G_1 phase by inhibiting cyclin D-dependent protein kinase (CDK)4 and CDK6 (62). It functions as a tumor suppressor and can be inactivated by hypermethylation in the gene-promoter region (63). Kanyama *et al* (64) investigated the promoter methylation status of the p16 gene in paired tumor and serum samples from patients with GC. p16 hypermethylation was found in primary GC samples but in none of the corresponding gastric mucosae. Consistently, Ichikawa *et al* (65) reported that hypermethylation of the promoter region in the p16 and E-cadherin genes was detected in serum DNA samples from patients with GC, but not from healthy volunteers. Recently, Guo *et al* (66) detected promoter methylation of the p16 gene in peripheral blood samples from patients with GC and healthy controls using methylation-specific PCR (MSP) analysis, and found that the ratio of methylated p16 was significantly higher in GC samples compared with the control group (P<0.01). However, the reported ratios of circulating p16 methylation in GC vary remarkably across different studies (67). Thus, further validations are needed to determine whether the variation in positive methylation ratios is due to assay aberrations or primary sample differences.

Besides *p16*, Runt-related transcription factor 3 (*RUNX3*) has also been reported as a candidate tumor suppressor in GC (68). Hypermethylation of *RUNX3* CpG islands was observed in GC cell lines and primary gastric carcinoma, with significantly higher ratios than in non-malignant gastric disorders (69). Consistently, high levels of methylated *RUNX3* sequences were also detected in the peripheral circulation of patients with GC. The *RUNX3* methylation index was concordant with cancer stage, histology, and lymphatic and vascular invasion, and was more sensitive than CEA as a biomarker (70). Applying a real-time MSP assay, Lu *et al* investigated *RUNX3* methylation levels in serum samples from normal individuals without any gastric lesions or *H. pylori* infection, patients with benign lesions and patients with GC (71). Notably, the serum detection of *RUNX3* methylation was negative in almost all benign and normal samples, except for two patients who had severe dysplasia. However, circulating methylated *RUNX3* was detected in almost all patients with GC who had detectable *RUNX3* methylation in tissue samples with significant accordance (k=0.887; P<0.001) (71). Recently, Lin *et al* (72) measured the methylation status of three selected genes in blood samples of patients with GC and precancerous lesions using an MSP assay, and found that the methylation rate of *ZIC1*, *HOXD10* and *RUNX3* increased significantly during the progression of gastric carcinogenesis. For predicting GC and intraepithelial neoplasia, the combined detection of

Table IV. Methylated DNA as biomarker for detection of gastric cancer.

cfDNA	Biomarker candidate	Sample type	Method	Diagnostic value, % (n)		(Refs.)
				Sensitivity	Specificity	
MethyDNA	P16	Serum	MSP	26 (6/23)	100 (16/16)	(64)
		Serum	MSP	18 (20/109)	100 (10/10)	(65)
		Blood	MSP	72.6 (77/106)	94.4 (17/18)	(93)
		Serum	MSP	51.9 (28/54)	100 (30/30)	(98)
		Plasma	MSP/HPLC	14.3 (12/84)	100 (15/15)	(110)
		Serum	MSP	26.9 (14/52)	100 (29/29)	(67)
		Serum	MSP	22 (9/41)	100 (10/10)	(111)
	P16/E-Cadherin	Serum	MSP	37 (40/109)	100 (10/10)	(65)
	E-Cadherin	Serum	MSP	24 (26/109)	100 (10/10)	
		Serum	MSP	22 (9/41)	100 (10/10)	(111)
	p16/E-cadherin/RARbeta	Serum	MSP	44 (18/41)	100 (10/10)	
	RUNX3	Tissues and cell lines	MSP	64 (48/75)		(69)
		Serum	rt-MSP	70.8 (143/202)	99.8 (848/850)	(71)
		Serum	qMSP	95.5	62.5	(70)
		Plasma	MSP	42.7 (56/131)	100 (34/34)	(72)
	Plasma ZIC1, HOXD10 and RUNX3	Plasma	MSP	91.6	50.0	
	Zic1	Plasma	MSP	69.5 (91/131)	100 (34/34)	
	RASSF1A	Serum	MSP	34 (16/47)	100 (30/30)	(74)
		Serum	MSP	68.5 (50/73)	100 (20/20)	(75)
		Plasma	MSP	83.2 (84/101)	94.55 (191/202)	(76)
	PCDH10	Plasma	MSP	94.1 (95/101)	97.03 (142/202)	
	Reprimo	Plasma	MSP	95.3 (41/43)	90.3 (28/31)	(79)
		Plasma	MSP	86.3 (44/51)	97.6 (48/49)	(81)
		Plasma	MSP	62 (31/50)	100 (30/30)	(80)
	hMLH1	Plasma	MSP	48 (24/50)	96.7 (29/30)	

P16, Cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1; E-Cadherin, epithelial cadherin; RARbeta, retinoic acid receptor beta; RUNX3, Runt-related transcription factor 3; ZIC1, zinc finger of the cerebellum 1; RASSF1A, Ras association domain-containing protein 1; PCDH10, protocadherin-10 gene; hMLH1, MutL homolog 1; MSP, methylation-specific polymerase chain reaction; HPLC, high performance liquid chromatography.

these three genes showed a synergistic effect compared with that of testing a single biomarker.

Ras-association domain family 1A (*RASSF1A*) is a putative tumor suppressor gene, which is often silenced by hypermethylation of its promoter region in a number of human tumors such as GC (73). Previous studies conducted by Wang *et al* (74) and Balgkouranidou *et al* (75) reported that *RASSF1A* methylation was detected in the serum samples of patients with GC, but not in the healthy control samples (P<0.001). Recently, Pimson *et al* (76) investigated the promoter methylation statuses of *RASSF1A* and *PCDH10* (another tumor suppressor gene in the protocadherin family) in GC. The authors found that hypermethylation of *PCDH10* and *RASS1A* was detectable in plasma samples from patients with GC, and aberrant *PCDH10* and *RASSF1A* promoter methylation in plasma DNA was associated with worse clinical outcome.

Reprimo (*RPRM*) is another tumor suppressor gene involved in the development of numerous malignant tumors, including GC (77,78). Bernal *et al* (79) evaluated the DNA methylation patterns of 24 genes by MSP in primary tissues from patients with GC. In >50% of cases, hypermethylation was detected in ≥1 of 11 genes, including *APC*, *SHP1*, *E-cadherin*, *ER*, *Reprimo*, *SEMA3B*, *3OST2*, *p14*, *p15*, *DAPK* and *p16*. The most frequently hypermethylated genes were further evaluated in primary tissues and plasma samples from prospectively collected GC cases, as well as in plasma samples from asymptomatic age- and gender-matched controls (which formed the validation group). The results confirmed a high methylation frequency of seven genes (namely, *APC*, *SHP1*, *E-cadherin*, *ER*, *Reprimo*, *SEMA3B* and *3OST2*) in GC. Notably, the prevalence of *Reprimo* methylation was significantly higher in GC tumor and plasma samples than in asymptomatic control samples (P<0.001). In another study (80), the DNA methylation

Table V. Methyated genes with limited sample sizes and method variants.

Type	Biomarker candidate	Sample type	Method	Diagnostic value, % (n)		(Refs.)
				Sensitivity	Specificity	
MethyDNA	Zic1	Plasma	MSP	60.6 (63/104)	100 (20/20)	(83)
	RASSF10	Serum	BSP	81.71 (67/82)	89.5 (85/95)	(84)
	RNF180	Plasma	q-MSP	56 (18/32)	100 (64/64)	(85)
	SFRP1	Serum	MSP	30.95 (13/42)	93.2 (41/44)	(86)
	IRX1	Plasma	MSP	73.3 (11/15)	90 (9/10)	(112)
	CYP26B1+KCNA4	Serum	MSP	91.3	92.1	(113)
	SLC19A3	Plasma	MSRED-qPCR	85 (17/20)	85 (17/20)	(114)
	FAM5C/MYLK	Serum	MSP	77.6 (45/58)	90 (27/30)	(115)
	ATP4B	Plasma	MSP	64 (16/25)	100 (9/9)	(116)
	XAF1	Serum	rt-MSP	83.9 (141/168)	94.3 (83/88)	(117)
	SOX17	Serum	MSP	58.9 (43/73)	100 (20/20)	(118)
	SPG20	Blood	MSP	48.8 (20/41)	100 (21/21)	(119)
	FLNC/THBS1/ UCHL1/DLEC1	Serum	q-MSP	FLNC: 67 (55/82), THBS1: 63.4 (52/82), UCHL1: 56.1 (46/82), DLEC1: 80.5 (66/82),	FLNC: 93.0 (80/86), THBS1: 94.2 (81/86), UCHL1: 89.5 (77/86), DLEC1: 93.0 (80/86)	(120)
	OSR2/VAV3/PPFIA3	Serum	MSP	OSR2: 62.5 (30/48), VAV3: 45.8 (22/48), PPFIA3: 56.3 (27/48), Combined: 83.3 (40/48)	OSR2: 92 (23/25), VAV3: 100 (25/25), PPFIA3: 96 (24/25), Combined: 88 (22/25)	(121)
	TFPI2	Tissue	q-MSP	68	83	(122)

RASSF10, Ras-association domain family 10; RNF180, ring finger protein 180; SFRP1, secreted frizzled-related protein 1; IRX1, iroquois homeobox protein 1; CYP26B1, cytochrome P450 26B1; KCNA4, potassium voltage-gated channel subfamily A member 4; SLC19A3, solute carrier family 19 member 3; FAM5C, family with sequence similarity 5, member C; MYLK, myosin light chain kinase; ATP4B, ATPase H+/K+ transporting beta subunit; XAF1, XIAP-associated factor 1; SOX17, a member of the Sox family of transcription factors; SPG20, spastic paraplegia-20; FLNC, filamin-C; THBS1, thrombospondin 1; UCHL1, ubiquitin carboxy-terminal hydrolase L1; DLEC1, deleted in lung and esophageal cancer1; OSR2, protein odd-skipped-related 2; VAV3, guanine nucleotide exchange factor; PPFIA3, PTPRF-interacting protein alpha-3; TFPI2, tissue factor pathway inhibitor 2.

statuses of the *Reprimo* and *hMLH1* genes in tissue and plasma samples of patients with GC, dysplasia, chronic AG and normal controls were investigated by MSP. The results showed that plasma hypermethylation of *Reprimo* was detectable in GC, AG and dysplasia, but not in normal controls. Meanwhile, methylated *hMLH* was also detected in a higher percentage of GC and dysplasia samples compared with that of normal controls. Consistently, Lai *et al* (81) examined *Reprimo* gene methylation in GC tissues and plasma samples by bisulfite sequencing, and found that the *Reprimo* gene-promoter region was hypermethylated in GC tissues, plasma and cell samples. This was correlated with a decrease in *Reprimo* gene expression, thus supporting the potential utility of *Reprimo* methylation as a diagnostic biomarker for GC.

Of note, there is obvious heterogeneity in gene methylation frequencies between different studies, which might be attributable to differences in samples size, technique variations and geographical differences. To obtain a better understanding of this variance, Hu *et al* (82) recently performed a meta-analysis to evaluate the pooled sensitivity and specificity of the results of 32 studies, which included 4,172 patients with GC and 2,098 controls. Collectively, the overall sensitivity of DNA methylation-based blood test for detecting GC was 57% (95% CI, 50-63%), while the specificity was 97% (95% CI, 95-98%). The sensitivity and specificity of tests covering multiple methylated genes were 76% (95% CI, 64-84%) and 85% (95% CI, 65-95%), respectively. These results indicate that blood-based DNA methylation tests have high specificity but modest sensitivity for detecting GC. Evaluating multiple methylated genes or using plasma samples seems to improve diagnostic sensitivity.

Besides the methylation markers described above, increased methylation of numerous other genes in cfDNA has also been reported in GC (83-86) (Table V). However, due to the limited sample sizes and method variants, further studies are needed to demonstrate their analytical and clinical validity.

5. Current challenges

Although the genetic landscape for GC has been well researched and a large number of candidate biomarkers have been detected in blood samples in the past decades, none of

these have yet progressed into clinical assays for GC. A number of challenges account for this delay.

First and foremost is the lack of biomarker validation studies demonstrating acceptable sensitivities and specificities for clinical use. In fact, most of the proposed biomarkers were identified or validated in retrospective studies with limited sample sizes. Quantification of plasma cfDNA alone, for example, is insufficient as a clinical biomarker due to its lack of specificity. On the other hand, detection of mutations or rearrangements of ctDNA seems more intriguing due to their biological relevance for tumor initiation and development. However, even the most commonly mutated genes, such as *TP53* and *KRAS*, are typically aberrant in <50% of the cases in any particular cancer type. On this context, it is assumed that multigene panel analysis of ctDNA could lead to increased test sensitivity. However, the mutations of these genes are often located in different exons, and their abundance in circulation is generally elevated only in late-stage cancers. This impairs the detection of DNA-based sequence variations, as well as their utility in early cancer detection. For instance, in the recent CancerSEEK study, detecting mutations of 16 genes only achieved 40% sensitivity for early-stage tumors (38). By contrast, assaying DNA methylation (which is the epigenetic modification of CpG dinucleotides) is more robust and consistent than testing genetic alterations. There is accumulating evidence that cfDNA gene hypermethylation is more readily detectable than genetic mutations in patients with GC or pre-cancerous diseases such as intestinal metaplasia and dysplasia. For example, the hypermethylation of a number of genes, including *RunX3*, *RPRM* and *RASSF1A*, was significantly elevated in plasma samples of patients with early-stage GC. Aside from Epi proColon, the first FDA-approved gene methylation-based GC assay, Epi proLung, which tests for plasma *SHOX2* and *PTGER4* gene methylation, recently received a CE-IVD mark in Europe for lung cancer detection as well (87). These developments demonstrate the current utility and future potential of cfDNA methylation assays for cancer detection.

In addition to testing sensitivity and specificity, tracking tumor location is another challenge for cfDNA-based tests. For instance, although hypermethylation of Septin 9 is preferentially detected in patients with CRC, it is also present in some patients with primary lung and stomach cancer (88,89). In fact, even CancerSEEK had to rely on conventional protein tumor biomarkers to track the tissue of origin. However, in patients with early-stage cancer, conventional protein tumor biomarkers can be difficult to detect. Recent studies using computer based-analyses of genome-wide methylation signatures have demonstrated certain potential for identifying the presence, type and location of tumors (90-93). For instance, methylated haplotype load, an analysis of tissue-specific methylation haplotype blocks using whole-genome bisulfite sequencing (WGBS) data, can help to identify cancer-associated biomarkers in both tissue and plasma samples (94). Preliminary data from this analysis demonstrated its potential in determining tissue of origin as well as in predicting cancer development and progression from plasma samples of patients with lung cancer and CRC. Similarly, Kang *et al* (92) developed CancerLocator, a probabilistic approach to WGBS analysis that is used to predict disease burden and the tissue of origin of ctDNA based on the genome-wide methylation profile of its cfDNA. However, further prospective studies with larger sample sizes are required to validate its utility in clinical settings.

Besides biomarker validation, another challenge for developing cfDNA-based tests in GC is the lack of a standard consensus for experimental procedures, including sampling, storage conditions, cfDNA isolation and enrichment, data analysis and results interpretation (95). Currently, the technologies used for cfDNA detection include qPCR, next-generation sequencing (NGS), dPCR and ultra-sensitive amplification refractory mutation system (ARMS) PCR. Each of these methods has its advantages and drawbacks (96).

Recently, liquid biopsy testing by qPCR (Cobas and Therascreen) was FDA-approved for *EGFR* exon 19 deletions, *EGFR* L858R and *EGFR* T790M in patients with NSCLC. However, these kits have only been validated for allele frequencies of >1%, which is not sufficient when attempting to detect tumors at early stages (97-99).

By contrast, dPCR has the highest testing sensitivity and suitability in liquid biopsies (100,101). Compared with the characteristics of NGS, dPCR is more cost-effective and has faster turnaround times. However, it has a lower throughput, and can only detect a limited number of known mutations at a time (102). NGS, on the other hand, is theoretically able to detect numerous gene mutations, amplifications and fusions in parallel with higher throughput, and has already been approved for tumor-tissue profiling in the clinic (103,104). Despite this, conventional NGS has relatively low-detection sensitivity and a high-error rate, thus limiting its usefulness for analyzing cfDNA, which occurs in low abundance in plasma samples. New targeted- and genome-wide-NGS approaches for liquid biopsy testing have been developed with improved sensitivity and error-suppression rates (105,106). However, the complicated process, quality control and cost-effectiveness of NGS still need to be improved for clinical applications.

On the other hand, an ultra-sensitive ARMS PCR assay (Udx-PCR, Super-ARMS) was developed, which can detect mutant ctDNA at an allele frequency of 0.1-0.02% in the background of 10-50 ng wild-type DNA (46,107). This is comparable to the detection sensitivity of dPCR, but with significantly improved robustness, cost-effectiveness and procedural ease.

6. Conclusion

Recent milestones in cfDNA analysis as a liquid biopsy for early cancer detection pave the way for its adoption in clinical practice. Among them, Epi proColon is the first population-based CRC screening product that detects gene methylation in plasma samples. Another study led by Chan *et al* (108) indicated that detection of Epstein-Barr virus DNA in plasma is effective for the screening of early asymptomatic nasopharyngeal carcinoma. Additionally, CancerSEEK has demonstrated how a single assay can screen multiple cancer types by combining ctDNA and protein biomarkers (38). Liquid biopsy draw the attention of independent libraries and commercial companies. In a recent research by GRAIL, which focused on applying cfDNA liquid biopsy to cancer early-stage diagnosis, the sensitivity

of stage I-III was 67.3% (CI, 60.7-73.3%) in a pre-specified set of 12 cancer types (such as anus, bladder, colon/rectum, esophagus, head and neck, liver/bile-duct, lung, lymphoma, ovary, pancreas, plasma cell neoplasm and stomach), and 43.9% (CI, 39.4-48.5%) in all cancer types (54). In other studies on breast cancer, 358 cancer and 452 normal cases were included. The results indicated that for three types of breast cancer (triple negative, HER2-positive/hormone receptor-positive and HER2-negative), the sensitivity was 58, 40 and 15%, respectively. This sensitivity shows that cfDNA liquid biopsy is still far from ready-to-use for clinic diagnosis (109). Nonetheless, since non-invasive and low-cost cfDNA testing still plays an important role in consumer-grade cancer diagnosis and cancer treatment management, such as personalized medicine and cancer prognosis. In those studies, two available approaches for early cancer detection using liquid biopsy were presented: i) One assay for one cancer (one-to-one); and ii) one assay for multiple cancer types (one-to-many) by utilizing genome-wide profiling or a large genetic signature panel.

Although numerous potential biomarkers have already been identified in GC, the development of a new generation of minimally invasive cfDNA-based tests for GC early detection must consider their clinical validity and utility. These require collaborative efforts in two areas: i) Developing new assays with improved sensitivity, reproducibility, procedural standardization and cost-effectiveness; and ii) validating emerging biomarkers in larger prospective clinical studies. Although the 'one-to-many' liquid biopsy approach is more attractive in the long term, it presents greater challenges than the 'one-to-one' approach. With these recent advances in cancer genetics and assay modalities, particularly the clinical implementation of circulating methylated DNA-based CRC and lung cancer screening tests, it is expected that a new, clinically effective, liquid biopsy assay for early detection of GC will be available in the near future.

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ZH performed researched data and wrote this manuscript, HZ contributed to discussions of content and helped to draft the manuscript. BY revised the draft of the manuscript. DY designed the study and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

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Competing interests

The authors declare that they have no competing interests.

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