



2015 Advances in Gastric Cancer

## Emerging blood-based biomarkers for detection of gastric cancer

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**Author contributions:** Kalniņa Z and Meistere I equally contributed to the collection of data and writing the manuscript; Kikuste I and Tolmanis I contributed to collection of data and writing individual sections of the manuscript; Zayakin P assisted in collection of data; Linē A supervised the work and revised the manuscript.

Supported by ERDF project, No. 2013/0052/2DP/2.1.1.1.0/13APIA/VIAA/019.

**Conflict-of-interest statement:** The authors have no conflicts of interest to report.

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Received: May 9, 2015  
Peer-review started: May 11, 2015  
First decision: June 23, 2015  
Revised: July 8, 2015

Accepted: September 30, 2015  
Article in press: September 30, 2015  
Published online: November 7, 2015

### Abstract

Early detection and efficient monitoring of tumor dynamics are prerequisites for reducing disease burden and mortality, and for improving the management of patients with gastric cancer (GC). Blood-based biomarker assays for the detection of early-stage GC could be of great relevance both for population-wide or risk group-based screening programs, while circulating biomarkers that reflect the genetic make-up and dynamics of the tumor would allow monitoring of treatment efficacy, predict recurrences and assess the genetic heterogeneity of the tumor. Recent research to identify blood-based biomarkers of GC has resulted in the identification of a wide variety of cancer-associated molecules, including various proteins, autoantibodies against tumor associated antigens, cell-free DNA fragments, mRNAs and various non-coding RNAs, circulating tumor cells and cancer-derived extracellular vesicles. Each type of these biomarkers provides different information on the disease status, has different advantages and disadvantages, and distinct clinical usefulness. In the current review, we summarize the recent developments in blood-based GC biomarker discovery, discuss the origin of various types of biomarkers and their clinical usefulness and the technological challenges in the development of biomarker assays for clinical use.

**Key words:** Gastric cancer; Biomarker; Liquid biopsy; Cell-free DNA; Cell-free RNA; Extracellular vesicles; Autoantibodies; Proteomics

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**Core tip:** The identification of blood-based biomarkers that could reliably detect the presence of early-stage gastric cancer or provide means to monitor the tumor dynamics is an unmet clinical need. Recently, considerable effort has been devoted to discovering various types of cancer-associated molecules in the blood of gastric cancer patients, and this has resulted in establishing biomarker models with remarkably high sensitivity and specificity. However, a validation in large-scale studies and a head-to-head comparison of the biomarker models and technologies are required before these biomarkers can be used in routine clinical practice.

Kalniņa Z, Meistere I, Kikuste I, Tolmanis I, Zayakin P, Linē A. Emerging blood-based biomarkers for detection of gastric cancer. *World J Gastroenterol* 2015; 21(41): 11636-11653 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i41/11636.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i41.11636>

## INTRODUCTION

Although the incidence of gastric cancer (GC) has decreased in most parts of the world, with estimated 952000 new cases and 723000 deaths from GC in 2012, it still accounts for approximately 6.8% of all cases and 8.8% of cancer-related deaths worldwide<sup>[1]</sup>. The incidence rates vary significantly across the globe, being the highest in Eastern Asia, followed by Central and Eastern Europe and rates are the lowest in North America and Western Africa<sup>[1,2]</sup>. The main type of GC is adenocarcinoma (approximately 95%), which can be further categorized into an intestinal and a diffuse type according to Lauren's classification<sup>[3]</sup>. Intestinal-type gastric adenocarcinoma, the most common subtype of GC, develops through a well-described sequence of histopathological stages from normal mucosa to chronic gastritis, chronic atrophic gastritis followed by intestinal metaplasia, dysplasia and finally to adenocarcinoma, with *H. pylori* infection, which is recognized as the main underlying cause of pan-gastric mucosal inflammation<sup>[4,5]</sup>. Thus, the main risk factors for GC are chronic infection with *H. pylori* and the presence of the above-listed precancerous lesions, whereas a relatively smaller proportion of GC cases are linked to a genetic predisposition and dietary factors<sup>[6,7]</sup>.

The high mortality rate in GC mostly results from its detection at late stages. Most GC cases are detected at stage IIIA-IV, when the estimated 5-year survival ranges from 7%-27% and the median survival is less than 12 mo<sup>[8,9]</sup>. On the contrary, early GC that is limited to the submucosal layer is curable by endoscopic mucosal dissection or minimally-invasive surgery<sup>[10]</sup>. Early GC detection, however, is hampered by the lack of specific symptoms before it

has spread beyond the original site. Thus, organized screening programs that aim to detect pre-cancerous lesions and early-stage GC seem to be a main tool for reducing GC-related mortality, but such programs have been implemented only in some Asian countries<sup>[11]</sup>. Upper endoscopy is the primary screening technique in most of the programs and the gold standard for confirmation of the diagnosis<sup>[6,11]</sup>. However, endoscopy is an invasive technique with uncommon but serious side effects and a relatively high cost, and the results are highly dependent on the skill of the endoscopist<sup>[6]</sup>. Therefore, GC screening in low GC incidence areas and low-income countries is not practical, and is likely to be associated with low participation rates in the screening programs.

Currently, the only non-invasive test that has been used for GC detection is the pepsinogen (PG) test. PGs are pro-enzymes that are converted into the proteolytic enzyme pepsin. PGs are mainly synthesized and secreted by the gastric chief cells and their serum levels indirectly reflect secretion in the stomach<sup>[12]</sup>. PGI is exclusively produced by the corpus mucosa, while PGII is also secreted by the cardiac and pyloric glands and the proximal duodenal mucosa<sup>[13]</sup>. Low PGI levels and a low PG I /PG II 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%<sup>[14-16]</sup>, whereas the sensitivity for GC detection ranges from 36.8%-62.3%<sup>[17-19]</sup>, which is not acceptable in population-based screening settings. Thus, the PG test can be administered in a two-stage screening approach as a primary screening test to identify individuals who are at an elevated GC risk, and these high-risk individuals are then referred for endoscopic examination followed by the histological analysis of gastric biopsy<sup>[11]</sup>.

In recent years, considerable effort has been devoted to the discovery of novel blood-based biomarkers that are suitable for the development of non-invasive tests to detect GC at an early stage or to monitor tumor dynamics. Such biomarkers may include quantitatively- or structurally-altered proteins, cancer-associated autoantibodies, cell-free nucleic acids (cfNAs), circulating tumor cells (CTCs), cancer-derived extracellular vesicles (EVs) and metabolites. In the current review, we provide an overview of recently-discovered blood-based GC biomarkers, and discuss their origin and mechanisms of release into the bloodstream, and also their potential clinical usefulness.

## CRITERIA FOR BIOMARKERS APPLICABLE TO CANCER CONTROL PROGRAMS

In 2013, a Working Group of international experts established by the International Agency for Research on Cancer made recommendations for GC control

and concluded that a decisive public health action to include GC in cancer control programs is required; however, interventions should be tailored to the local conditions, taking into account the prevalence, cost-benefit ratio and adverse consequences<sup>[20]</sup>. Prevention strategies should aim to reduce both GC incidence and mortality. Primary prevention strategies are focused on preventing exposure to GC risk factors, for example, by eradicating *Helicobacter pylori* (*H. pylori*) infection or modifying patients' diet and lifestyle, while secondary prevention strategies aim to identify patients with early-stage GC or precancerous lesions, who would then undergo endoscopic surveillance<sup>[6,11]</sup>. Tertiary prevention aims to control the symptoms and morbidity of established cancer. Blood-based biomarkers for the detection of early-stage, residual or recurrent cancers could be highly relevant for both secondary and tertiary prevention strategies.

Ideally, a biomarker that is used in population-wide screening programs should be stable and robustly measurable in plasma or serum using routine laboratory equipment, appear in the bloodstream before the clinical signs and symptoms arise, should discriminate between cancer and inflammatory diseases and should have high positive and negative predictive values. However, the relatively low prevalence of GC in most parts of the world, except for Eastern Asia, suggests that even biomarker assays with high sensitivity and specificity would have a low positive predictive value (PPV). For example, if a hypothetical biomarker assay with a sensitivity of 95% and specificity of 98% would be applied to screen 100000 asymptomatic individuals in a medium-incidence area such as Eastern Europe (with GC prevalence of 0.04%), 38 true positives, 2 false negatives and approximately 2000 false positives would be detected, thus yielding PPV of only 1.87%.

A biomarker for detecting residual or recurrent cancer, however, must reflect the tumor dynamics. For example, it should be rapidly cleared from the circulation after complete tumor removal, and it should be able to detect incompletely-resected tumor and to increase in the circulation before the clinical signs of recurrence.

## PROTEOMIC BIOMARKERS

Proteomic analyses can provide information on a complex composition of proteins that are differentially expressed in blood specimens from cancer patients and healthy donors that could be used for cancer biomarker discovery. The flowchart of serum proteomic analysis usually consists of protein extraction and separation performed by 2-dimensional gel electrophoresis (2-DE), difference gel electrophoresis (2D-DIGE), surface-enhanced laser desorption/ionization (SELDI), Liquid Chip and other approaches. These are followed by diagnostic model determination or protein identification through MS and bioinformatics, after which identified

proteins are verified using conventional techniques such as Western blot and ELISA (technology approaches reviewed by Liu *et al.*<sup>[21]</sup>). The current challenges in blood-borne biomarker discovery include variability of sample preparation and pre-treatment as well as inter-laboratory analytical variability of different instruments used in discovery and validation studies. Another issue is the choice of sample type used for proteome analyses - serum seems to be the most common choice because of its availability in biobanks and thus, it is frequently used in studies. However, the Human Proteome Organisation recommends the use of plasma for proteomic studies to reduce the variability caused by the coagulation process<sup>[22]</sup>.

Many proteomic studies of serum biomarkers for GC detection have been published in the last 10 years (reviewed in detail by Liu *et al.*<sup>[21]</sup> and Lin *et al.*<sup>[23]</sup>), and examples of biomarker models are listed in Table 1. In one of the pioneering studies, Ebert *et al.*<sup>[24]</sup> analyzed serum from GC patients with SELDI-TOF-MS and Protein-Chip technology in combination with a pattern-matching algorithm and built a classifier ensemble that consists of 50 decision trees that achieved 100% sensitivity and 96.7% specificity (including both, intestinal and diffuse type GC). Moreover, this classifier could detect early stage GC with sensitivity of 89.9%. Liu *et al.*<sup>[25]</sup> showed that there were three differentially-expressed peaks identified by screening serum samples from 65 GC and 53 cancer-free individuals, including patients with chronic superficial gastritis and chronic atrophic gastritis. The combined use of the three biomarkers, which were identified as fibrinogen  $\alpha$  chain, apolipoprotein A-II and apolipoprotein C-I, distinguished the cancer group from the control group with a sensitivity of 93.85% and a specificity of 94.34% in an independent validation set. In another study, Li *et al.*<sup>[26]</sup> found a six-feature proteomic model by applying SELDI-TOF-MS analysis that effectively distinguished GC samples from control samples with a sensitivity of 93.5% and specificity of 91.6%. In addition, they observed that three of the peaks were differentially expressed between patients with stage I GC and advanced GC (accuracy 88.9%). Other groups have reported using the SELDI-MS application to analyze the serum profile from GC patients, and they showed an overall high sensitivity and specificity (over 90% and 80%, respectively)<sup>[27-32]</sup>. However, these promising results have to be validated in larger multicenter studies because the SELDI-MS approach has several disadvantages, as follows: the results lack consistency among research groups, the reproducibility is low and it cannot directly identify proteins<sup>[33]</sup>.

Other approaches besides SELDI-MS have been used. Yang *et al.*<sup>[34]</sup>, using magnetic beads, separated peptidome from GC patients' serum using matrix-assisted laser desorption/ionization - time of flight (MALDI-TOF) MS, and they found 11 differentially-expressed proteins and the two most promising of them could detect GC patients with 95.2% sensitivity

**Table 1** Proteomics-based biomarker models for detection of gastric cancer

Biomarker model	Approach used	Sample size and type (cancer/controls)	Diagnostic value <sup>1</sup>	Ref.
Five peaks - 3316, 6629, 3217, 3952, 6431 Da	MB-WCX, MALDI-TOF-MS	T: GC = 32/HC = 32 V: GC = 30 (GC I - II = 8)/HC = 30	AUC = 0.86-0.99 for individual features ( $P < 0.001$ ), Sn = 79.3%, Sp = 86.5% Sn = 71.7% for early stage GC	Yang <i>et al.</i> <sup>[148]</sup> , 2012
1546 Da (SERPINA1) 5335 Da (ENOSF1)	MB-WCX, MALDI-TOF-MS; ELISA for validation	T: GC = 70/HC = 72 V: GC = 36/HC = 36, BGD = 30, other cancers = 108	AUC (1546 Da) = 0.83 ( $P < 0.001$ ), AUC (5335 Da) = 0.87 ( $P < 0.001$ ) - calculated for training set; in validation set SERPINA1 concentration was significantly higher for GC patients than for all other controls ( $P < 0.001$ ) and ENOSF1 concentration was significantly higher for GC patients than HC ( $P < 0.001$ )	Yang <i>et al.</i> <sup>[34]</sup> , 2015
Fibrinogen $\alpha$ -chain, apolipoprotein A- II and apolipoprotein C- I	HPLC, LC-MS/MS	T: GC = 65/HC = 30, BGD = 23 V: GC = 44/ HC = 30, BGD = 23	Sn = 90.9%, Sp = 90.6% ( $P = NA$ )	Liu W <i>et al.</i> <sup>[37]</sup> , 2012
Six peaks at 2873, 3163, 4526, 5762, 6121 and 7778 m/z; For stage I three peaks at 2873, 6121 and 7778 m/z	Protein Chip SELDI-TOF-MS	GC = 169 (GC I = 27)/ HC = 83	Sn = 93.5%, Sp = 91.6% Accuracy for stage I - 88.9%, ( $P = NA$ )	Li <i>et al.</i> <sup>[26]</sup> , 2012
EGFR, proApoA1, ApoA1, TTR, RANTES, VN, DD, IL-6, A2M, CRP, PAI1	xMAP (Luminex), ELISA	T: GC = 120/BGD = 101, HC = 19 V: GC = 95 (GC I - II = 75)/BGD = 43, HC = 8	AUC = 0.95, ( $P < 0.05$ ) Sn = 88.8%, Sp = 89.7% Sn (I - II) = 92.3% Sn (tumor size $\leq 2$ cm) = 81.8%	Ahn <i>et al.</i> <sup>[35]</sup> , 2012
Four peaks at 1867 (tubulin beta chain), 2701 (thymosin beta4 like protein3), 2094 (cytochrom b-c1 subunit), 1467 Da	MB-WCX, MALDI-TOF-MS	T: GC = 40/HC = 39 V: GC = 40/GA = 30, HC = 39	AUC (1867 Da) = 1, AUC (1467 Da) = 0.83 AUC (2701 Da) = 0.71 AUC (2094 Da) = 0.70 ( $P < 0.05$ ) Sn = 95.0%, Sp = 97.1% Sn = 100%, Sp = 96.7%	Fan <i>et al.</i> <sup>[30]</sup> , 2013
50 decision trees, 28 masses	Protein Chip SELDI-TOF-MS,	T: GC = 41/HC = 49 V: GC = 28; GC I = 9/HC = 30	For stage I Sn = 89.9% ( $P = NA$ )	Ebert <i>et al.</i> <sup>[24]</sup> , 2004
Three peaks at 3946, 3503 and 15958 Da			Sn = 92.8%, Sp = 86.7% For stage I Sn = 89.9% ( $P = NA$ )	

<sup>1</sup>Diagnostic values listed for validation set, if not otherwise stated. A2M: Alfa 2 macroglobulin; Apo: Apolipoprotein; AUC: Area under the curve; BGD: Benign gastric diseases; CRP: C reactive protein; DD: D-dimer; ENOSF1: Isoform 2 of mitochondrial enolase superfamily member 1; EGFR: Epidermal growth factor receptor; GA: Gastric adenoma; GC (I-IV): Gastric cancer (TNM stages); HC: Healthy control; HPLC: High performance liquid chromatography; IL-6: Interleukin 6; LC: Liquid chromatography; MALDI-TOF-MS: Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry; MB-WCX: Magnetic bead based weak cation-exchange chromatography; NA: Not available; PAI1: Plasminogen activator inhibitor 1; proApo: Proapolipoprotein; RANTES: Regulated upon activation, normally T-expressed and presumably secreted; SELDI-TOF-MS: Surface-enhanced laser desorption/ionization-time of flight-mass spectrometry; SERPINA1: Serpin peptidase inhibitor clade A (alpha-1 antitrypsin), member 1; Sn: Sensitivity; Sp: Specificity; T: Training set; TTR: Transthyretin; V: Validation set; VN: Vitronectin.

and 93.6% specificity. In another study, Ahn *et al.*<sup>[35]</sup> constructed a 29-plex array platform based on antibodies against 11 proteins discovered using proteomic approaches and 18 known cancer-associated proteins, and used it to examine serum from 120 GC patients and 120 non-cancerous individuals including 98 gastritis or ulcer patients. They used multivariate classification analysis including 11 analytes (listed in Table 1) that differed between the above-mentioned groups ( $P$  value  $< 0.001$ ). They obtained an accuracy  $> 85\%$  in an independent validation sample set (95 GC and 51 controls).

By evaluating the known individual serum proteins identified using proteomic approaches from the cancer biomarker perspective, the complexity of the plasma proteome has to be taken into account; it has a wide dynamic range covering 10 orders of magnitude star-

ting from albumins as the most abundant proteins and ending with cytokines and interleukins<sup>[36]</sup>. Some groups have tried to reduce the plasma proteome complexity by depleting highly abundant protein fractions using different means; however, the results obtained are rather ambiguous. For example, to focus on lower-abundance proteins that might be relevant to cancer, Liu *et al.*<sup>[37]</sup> depleted serum of predominant protein fractions and compared GC and healthy donor specimens using 2D-DIGE followed by MS. They detected 12 differentially expressed proteins including plasminogen, apolipoprotein A-IV, kininogen-1, clusterin and complement component C4A. Chong *et al.*<sup>[38]</sup> used a combination of proteomic techniques that included highly abundant protein removal and found that plasma protein C9 was significantly increased in GC patients compared with



the healthy donor group. Increased C9 levels have also been reported in serum samples from patients with acute leukemia and sarcoma as well as autoimmune diseases<sup>[38]</sup>. Ebert *et al.*<sup>[39]</sup> used MALDI-TOF-MS for screening whole serum samples from 14 GC patients and 14 healthy individuals and found that a peptide fragment increased in cancer patients' serum; the peptide was later identified as fibrinopeptide A (FpA). The authors confirmed its level in serum using ELISA in a larger cohort of GC patients ( $n = 99$ ), high-risk individuals ( $n = 13$ ) and controls ( $n = 111$ ), and they observed increased levels in cancer patients and high-risk individuals compared to normal controls. FpA is a blood coagulation protein that is also reported to be a putative biomarker for GC staging<sup>[32,40]</sup>. The above-mentioned apolipoprotein C- I together with C-III have been previously reported as diagnostic biomarkers for GC, and the analysis of serum from 103 GC patients and 54 cancer free controls showed decreased expression in the cancer group versus the control group; these results were confirmed using ELISA. The level of apolipoproteins in blood has been reported to be a potential biomarker for various cancers<sup>[41]</sup>. Yang *et al.*<sup>[34]</sup> identified two peptides that were later characterized as fragments of SERPINA1, an inflammation acute phase protein, and ENOSF1 as the most significantly increased peptides in GC patients. Generally, most proteins mentioned above represent highly abundant plasma proteins and their roles as GC-specific diagnostic markers have to be interpreted with caution, because they are known to be part of a blood coagulation system or represent acute phase inflammatory proteins and they have been reported to be associated with other types of cancer. Evidence from a study using a mouse model of breast cancer showed that the host cell and tumor microenvironment-derived protein signature in plasma differs from the signature associated with inflammatory conditions that are not related to cancer, and therefore could be used for early stage cancer detection<sup>[42]</sup>.

Some studies are focused on posttranslational modification of the serum proteome, such as protein glycosylation<sup>[43-47]</sup>, because it is known that alterations in protein glycosylation are a common feature of tumor cells. Bones *et al.*<sup>[43]</sup>, using a combination of glycomic techniques and 2D-DIGE, demonstrated an increased level of sialyl Lewis X epitopes that are presented on triantennary glycans in serum from 80 GC patients compared with 10 patients who had benign gastric diseases and 20 healthy donors, and core fucosylated biantennary agalactosyl glycans were present on extracted immunoglobulin G molecules that were associated with increased TNM stage. Ozcan *et al.*<sup>[45]</sup>, by analyzing serum N-glycan profiles using MALDI-TOF-MS, identified 19 glycans that were differentially expressed among patients with GC, non-atrophic gastritis and duodenal ulcers. The glycan profile of the duodenal ulcer group was similar to that in the GC group. In another study, the serum immunoglobulin

G glycosylation profile was analyzed using Nano-LC-MS, and eight glycans that can distinguish GC from non-atrophic gastritis, eight glycans that differed between GC and duodenal ulcer and three glycans that differentiated between the non-atrophic gastritis and duodenal ulcer groups were identified<sup>[47]</sup>. Roy *et al.*<sup>[46]</sup> used an on-chip lectin microarray-based glycomic approach to analyze tissue and serum samples from patients with GC, chronic gastritis and healthy individuals. They showed that the glycoprofile obtained from the tissue samples deviated more than that from the serum samples. It is likely that the altered glycan profile in serum from cancer patients is related to the inflammatory processes and the host defense response mechanisms during carcinogenesis in general<sup>[43,45,47]</sup>.

Although highly promising proteomic diagnostic biomarkers have been identified, especially for early GC diagnosis, there are currently no proteomic-based serum biomarker tests available for clinical application. It has become apparent that large-scale validation studies are critical to evaluate the accumulated proteomic data. Currently, the field of proteomic techniques is rapidly evolving, and continuously-improving technical performance provides constant and reliable high throughput analysis and increasing technical sensitivity for low concentration plasma protein measurements<sup>[36,48]</sup>.

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## CANCER-ASSOCIATED AUTOANTIBODIES

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The human immune system senses the presence of cancer before manifestation of the disease<sup>[49]</sup>. High-titer IgG class autoantibodies against specific tumor associated antigens (TAAs) have been found in patients' blood even up to five years before clinical diagnosis, thus demonstrating their potential for the detection of early stage cancer<sup>[50-52]</sup>. In addition, autoantibodies have other promising biomarker qualities: they are found in all tumor types that have been analyzed so far<sup>[53,54]</sup> and they are highly stable, antigen specific. Unlike the known GC biomarkers such as pepsinogens, CEA and CA19-9, autoantibodies are qualitative, not quantitative, biomarkers. Testing autoantibody reactivity against panels of TAAs using multiplex immunoassays has been shown to be feasible<sup>[55]</sup> and this aspect might substantially foster their transition from experimental to clinical medicine.

Accumulating evidence has shown that any individual cancer-associated autoantibody biomarker has a limited diagnostic value. Autoantibody repertoires in cancer patients are diverse, and the frequency of antibodies against any particular antigen typically ranges from 1%-15%<sup>[56-58]</sup>. Among the most studied individual markers in GC, there are autoantibodies against well-known TAAs such as p53 (*e.g.*, 13 studies summarized by Werner *et al.*<sup>[58]</sup> report a biomarker sensitivity range of 8.1-32.1% and specificity range of

**Table 2** Autoantibody signatures with diagnostic value for gastric cancer

Biomarker signature description	Technology	Study design	Sample size (GC/controls)	Diagnostic value	Ref.
2 TAAs – p62, Koc	ELISA	GC vs HC	135/82	Sn = 19.3%, Sp = 97.6%, $P < 0.01$	Zhang <i>et al.</i> <sup>[62]</sup> , 2001
3 TSAs - IQGAP3, KRT23 and REG3A	PARSE assay	GC vs HC (age and sex matched)	48/46	Sn = 22.9%, Sp = 100%, $P < 0.001$	Xu <i>et al.</i> <sup>[149]</sup> , 2012
3 TAAs – p16, p53, c-myc	ELISA	GC vs HC	74/82	Sn = 21.6%, Sp = 97.6%; $P < 0.001$	Looi <i>et al.</i> <sup>[150]</sup> , 2006
7 TAAs - p53, C-myc, p16, IMP1, Koc, p62 and Survivin	ELISA	Cardia GC vs HC	88/140	AUC = 0.73, Sn = 64%, Sp = 87%, $P < 0.001$	Zhou <i>et al.</i> <sup>[68]</sup> , 2015
7 TAAs - C-myc, Cyclin B1, IMP1, Koc, P53, p62 and Survivin	ELISA, fixed cut-off	GC vs HC	91/346	Sn = 52.7%, Sp = 89.9%, $P < 0.01$	Zhang <i>et al.</i> <sup>[63]</sup> , 2003
	ELISA, individual cut-off (recursive partitioning)	GC vs HC	91/346	Sn = 98.9%, Sp = 93.1%, $P < 0.001$	Koziol <i>et al.</i> <sup>[151]</sup> , 2003
45 T7 phage-displayed TAA clones (including NY-ESO-1, DDX53, MAGE antigens <i>etc.</i> )	T7 phage displayed TAA microarray	GC vs HC (age and sex matched)	T:100/100 V:235/213	AUC = 0.79, Sn = 59%, Sp = 90%, $P < 0.001$	Zayakin <i>et al.</i> <sup>[56]</sup> , 2013
		GC vs gastritis	235/100	AUC = 0.64, Sn = 58.7%, Sp = 55%, $P < 0.001$	
		GC vs gastric ulcer	235/54	AUC = 0.76, Sn = 58.7%, Sp = 81.5%, $P < 0.001$	

AUC: Area under the curve; GC: Gastric cancer; HC: Healthy controls; ND: Not determined; Sn: Sensitivity; Sp: Specificity; TAA: Tumor associated antigen; TSA: Tumor specific antigen; T: Training; V: Validation.

95.25%-100%), NY-ESO-1<sup>[59,60]</sup>, MUC1<sup>[61]</sup>, Koc, p62<sup>[62]</sup>, C-myc and Survivin<sup>[63,64]</sup> and others<sup>[58]</sup>.

The development of high-throughput proteomic techniques, such as various native and recombinant protein microarrays and bead-based technologies (reviewed by Meistere *et al.*<sup>[65]</sup>), has enabled the simultaneous detection of autoantibodies against many different TAAs. This has allowed systematic analysis and comparison of the heterogeneous repertoires of circulating autoantibodies within large patient cohorts, which has resulted in selection for cancer-associated biomarker signatures and discarding of those that are induced by other immune processes such as tissue damage, viral infections or possible autoimmune conditions<sup>[66,67]</sup>. To the best of our knowledge, seven studies have been published on the diagnostic values of different GC-associated autoantibody biomarker combinations (overviewed in Table 2). Within these studies, the identified biomarker signatures could discriminate GC from healthy controls with relatively high specificity (ranging from 87-100%) but with variable sensitivity (19.3%-98.9%). AUC was reported in only two studies: Zhou *et al.*<sup>[68]</sup> showed that autoantibody reactivity against seven known TAAs was able to distinguish between patients with cardia GC from healthy controls with an AUC of 0.73, while Zayakin *et al.*<sup>[56]</sup> reported that 45 GC-associated autoantibody classifiers distinguished GC (all stages with similar sensitivity) from healthy controls with an AUC of 0.79. However, these studies vary greatly in regard to various important aspects, such as the multiplexing level (2-45 autoantibodies), the method used for autoantibody detection, definition of appropriate control group(s), and approaches used for data normalization and cut-off definition. Altogether, these issues may greatly hamper the introduction of the identified biomarkers into clinical practice.

The most relevant biomarkers for early GC diag-

nosis would be those capable of detecting cancer in high-risk individuals. Only some studies have addressed the GC-associated autoantibody repertoire overlap with that found in patients with benign gastric lesions. For example, a study by Zayakin *et al.*<sup>[56]</sup> found that, within the diagnostic 45-autoantibody signature, the identified biomarker pattern was partially shared between GC and gastritis patients, and was not found in patients with peptic ulcer and healthy controls. Two smaller studies addressed the p53 autoantibody specificity regarding benign gastric diseases and in both cases it was shown that this biomarker specifically detect GC in approximately 32% of the cases and that it is not found in the control patients<sup>[69,70]</sup>. Another issue is that GC-associated serologically active antigens have been shown to elicit B cell responses in variety of other malignancies<sup>[71]</sup>. The overlap of the identified GC-associated autoantibody signatures with those found in patients with other (gastrointestinal) cancer types has been addressed only partially and remains to be systematically analyzed within further studies to ascertain their clinical value.

In summary, cancer-associated autoantibody biomarkers have been shown to have high specificity, but moderate sensitivity, which would hinder their use in clinical practice for population-based screening. The limitations of the autoantibody biomarker sensitivity from the biological point of view are currently unknown. In a previous study, we analyzed autoantibody responses against 45 TAAs in 235 GC patients and found no serum-reactivity in 41% of the patients<sup>[56]</sup>. We then performed extensive screening of cDNA expression libraries with serum samples that did not react against the 45 TAA panel. The screening results showed that up to 10% of the GC patients either generally do not mount an antibody response against tumor antigens or did not have detectable autoantibody levels at the given time

point (unpublished results), thus demonstrating the biological limits for the sensitivity of autoantibody-based diagnostic assays. In addition, heterogeneity of TAA repertoires between cancer patients is high, and each individual autoantibody biomarker generally has a low frequency of detection. Thus, currently-published studies are most likely statistically underpowered. Rare cancer-specific autoantibodies that individually do not reach statistical significance, but are incorporated into the diagnostic biomarker panels, lead to the low reproducibility of initially-obtained results and this lowers the diagnostic value of a diagnostic autoantibody signature, which may be improved by analyzing the proposed biomarker combinations within cohorts with sufficient statistical power.

However, autoantibodies may be important players in the stratification of risk group patients. One of their strengths over other biomarker classes is that the adaptive immune system senses the tumor development early on<sup>[49]</sup> and can mount high titer antibody responses even to minute amounts of antigen while the presence of other biomarkers (*e.g.*, circulating tumor cells, protein biomarkers, cancer exosomes, cell-free nucleic acids) is gradually increasing in circulation during the progression of cancer. Moreover, the autoantibody repertoires elicited by GC have not been previously analyzed in the context of IgG subclasses. This may be an important aspect because each of the IgG1-4 subclasses have different affinities for activating and inhibiting Fcγ receptors, which eventually has an impact on the activating/inhibitory balance of the infiltrating immune effector cells. This may result in either host-protective or tumor-promoting immune responses, and thus the diagnostic value could be assigned to the specific IgG subclass itself and not only to the antigen specificity of total IgG, as was shown for melanoma<sup>[72]</sup>. In addition, the analyses of a TAA-specific secreted IgA repertoire might reveal possible novel biomarker candidates because mucosal linings are known to produce more IgA than all other types of antibodies combined.

## CELL-FREE NUCLEIC ACIDS

Although the presence of cell-free nucleic acid (cfNA) in human blood was first described by Mandel and Métais<sup>[73]</sup> in 1948, researchers only began to realize the clinical significance of this finding half a century later<sup>[74]</sup>. During the past decade, the idea that cfNAs could serve as blood-based biomarkers of cancer has attracted increasing attention. cfNAs may serve as a “liquid biopsy” of cancer reflecting the genetic make-up of tumors, thus enabling detection of drug targets and tracking evolving genetic alterations throughout the course of the disease. Numerous studies have investigated the diagnostic and prognostic potential of total cfDNA levels, gene copy number, DNA integrity, cancer-associated DNA methylation markers or

somatic mutations and expression levels of mRNAs, miRNAs and other non-coding RNAs in the blood of cancer patients.

cfNAs can be released into the circulation *via* various forms of cell death such as apoptosis, necrosis, autophagy and necroptosis<sup>[74-76]</sup> or actively secreted by packaging into extracellular vesicles (EVs)<sup>[77-80]</sup>. Most of the cfDNA is fragmented and the size distribution of the fragments varies from 150-350 bp to > 10000 bp<sup>[81]</sup>. The shorter fragments correspond to the mono- and dinucleosomal DNA fragments released from apoptotic cells, while the larger fragments are likely to be released from necrotic cells<sup>[81]</sup>. Increased cfDNA integrity (*i.e.*, higher ratio of longer to shorter DNA fragments), presumably reflecting an increased rate of necrotic cell death in cancer, has been found in several types of cancer and has been shown to have a diagnostic relevance<sup>[82-84]</sup>. However, the fraction of tumor-derived DNA has been shown to vary from only 3% to as much as 93% of total cfDNA in different patients<sup>[81]</sup> and the cellular source of cfDNA is still controversial.

Circulating cfRNA, in particular miRNA, has been found to be remarkably resistant to endogenous and exogenous RNase activity, extreme pH conditions and freeze-thaw cycles<sup>[85]</sup>. This suggests that cfRNA may be protected from degradation by packaging into various EVs, including exosomes, microvesicles and apoptotic bodies. Studies evaluating the proportion of vesicle-enclosed and vesicle-free miRNA in human plasma, however, have come to controversial conclusions: several studies have showed that the majority of circulating miRNAs are concentrated in exosomes and exosome isolation improves the sensitivity and consistency of miRNA analysis in biofluids<sup>[86,87]</sup>, while other studies showed that only a few miRNAs are enclosed into exosomes<sup>[88]</sup> and, on average, there is less than one molecule of a given miRNA per exosome<sup>[89]</sup>. Currently, the reason for such a discrepancy is unclear and more detailed studies on the content, localization and stoichiometry of various RNA species in distinct EV subtypes are required.

### **Total cfDNA level**

Several studies have reported increased levels of total cfDNA in plasma of GC patients compared with healthy controls<sup>[90-93]</sup> (Table 3). The cfDNA levels could distinguish between GC and control plasma with an AUC varying from 0.75<sup>[90]</sup> to 0.991<sup>[92]</sup>. Because the measurement of cfDNA levels does not require any *a priori* knowledge of genetic alterations in the tumor tissue, such an approach could be highly relevant to the development of non-invasive assays for the early detection of GC. However, the size of patient cohorts was relatively small in all of these studies, and therefore validation of the findings in large, well characterized cohorts is required to draw conclusions about clinical utility of cfDNA levels. In addition,

**Table 3** Cell-free DNA as biomarkers for detection of gastric cancer

Candidate biomarkers	Sample size and type	Method/technology	Diagnostic value/outcome	Ref.
Total cell-free DNA level				
β-actin (total cfDNA level)	GC = 53, HC = 21, plasma	qPCR	AUC = 0.75, $P < 0.0001$	Sai <i>et al</i> <sup>[90]</sup> , 2007
DNA integrity		qPCR (ratio of long vs short b-actin amplicons)	No significant difference between GC and HC	
Alu DNA sequences	GC = 54, HC = 59; plasma	Alu81-qPCR	AUC = 0.784, Sn = 75%, Sp = 63%	Park <i>et al</i> <sup>[101]</sup> , 2012
Total cfDNA level	Early GC = 16; advanced GC = 14; HC = 34; plasma	Measurement of cfDNA concentration	AUC = 0.991, Sn = 96.67%, Sp = 94.11% for GC vs HC	Kim <i>et al</i> <sup>[102]</sup> , 2014
Gene amplification				
<i>MYC</i> gene copy number ( <i>MYC</i> / <i>GAPDH</i> ratio)	GC = 57, HC = 39; tissues and plasma	qPCR	AUC = 0.816; strong positive correlation between <i>MYC</i> levels in GC tissues and plasma ( $r = 0.342$ ; $P = 0.009$ )	Park <i>et al</i> <sup>[101]</sup> , 2009
<i>HER2</i> gene copy number ( <i>HER2</i> / <i>RPPH1</i> ratio)	Discovery: GC = 52 (pre and post-operative treatment), HC = 40; plasma and tissues  Validation: GC = 25 plasma	qPCR	AUC = 0.746, Sn = 53.9%, Sp = 96.7%; Positive correlation between GC tissues and plasma ( $r = 0.424$ ; $P = 0.00721$ ); decrease in post-treatment plasma in <i>HER2</i> + GC cases  Sn = 66.7%, Sp = 100%	Shoda <i>et al</i> <sup>[100]</sup> , 2014
DNA methylation markers				
<i>RPRM</i> (Reprimo)	GC = 43, HC = 31; GC tissues and plasma	MSP	95.3% GC, 9.7% HC, $P < 0.00001$ ; Strong correlation between methyl status in tissues and plasma	Bernal <i>et al</i> <sup>[107]</sup> , 2008
<i>RUNX3</i>	GC (preoperative) = 65, GC (postoperative) = 43, HC = 50, tissues and serum	qMSP	AUC = 0.8651, Sn = 95.5%, Sp = 62.5%; decrease after surgical resection	Sakakura <i>et al</i> <sup>[152]</sup> , 2009
<i>KCNA4</i> + <i>CYP26B1</i>	GC = 46, GPL = 46, HC = 30; serum	Discovery: Methylation microarray in tissues; Testing: MSP	AUC = 0.917, Sn = 91.3%, Sp = 92.1%	Zheng <i>et al</i> <sup>[108]</sup> , 2011
<i>SLC19A3</i>	Discovery: GC = 45, HC = 60; plasma Validation: GC = 20, HC = 20	MSRED-qPCR	Increased in GC, $P < 0.0001$  AUC = 0.82, Sn = 85%, Sp = 85%	Ng <i>et al</i> <sup>[153]</sup> , 2011
<i>FAM5C</i> + <i>MYLK</i>	GC = 58, GPL = 46, HC = 30; serum	Discovery: MeDIP in cell lines; Testing: MSP	AUC = 0.838, Sn = 77.6%, Sp = 90% for GC vs HC; Sn = 30.4% for GPL vs HC; decrease after surgical resection	Chen <i>et al</i> <sup>[154]</sup> , 2012
<i>XAF1</i>	GC = 202, HC = 88, tumor tissues and serum	qMSP	AUC = 0.909, $P < 0.0001$ ; 83.9% concordance between GC tissues and serum	Ling <i>et al</i> <sup>[108]</sup> , 2013

AUC: Area under the curve; GC: Gastric cancer; GPL: Gastric precancerous lesions; HC: Healthy controls; MeDIP: Methylated DNA immunoprecipitation; MSP: Methylation-specific PCR; MSRED-qPCR: Methylation-sensitive restriction enzyme digestion and real-time quantitative PCR; Sn: Sensitivity; Sp: Specificity.

elevated cfDNA levels have also been detected in patients with inflammatory diseases<sup>[94]</sup>, infections<sup>[95]</sup> and cardiovascular disorders<sup>[96]</sup> and in healthy individuals after exercise<sup>[97]</sup>, thus indicating that this phenomenon is not strictly cancer-specific. Similarly, a recent study by Hamakawa *et al*<sup>[98]</sup> demonstrated that the quantity of DNA fragments harboring cancer-specific somatic mutations in *TP53* gene (circulating tumor DNA, ctDNA) did not correlate with the level of total plasma cfDNA, and only ctDNA showed a good correlation with the GC disease status.

### Cancer-specific gene amplification

More specific approaches for measuring total cfDNA levels could be the assessment of cancer-specific genetic alterations in the circulating cfDNA. Several studies have used qPCR to quantify the copy number of genes known to be amplified in GC tissues, such as *MYC*<sup>[99]</sup> and *HER2*<sup>[100,101]</sup>, in cell-free plasma from GC patients (Table 3). An increased *MYC*/*GAPDH* ratio in plasma significantly correlated with that in the GC

tissues and could distinguish between GC patients and healthy controls with an AUC of 0.816<sup>[99]</sup>. Similarly, the *HER2* level showed a high correlation in plasma and GC tissues, when quantified using qPCR, and had an AUC of 0.746 for detecting GC<sup>[100]</sup>. Meanwhile, Lee *et al*<sup>[101]</sup> reported that the *HER2* copy number in tumor tissues determined by FISH was not significantly associated with the plasma *HER2* level, thus calling into question how well ctDNA levels reflect gene copy numbers in the tumor tissue. The diagnostic usefulness of such tests is limited to detecting GC in patients who harbor the respective genetic amplifications, and therefore they are unlikely to be widely implemented in routine diagnostic examinations. However, they might prove to be highly relevant for detecting the presence or loss of therapeutic targets, and for monitoring treatment efficacy and the course of the disease. Further studies are needed to assess to what extent the ctDNA levels reflect the intratumoral heterogeneity and what factors affect the stability and half-life of the DNA fragments in the plasma.



**DNA methylation markers**

Several other studies have explored the possibility of detecting cancer-associated hypermethylated DNA fragments in the cfDNA of cancer patients. Methylation markers in the bloodstream were first discovered in breast and lung cancer patients in 1999<sup>[102,103]</sup>. Lee *et al.*<sup>[104]</sup> demonstrated, for the first time, the feasibility of detecting aberrant methylation in serum from GC patients. This study reported that promoter region hypermethylation of genes encoding DAP-kinase, E-cadherin, GSTP1, p15 and p16 was detected in serum of 48.1%, 57.4%, 14.8%, 55.6% and 51.9% of GC patients, respectively. Subsequently, multiple studies showed hypermethylated genes in the plasma or serum of GC patients. These studies have been systematically summarized in recent reviews by Tsujiura *et al.*<sup>[105]</sup> and Toyama *et al.*<sup>[106]</sup> and examples of key studies are given in Table 3. Hypermethylated genes showing the highest diagnostic value for detecting GC include *RPRM*<sup>[107]</sup>, *XAF1*<sup>[108]</sup> and a combination of *KCNA4* and *CYP26B1*<sup>[109]</sup>. *RPRM* encodes Reprimo, a TP53-dependent cell cycle regulator, and is frequently silenced in GC *via* methylation of its promoter<sup>[110]</sup>. Bernal *et al.*<sup>[107]</sup> reported that methylated *RPRM* was detected in plasma from 95.3% of GC patients but in only 9.7% of healthy controls, thus yielding a sensitivity of 95.3% and specificity of 90.3%. *XAF1*, a negative regulator of apoptosis inhibitor, has been shown to be downregulated by hypermethylation in cancer tissues of over 83% of GC patients and the agreement between the methylation status in tumor tissues and corresponding serum was 83.9%. Methylated *XAF1* promoter fragments were detected in the serum from 141 out of 202 GC patients, while all 88 cancer-free controls were negative (AUC, 0.909; 95%CI: 0.875-0.942,  $P < 0.0001$ )<sup>[108]</sup>. Zheng *et al.*<sup>[109]</sup> used methylation CpG island microarray technology to search for hypermethylated genes in GC tissues and then selected five candidate genes in the serum of 46 GC patients, 46 patients with precancerous lesions and 30 healthy controls. A combination of two methylation markers, *CYP26B1* and *KCNA4*, could distinguish GC from the control serum with a sensitivity of 91.3%, specificity of 92.1% and AUC of 0.917 (95%CI: 0.858-0.976,  $P < 0.001$ ).

These studies have shown several promising methylation markers that warrant further validation in independent cohorts of patients to establish which of the individual markers or combination of markers has the highest diagnostic value. There are also several technical issues that have to be resolved before these assays could be used in a clinical setting. Most of the studies are based on the treatment of DNA with sodium bisulfite, which converts unmethylated cytosine residues to uracil but leaves methylated cytosines unaffected. The modified DNA is analyzed by methylation-specific PCR (MSP) or DNA sequencing. However, these techniques are prone to false-positive results arising mostly

from incomplete conversion of unmethylated cytosine residues to uracil<sup>[111,112]</sup>. Recently, several quantitative techniques for methylation analysis, such as MS-HRM, SMART-MSP, methyl-BEAMing and bisulfite pyrosequencing, have been established<sup>[112-114]</sup>, but their performance in a clinical setting still needs to be validated.

**Cell-free RNAs**

In 2008, Mitchell *et al.*<sup>[85]</sup> used a mouse model to demonstrate that miRNAs originating from human prostate cancer xenografts enter the blood circulation, thus providing proof of principle that cancer cells release miRNAs that can be detected in the blood. Chen *et al.*<sup>[115]</sup> reported results obtained by deep sequencing of serum miRNAs in patients with diabetes, lung and colorectal cancer and healthy individuals. This study revealed that serum from patients had distinct patterns of disease-specific miRNAs that were absent in the healthy controls and suggested that several diseases may leave specific miRNA-fingerprints in the blood of patients. Recently, more than 20 studies<sup>[116]</sup> have explored the usefulness of circulating miRNAs for detecting GC. Examples of key studies are given in Table 4. Most of these studies were focused on candidate miRNAs that were selected from previous analysis of GC tissues, while others used a hypothesis-free approach, where miRNA profiling is performed in a discovery sample set using high throughput techniques such as TaqMan arrays, microarrays or deep sequencing, and the diagnostic value of the selected candidate miRNAs is then determined using qRT-PCR in an independent validation set.

Tsujiura *et al.*<sup>[117]</sup> for the first time demonstrated the usefulness of circulating miRNAs for diagnosing and monitoring GC. The levels of five GC-associated miRNAs (miR-17-5p, miR-21, miR-106a, miR-106b and let-7a) were studied in plasma from GC patients and the results showed that the former four miRNAs were present at significantly higher levels while let-7a was decreased in the plasma from GC patients compared to the controls, and the miR-106a/let-7a ratio could distinguish between patients and controls with an AUC of 0.879. Although the authors found relatively good correlation between the miRNA expression levels in the blood and tumor tissue, several subsequent studies showed that only a subset of miRNAs that are highly expressed in tumors show elevated levels in serum or plasma, while other miRNA species are selectively released or retained by the cell<sup>[118]</sup>. The same group then compared miRNA profiles in pre- and post-operative plasma samples from GC patients using microarray analysis and identified a list of miRNAs that were markedly decreased in post-operative plasma and therefore are likely to be associated with the presence of cancer<sup>[119]</sup>. Two candidate miRNAs, miR-451 and miR-486, were tested in a cohort of 56 GC patients and 30 healthy controls, and the ROC curve analyses

Table 4 Cell-free RNAs as biomarkers for detection of gastric cancer

Candidate biomarkers	Sample size and type	Method/technology	Diagnostic value/outcome	Ref.
Circulating cell-free miRNAs				
miR-106a/let-7a ratio	GC = 69, HC = 30; plasma	qRT-PCR	AUC = 0.879, Sn = 85.5%, Sp = 80%	Tsujiura <i>et al</i> <sup>[117]</sup> , 2010
5-miRNA signature: miR-1, miR-20a, miR-27a, miR-34, miR-423-5p, miR-451	Discovery: GC = 20, HC = 20; Validation: GC = 142, HC = 105; Serum	Discovery: Solexa sequencing; Testing: qRT-PCR	AUC = 0.831 (validation set)	Liu <i>et al</i> <sup>[155]</sup> , 2011
miR-486	Discovery: pre- and post-operative plasma, GC = 3; Validation: GC = 56, HC = 30	Discovery: microarray; Testing: qRT-PCR	AUC = 0.96, Sn = 96%, Sp = 100%; decreased in 90% of post-operation plasma samples AUC = 0.92, Sn = 86%, Sp = 97%; decreased in 93% of post-operation plasma samples	Konishi <i>et al</i> <sup>[119]</sup> , 2012
miR-378	Discovery: GC = 7, CRC = 7, HC = 10; Validation: GC = 40, HC = 41; serum	Discovery: microarray; Testing: qRT-PCR	AUC = 0.861, Sn = 87.5%, Sp = 70.73%; No significant differences across stages I-IV	Liu <i>et al</i> <sup>[122]</sup> , 2012
miR-223	Test set: GC = 10, HC = 10;	qRT-PCR	AUC = 0.9089	Li <i>et al</i> <sup>[121]</sup> , 2012
miR-21	Validation: GC = 60, HC = 60;		AUC = 0.7944	
miR-218	plasma		AUC = 0.7432	
3 miRNA combined			AUC = 0.9531, Sn = 84.29%, Sp = 92.86% No significant differences across stages I-IV	
3-miRNA signature: miR-221, miR-744, and miR-376c	Discovery: GC = 14, HC = 14; Validation I : GC = 68, HC = 68; Validation II : DYS = 46, HC = 46	Discovery: TaqMan array, validation: qRT-PCR	Sn = 82.4%, Sp = 58.8% (for GC vs HC) Sn = 73.3% (for early GC) miR-221 elevated in DYS, no difference from HC for miR-376c and miR-744; Increase during GC development; Sn = 79.3% (for GC 2-5 years before diagnosis)	Song <i>et al</i> <sup>[124]</sup> , 2012
miR-106b	Discovery: GC = 30, HC = 30	qRT-PCR	AUC = 0.773 (all in validation set)	Cai <i>et al</i> <sup>[156]</sup> , 2013
miR-20a	Validation: GC = 60, HC = 60;		AUC = 0.859	
miR-221	plasma		AUC = 0.796	
miR-223	GC = 50, HC = 47; serum	qRT-PCR	AUC = 0.85, Sn = 81%, Sp = 78%; Increased in advanced stages AUC = 0.90, Sn = 79%, Sp = 78% AUC = 0.71, Sn = 71%, Sp = 58% Increased in advanced stages	Wang <i>et al</i> <sup>[157]</sup> , 2014
miR-16			AUC = 0.768 (all in validation set)	
miR-100			AUC = 0.694	
miR-16	Discovery: stage I non-cardia GC = 40, HC = HC;	Discovery: TaqMan array, validation: qRT-PCR	AUC = 0.732	Zhu <i>et al</i> <sup>[120]</sup> , 2014
miR-25	Validation: stage I non-cardia GC = 48, HC = 102	PCR	AUC = 0.790	
miR-92a			AUC = 0.779	
miR-451			AUC = 0.812, Sn = 72.9%, Sp = 89.2%; In vitro evidence that miR-16, miR-25 and miR92a but not miR-451 and miR486-5p are secreted from cancer cells	
miR-486-5p				
5 miRNA combined				
miR-222	GC = 114, HC = 56; plasma	qRT-PCR	AUC = 0.850, Sn = 66.1%, Sp = 88.3%	Fu <i>et al</i> <sup>[158]</sup> , 2014
miR-18a	GC = 82, HC = 65, plasma	qRT-PCR	AUC = 0.907, Sn = 80.5%, Sp = 84.6%; no association with stage	Su <i>et al</i> <sup>[123]</sup> , 2014
miR-18a	GC = 104, HC = 65, plasma and GC tissues	qRT-PCR	AUC = 0.8059, Sn = 84.6%, Sp = 69.2% Overexpressed in GC; in vitro evidence that miR-18a is released by cancer cells; decreased in postoperative plasma	Tsujiura <i>et al</i> <sup>[159]</sup> , 2015
Circulating cell-free mRNAs and long non-coding RNAs				
hTERT mRNA	GC = 118, CAG = 40, HC = 58; plasma	qRT-PCR	AUC = 0.891, Sn = 66%, Sp = 87%; strong positive correlation with advanced stage of GC	Kang <i>et al</i> <sup>[125]</sup> , 2013
MACC1 mRNA	GC = 76, HC = 54, plasma	qRT-PCR	Sn = 68%, Sp = 89%	Burock <i>et al</i> <sup>[160]</sup> , 2015
LINC00152	Pre- and post-operative plasma GC = 79, GED = 31, HC = 81	qRT-PCR	AUC = 0.657, Sn = 48.1%, Sp = 85.2%	Li <i>et al</i> <sup>[131]</sup> , 2015

AUC: Area under the curve; DYS: Intestinal dysplasia; GC: Gastric cancer; HC: Healthy controls; Sn: Sensitivity; Sp: Specificity.

showed an AUC of 0.96 and 0.92, respectively, thus demonstrating their relevance for diagnosing GC and monitoring the course of the disease. However, Zhu *et al*<sup>[120]</sup> found that these two miRNAs had a lower diagnostic performance (AUC of 0.790 and 0.779, respectively) for detecting early stage non-cardia GC.

Surprisingly, both miRNAs were downregulated in GC tissues compared with adjacent normal tissues<sup>[119]</sup>, and their cellular source and the mechanism of release into the circulation remains unknown<sup>[120]</sup>.

Subsequent studies have resulted in the identification of several individual miRNAs or miRNA signatures

that show significant diagnostic values, with an AUC as high as 0.953<sup>[121]</sup>. Some of the studies report no significant differences in the miRNA levels across GC stages, thus suggesting that these miRNA biomarkers appear in patients' blood at an early stage of cancer development and could be suitable for the detection of early GC<sup>[121-123]</sup>. A retrospective study by Song *et al.*<sup>[124]</sup> demonstrated an increasing trend in expression of three serum miRNAs (miR-221, miR-744 and miR-376c) over a 15-year timeframe before GC diagnosis and showed that the 3-miRNA panel could classify serum samples collected 2-5 years before the clinical diagnosis of GC with 79.3% accuracy.

Several other studies have explored the possibility of using circulating mRNAs, long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) for the detection of GC. Despite the presence of RNases in human blood, all these RNA species turned out to be stable and robustly detectable in plasma or serum samples and some of them have shown a relatively high diagnostic value. For example, Kang *et al.*<sup>[125]</sup> reported that elevated *hTERT* mRNA levels could distinguish between GC and healthy controls, with an AUC of 0.891, sensitivity of 66% and specificity of 87%. lncRNAs and circRNAs are recently-discovered categories of non-coding RNAs that regulate gene expression at the transcriptional and posttranscriptional levels and accumulating evidence suggests that they may play key roles in the development of cancer<sup>[126,127]</sup>. Several recent studies reported that their expression is deregulated in GC tissues and some can be detected in patients' blood<sup>[128-132]</sup>, and thus, they may represent a novel source for circulating biomarker discovery. However, a deeper understanding in their biology, mode of action and mechanism of release into the circulation is required to evaluate their clinical significance.

However, there is a little overlap among the identified miRNAs in various studies and, with a few exceptions such as miR-223 or miR-18a, most of the results have not been reproduced by other studies to date. One of the main reasons for variability and inconsistency among the findings is the approach used to normalize qRT-PCR data. Currently, there is no consensus on housekeeping genes in serum or plasma that could be used as internal controls for this normalization. Several studies have used U6 snRNA or miR-16 as a normalization control, but other studies have shown large fluctuations in their levels in serum and plasma, and they concluded that these RNAs are not suitable as endogenous controls<sup>[133,134]</sup>. An alternative approach for controlling the technical variability is based on synthetic spike-ins. In this approach, miRNAs without a sequence homology to human miRNAs, such as cel-miR-39, are spiked into the serum/plasma samples before RNA extraction and amplified together with the target miRNAs. The target miRNA levels are then normalized to the sample volume and spike-ins, but this approach does not

control for the preanalytical variability. Hemolysis has been shown to alter miRNA content in plasma. For example, miR-16 and miR-451 have been shown to be released by red blood cells and their levels were proportional to the degree of hemolysis<sup>[135]</sup>. This suggests that assessing the degree of hemolysis is a crucial step in assays that quantify circulating RNA levels.

## OTHER POTENTIAL BIOMARKERS

### *Circulating tumor cells*

Detection of the presence of CTCs in the peripheral blood of cancer patients has a promising clinical value in the predictive and prognostic setting, but currently, it has a rather limited potential for detection of early stage cancer. Accumulating evidence shows variable overall GC detection rates based on CTC isolation and characterization of their mRNA expression (ranging from 9.6%-71.2%). Current results are summarized in recent review by Tsujiura *et al.*<sup>[105]</sup>. Studies have shown that the number of CTCs analyzed in peripheral blood from patients with metastatic gastrointestinal cancer is generally lower (1-2 CTCs/7.5 mL of blood) than that found in other malignancies, such as in patients with metastatic prostate cancer (3-5 CTCs/7.5 mL of blood) or breast cancer (6-7 CTCs/7.5 mL of blood)<sup>[136-138]</sup>. Although novel approaches for rare CTC detection in a small amount of peripheral blood are emerging, their sensitivity for early stage GC is still limited. For example Kolostova *et al.*<sup>[139]</sup> demonstrated that there are biologically inherent limitations to the CTC-based test application for GC detection.

To date, the CellSearch system (Veridex) is the first and only FDA approved test that has been shown to be useful for detecting CTCs in patients with metastatic breast, prostate or colorectal cancer. It enables the enumeration of CTCs of epithelial origin (CD45-, EpCAM+, and cytokeratins 8, 18+, and/or 19+) in whole blood. The usefulness of the CellSearch system in GC detection has recently been evaluated by Uenosono *et al.*<sup>[140]</sup>. The authors showed that the test could detect stage I and II GC patients in only in 1.6% (1/64) and 3.9% (1/26) of the cases, respectively ( $P = 0.0002$ ); however, the data indicated that CTC detection in peripheral blood may be a useful tool for predicting tumor progression, prognosis, and the effect of chemotherapy in patients with GC. Besides the CellSearch system, novel and more sensitive experimental approaches for rare CTC detection are being developed; however, the data on their sensitivity for early stage GC is still limited. For example, Kolostova *et al.*<sup>[139]</sup> used the MetaCell<sup>®</sup> approach, which is based on physical sorting and cultivation of isolated CTCs, to detect one out of three stage I GC and two out of four stage II GC cases.

Taken together, although numerous studies have been performed, the research on this type of "liquid biopsy" for GC detection remains in its infancy. Further

studies involving larger patient/control cohorts, a deeper understanding of CTC biology and significance and progress in techniques linked to CTC isolation and characterization could enhance their usefulness as biomarkers in future.

### **Cancer-derived extracellular vesicles**

Cancer-derived EVs are gaining increasing attention in the cancer biomarker field<sup>[141]</sup>. Currently, they are under intense investigation for their composition, biological functions and distribution, along with their diagnostic and therapeutic potential. Either secreted or shed from cancer cells, they are considered to be a liquid tumor biopsy because they are found in elevated levels in the circulation and they have been shown to carry cancer cell-derived lipids, proteins, mRNAs, non-coding and structural RNAs and even genomic DNA, which at least partially reflect parental cells and represents attractive shuttles for cancer biomarkers<sup>[142,143]</sup>. Studies from several groups have demonstrated the diagnostic potential of cancer-derived EV for the detection of various cancer types, including but not limited to melanoma, prostate, ovarian and colorectal cancer (reviewed by Zocco *et al.*<sup>[144]</sup>). However, there is little data on circulating GC EVs; to the best of our knowledge, only one study has been published regarding the analyses of circulating EVs in patients with stomach cancer. Baran *et al.*<sup>[145]</sup> attempted to characterize the EVs isolated from platelet-depleted plasma samples from 37 GC patients, compared to those from 10 healthy controls. They demonstrated that GC patients, compared with controls, have: (1) a significantly higher number of total circulating EVs (except for patients with stage I GC) ( $P < 0.001$ ); (2) EVs with significantly higher expression of GC-associated proteins MAGE-1 and Her-2/*neu*+ (only late stage patients analyzed,  $n = 13$ ;  $P < 0.05$ ); and (3) EVs with upregulated CRC6 and downregulated CXCR4 surface expression ( $P < 0.05$ ). However, they made no attempt to set a diagnostic value based on these findings. Considering the current advances in this field, further studies on EVs released in patients with GC are warranted.

## **CONCLUSION**

Over the last decade, considerable effort has been dedicated to discovering various types of cancer-associated molecules in the blood of GC patients. Several of the identified biomarkers have remarkably high sensitivity and specificity that greatly outperform the previously-known GC serum biomarkers such as PGs, CA 72-4, CA19-9 and CEA<sup>[146,147]</sup>, and therefore have the potential to complement or replace the existing endoscopy, X-ray or biopsy-based examinations. Each type of biomarker has a different origin, provides various types of information and has their own strengths and weaknesses, thus suggesting different clinical applications. For example, autoantibodies

against TAAs are qualitative and highly specific markers for the presence of cancer, and they have been identified in the circulation several years before the clinical manifestation of the cancer. Autoantibodies against TAAs, therefore, seem to be an excellent biomarker for the detection of early-stage cancer. However, there is a subset of GC patients with no humoral immunity against tumor antigens that limits the use of autoantibody-based assays for population-based screening programs. Moreover, antibodies are relatively stable and they may remain in the circulation for several months, even years; therefore, they likely have limited potential for monitoring the disease. However, detection of cancer-specific genetic or epigenetic alterations in the cfNA would provide an excellent tool for monitoring cancer dynamics, while their diagnostic use is limited to those patients who have the respective alterations. In addition, these assays may fail to detect evolving cancer cell clones that have lost the respective marker. Several of the proteomics-based biomarker models have demonstrated high sensitivity and specificity for detecting GC; however, it is not clear if most of these proteins are directly and causally involved in the development of cancer and therefore further mechanistic studies are required to validate them as cancer-associated biomarkers.

We suggest that new bio-fluid testing systems, which will combine various types of biomarkers, will be developed in the future and will allow collection of all the information on the disease status, genetic make-up of the tumor and the status of patients' immune system using a single blood test. However, there are several technical issues that have to be resolved before such a device could meet the regulatory requirements. Thus, the next goal would be to perform a head-to-head comparison of various biomarker models and technological platforms in large, well-characterized cohorts of patients and controls to select the biomarkers with highest clinical relevance. This would require a collaborative effort among the research groups to establish standardized pre-analytical and analytical procedures and guidelines for reporting the results.

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**P- Reviewer:** Huang KH, Smith SM **S- Editor:** Yu J

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