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CASE REPORT

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Editorial board member of *World Journal of Gastroenterology*, Gülsüm Özlem Elpek, MD, Professor, Department of Pathology, School of Medicine, Akdeniz University, Antalya 07070, Turkey

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Recent advances in gastric cancer early diagnosis

Laura Necula, Lilia Matei, Denisa Dragu, Ana I Neagu, Cristina Mambet, Saviana Nedeianu, Coralia Bleotu, Carmen C Diaconu, Mihaela Chivu-Economescu

ORCID number: Laura G Necula (0000-0001-6531-7939); Lilia Matei (0000-0003-4479-655X); Denisa L Dragu (0000-0002-5868-2636); Ana I Neagu (0000-0001-6126-8973); Cristina Mambet (0000-0001-8495-7704); Saviana Nedeianu (0000-0001-8751-524X); Coralia Bleotu (0000-0002-9031-338X); Carmen C Diaconu (0000-0002-2259-1425); Mihaela Chivu-Economescu (0000-0001-7512-4700).

Laura Necula, Lilia Matei, Denisa Dragu, Ana I Neagu, Cristina Mambet, Saviana Nedeianu, Coralia Bleotu, Carmen C Diaconu, Mihaela Chivu-Economescu, Department of Cellular and Molecular Pathology, Stefan S. Nicolau Institute of Virology, Bucharest 030304, Romania

Laura Necula, Faculty of Medicine, Titu Maiorescu University, Bucharest 040441, Romania

Corresponding author: Mihaela Chivu-Economescu, PhD, Research Assistant Professor, Research Scientist, Senior Researcher, Department of Cellular and Molecular Pathology, Stefan S. Nicolau Institute of Virology, 285 Mihai Bravu Ave, Bucharest 030304, Romania. mihaela.chivu@gmail.com
Telephone: +40-21-3242590
Fax: +40-21-3242590

Abstract

Gastric cancer (GC) remains an important cause of cancer death worldwide with a high mortality rate due to the fact that the majority of GC cases are diagnosed at an advanced stage when the prognosis is poor and the treatment options are limited. Unfortunately, the existing circulating biomarkers for GC diagnosis and prognosis display low sensitivity and specificity and the GC diagnosis is based only on the invasive procedures such as upper digestive endoscopy. There is a huge need for less invasive or non-invasive tests but also highly specific biomarkers in case of GC. Body fluids such as peripheral blood, urine or saliva, stomach wash/gastric juice could be a source of specific biomarkers, providing important data for screening and diagnosis in GC. This review summarized the recently discovered circulating molecules such as microRNAs, long non-coding RNAs, circular RNAs, which hold the promise to develop new strategies for early diagnosis of GC.

Key words: Biomarkers; Gastric cancer; Early diagnosis; Genetic and epigenetic alterations; Circulating molecules

Core tip: Despite the fact that in the last decades, gastric cancer (GC) has shown a decreasing incidence, the five-year survival rate continues to remain poor mainly because most patients are asymptomatic until the disease progresses to advanced stages. Recent progress in molecular landscape of GC and improved detection methods may facilitate screening and diagnosis of GC in early stages. Numerous studies aim to identify specific non-invasive biomarkers from alternative sources such as peripheral blood, urine or saliva, stomach wash/gastric juice. This review summarized the recently

discovered circulating molecules which hold the promise to develop new strategies for early diagnosis of GC.

INTRODUCTION

Gastric cancer (GC) remains a challenge for oncology domain being the fifth most frequently diagnosed cancer (1033701 new cases in 2018) and the third leading cause of cancer death (782685 deaths) of all malignancies worldwide[1]. Although over the last decades GC has shown a decreasing incidence, the five-year survival rate continues to be poor, being estimated at 10% for patients with advanced GC. In the developed countries, like Japan, where early diagnosis of GC reaches 50%, the five-year survival rate attains 90%[2].

Currently, the most frequent tumor markers used in the clinic for early detection of GC comprise carcinoembryonic antigen (CEA), the carbohydrate antigens (CA) - CA19-9, CA72-4, CA125, CA24-2, CA50, and also pepsinogen and α-fetoprotein (AFP)[3]. However, the specificity and sensitivity of these serum biomarkers are poor and so far, none of them is unique for GC diagnosis[3,4]. Thereby, the development of improved detection method to diagnose CG in early stages is crucial, especially knowing that most patients are asymptomatic until the disease progresses to advanced stages. Moreover, GC is a complex, heterogeneous disease, involving multiple genetic and epigenetic alterations[5].

Recently, the use of high throughput technologies has brought new insights into the molecular pathogenesis, resulting in a new molecular classification of gastric adenocarcinoma into four subtypes, based on their genomic features. According to The Cancer Genome Atlas (TCGA), GCs are divided in Epstein-Barr virus (EBV)-infected tumors, microsatellite instability tumors (MSI), genomically stable tumors (GS), and chromosomally unstable tumors (CIN)[6]. The Asian Cancer Research Group (ACRG) categories GC into MSI tumors and Microsatellite Stable (MSS) tumors with either epithelial-to-mesenchymal transition (MSS/EMT), TP53 activity (MSS/TP53+), or TP53 inactivity (MSS/TP53-)[7,8]. This new classification opened the way for several clinical trials that are trying to define new therapeutic regimens combining immune checkpoint inhibitors with molecular targeted therapies, with promising results[9]. However, early diagnosis remains mandatory, and studies aiming to identify new biomarkers or genetic signatures are imperative.

Genetic alterations, including large chromosomal gain or loss, single nucleotide variations, and mutations, as well as epigenetic alterations, like aberrant DNA methylation, histone modification, microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) overexpression or down-regulation, were described as major aspects implicated in GC initiation and progression[10].

A better understanding of the molecular factors involved in gastric carcinogenesis can lead to the identification of novel biomarkers for early GC diagnosis or markers use for prognosis and for monitoring therapy response. This review aims to discuss the most important types of molecules secreted from the tumor tissues to the body fluids, which candidates as circulating biomarkers for early diagnosis of GC (Figure 1).

CIRCULATING PROTEOMIC BIOMARKERS IN EARLY GC

Although several circulating tumor-associated antigens have entered routine clinical practice for a long time their utility in early detection of GC remains elusive, due to the high incidence of false-positive and false-negative results[11,12]. CEA, CA19-9, and CA72-4 are the most frequently used conventional tumor markers in GC diagnosis, prognosis, therapeutic monitoring and detection of recurrences[13]. At diagnosis, both CEA and CA 19-9 levels can provide useful prognostic information regarding the depth of tumor invasion and the presence of metastases[14,15]. However, they do not
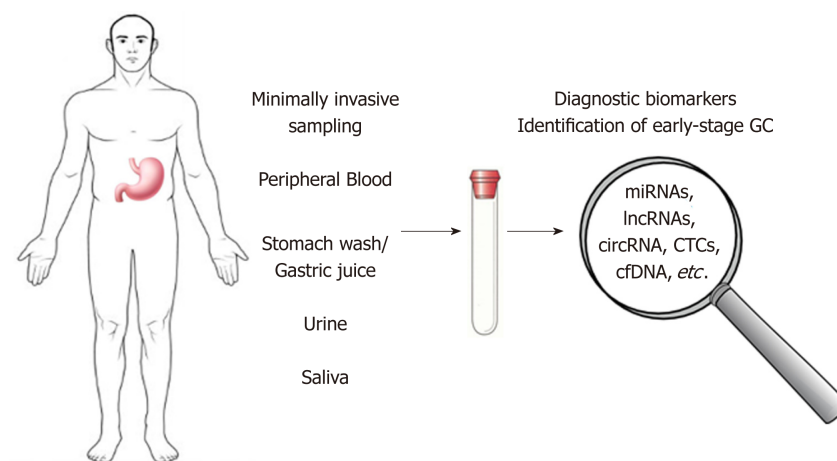
Figure 1 Possible non-invasive diagnostic biomarkers for early-stage gastric cancer. Genetic and epigenetic alterations, microRNAs, long non-coding RNAs and circular RNA, circulating tumor cells and tumor DNA represent promising candidates for the development of new non-invasive methods in early-diagnosis of gastric cancer. GC: Gastric cancer; miRNAs: MicroRNAs; lncRNAs: Long non-coding RNAs; circRNA: circular RNA; CTCs: Circulating tumor cells; cfDNAs: Cell-free circulating DNA.

represent effective tools for GC screening and early diagnosis as they do not display enough sensitivity and specificity under these circumstances[16,17]. CA72-4 was shown to exhibit higher sensitivity and accuracy than CEA, yet there are only few studies that investigated its relevance in GC screening[18]. Other tumor markers, such as AFP and CA125 proved to have very low positivity rates in early GC[19]. Also, CA50 is of limited diagnostic value[20].

To increase the diagnostic performance for GC different combinations of serological tumor markers were employed. In this respect, it was shown that by combining CEA, CA19-9, and CA72-4 with thymidine kinase 1 (TK1) - a biomarker of cell proliferation - a significant increase in sensitivity and specificity of GC detection was obtained, compared to the isolated use of the biomarkers[21]. Recently, a diagnostic model including the serum levels of CEA, CA72-4 and of three inflammatory cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8] was proposed for early GC detection. In the validation study, this model provided good discrimination between healthy controls, atypical hyperplasia of gastric mucosa, early-stage GC and advanced-stage GC groups[22].

Concerning the use of stomach-specific biomarkers, measurement of serum pepsinogens (PGs) is the most common non-invasive method employed for GC detection, although it identifies individuals with gastric precancerous lesions, rather than GC itself[23]. Thus, low levels of pepsinogen I (PGI) and a low pepsinogen-I to pepsinogen ratio (PGI/PGII) exhibit a good correlation with atrophic changes in the gastric corpus, while their accuracy for GC detection is low[24]. Additionally, gastrin-17 (G-17) was proposed as an indicator antral atrophy[25]. As shown recently, a biomarker panel comprising PGI, PGII, PGI/PGII, G-17 and IgG antibodies to *Helicobacter pylori* (*H. pylori*) represents a promising non-invasive tool to stratify individuals at high risk for GC development[26]. Also, serum levels of trefoil factor 3 (TFF3), a protein ectopically expressed in intestinal metaplasia of the stomach[27], was found to display better performance in GC detection than PGs[25], and the combination of TFF3 with PGs demonstrated even higher sensitivity for early GC[28].

Other potential circulating biomarkers for detecting early-stage GC include M2-pyruvate kinase, a tumor-associated metabolic marker; the adipocytokine leptin as an independent biomarker of intestinal metaplasia; p-53 autoantibody; the cell-cycle-related protein RegIV; the inflammatory signaling molecules olfactomedin 4 and vascular adhesion protein-1 (VAP-1)[29].

A different approach to identify early GC biomarkers has involved mass spectrometry for analyzing serological glycomic profiles in GC patients and non-cancer controls. Significant differences in serum N-glycans were observed between the two groups. Moreover, the decreased core fucose was validated as a potential biomarker for distinguishing early-stage GC patients from healthy controls[30].

ONCOGENES/TUMOR SUPPRESSORS IN GC

The development of state-of-the-art techniques holds the promise of new molecular markers identification that are able to diagnose early, predict the disease outcome and help access the appropriate therapy. Numerous studies showed an increased level of expression of oncogenes in GC. They stimulate tumor cell growth and cell cycle and inhibit apoptosis.

Recent studies identified several genes whose elevated expression level proved to be associated with GC and might be useful in early detection, such as *xpg*, interferon-induced transmembrane protein 1 (*iftim1*), matrix metalloproteinase-9 (*mmp-9*), pituitary tumor-transforming gene-1 (*pttg1*), *stc1*[31].

XPG/ERCC5 (13q33) Xeroderma pigmentosum group G/excision repair cross-complementing group 5, an enzyme from NER (nucleotide excision repair) system, is involved in repairing of DNA lesions caused by genomic instability. The gene expression level of *ercc5* was found to be significantly higher in GC compared to gastritis, and it was associated with tumor development and progression[32].

By microarray profiling methods, *ifitm1* was identified as a gene upregulated in tumor cell lines and GC tissues. Moreover, important differences in expression level in intestinal vs diffuse type of GC were observed. Although the role of this gene in tumorigenesis is not clearly understood, *ifitm1* raised expression was implicated in invasion and migration of GC cells[33] and was also related to increased inflammatory responses that may play a part in tumor progression.

MMP-9 is an enzyme that contributes to the degradation of the extracellular matrix, having a well-known role in tumor growth, invasion and metastasis in gastric carcinoma[34]. A study that evaluates both serum and tissue expression level of MMP-9, found out a correlation between serum concentration of MMP-9 before surgery and TNM staging. Although the *mmp-9* expression level in gastric tumor was higher compared to healthy tissue, and positively associated with depth of invasion, it did not correlate significantly with MMP-9 serum level[35].

A recently discovered proto-oncogene, *pttg1* can affect tumorigenesis, invasion, and metastasis of many cancer types. The expression of *pttg1* is upregulated in gastric tumor tissue compared to gastric intraepithelial neoplasia and normal mucosa (both mRNA and protein level) and it is an independent factor for survival. PTTG1 might represent a potential diagnostic marker and a therapeutic target[36].

STC1 and STC2, members of STC (stanniocalcin) family, were highly expressed in numerous cancer types. In GC, both STC1 and STC2 expression is upregulated, STC1 being significantly associated with tumor staging, metastasis, and progression-free survival. Serum level of STC1 was significantly elevated in preoperative cancer patients compared to benign gastric cases and decreased 7-10 d after surgery[37]. Arigami *et al*[38] reported a significantly higher number of *stc1* mRNA copies in the blood of GC patients *vs*. normal controls that correlates with tumor invasion and staging and has a greater sensitivity than CA 19-9 and CEA. These studies suggest the utility of serum STC1 as diagnosis and prognosis marker in GC.

Using gene microarray, our group also identified a panel of overexpressed genes associated with tumor progression: KRT17, COL10A1, KIAA1199, SPP1, IL11, S100A2, and MMP3. From these, COL10A1, KRT17, and SALL4 candidate as biomarkers for early detection having an increased expression in the early stages of gastric tumorigenesis[39]. COL10A1 was found elevated in serum of patients with colorectal cancer[40], proven to be a worthy circulating biomarker for early diagnosis. KRT17 was also demonstrated to be involved in tumor growth, motility, and invasion by *in vitro* and *in vivo* studies on gastric tumorigenesis[41].

Tumor suppressor genes can present loss of expression in GC patient samples that result in accelerated cell growth, the progression of the cell cycle, and decreased inhibition of the oncogene expression. These alterations were also studied in order to discover new diagnostic molecular markers for the early detection and progression of GC[31].

Using a gene microarray analysis, one study identified transmembrane protein with EGF like and two follistatin-like domains 2 (*tmeff2*) as a gene with significantly decreased expression in GC tissues, negatively correlated with the advanced cancer stage, large tumor size, and poor prognosis. The authors showed that the increase of *tmeff2* expression decrease cell proliferation by increasing apoptosis and by blocking the cell cycle in GC cells[42]. Moreover, modification of *tmeff2* expression in GC seems to be associated with *H. pylori* infection *via* STAT3 activation[43].

An interesting possible biomarker is gastrokine 1 (GKN1), a small protein significantly expressed in the surface lumen epithelial cell layer of gastric tissue, being involved in the maintenance of mucosal integrity and secreted into the stomach, but absent in GC[44]. It was also detected that GKN1 acts as a tumor suppressor and a modulator of apoptotic signals in GC, its lower expression might be considered an indicator of increased risk of gastric carcinogenesis[45].

Another study suggested the opportunity of detecting GC using the gene

expression profile of the blood. In this study, a four-gene panel discriminated GC with an accuracy of 95%, sensitivity of 92% and specificity of 96%. This four-gene panel for detection of GC includes two overexpressed genes: purine-rich element binding protein B (*purb*) and structural maintenance of chromosomes 1A (*smc1l1*), and two underexpressed genes: DENN/MADD domain containing 1B (*dennd1b*) and programmed cell death 4 (*pdcd4*)[46].

Next-generation deep sequencing was used to evaluate mutations of *tp53* in tumor biopsies, plasma and stomach fluids (gastric wash) obtained from GC patients. The results showed that *tp53* mutations were identified in 15/46 biopsies (32.6%), 7/46 gastric wash - (15.2%) and 6/46 plasma samples (13%). The authors suggested that gastric wash could be useful to detect DNA alterations using a comprehensive gene-panel designed for GC diagnosis[47].

METHYLATION PATTERN OF GC

In GC, epigenetic alteration by methylation occurs in specific genes involved in various processes such as cell cycle regulation (*p16nk4a, tcf4*), DNA repair (*hmlh1* and *mgmt*), cell growth/differentiation (*hoxd10, hai-2/spint2, ndrg2*), transcriptional regulation (*hltf, pax6, znf545, runx3*), cell adhesion/invasion/migration (*cdh1, cdh4, apc, flnc, lox, timp3, tsp1*), apoptosis (*bnip3, xiap, bnip3, bcl2, cacna2d3, dapk, gpx3, pcdh10, pcdh17, casp8, xaf1*), angiogenesis (*thbs-1* and *p73*), STAT pathway (*socs-1*), Ras pathway (*rassf1a, rassf2, hdab2ip, rkip*), Wnt pathway (*dkk-3, ctnnb1*), as well as in multidrug resistance genes (*mdr1, gstp1*)[48,49] and in genes associated with Epstein-Barr virus-type tumors (*pycard, bmpr1a,* and *pgr*) or *H. pylori* positive tumors (*brinp1, epha5, fli1,* and *sez6l*)[50]. The correlation of these biomarkers with tumor size, localization, differentiation, invasion, lymph node metastasis, distant metastasis, TNM stage, and prognosis is presented in Figure 2.

It was demonstrated previously that, in the case of gastric tumors, aberrant DNA methylation occurs more frequently than mutations[51], making DNA methylation a more specific assay in detection of such disease. Therefore, researchers started looking for an easier and less invasive method for the collection of cells and detection of DNA originated from gastric tumors. In serum/plasma DNA obtained from GC patient was observed a significantly higher methylation level of some biomarkers, such as *p16, cdh1, mgmt, rarb,* and *rnf180*[52].

Previously it was considered that DNA is denatured by stomach acidity[53], later on, it was demonstrated that this process is true in case of normal cells, but incorrect in case of DNA from tumor cells[45]. Collection of samples from stomach wash during endoscopy demonstrated that cancer cells from mucosal layers are easier exfoliated than normal cells into gastric juice and also that DNA isolated from such tumor cells is less degraded due to acidity[45] making it easy to be studied, offering a sensitive and quantitative method of detection.

Several genes were found to be methylated with higher frequency in gastric neoplasia versus normal condition and therefore were analyzed as possible biomarkers. Among them six methylated genes were most specific and sensitive for GC: *adam23, mint25, gdnf, prdm5, mlf1* and *rora*. The results have shown that the combination of the markers *mint25 + adam23 + gdnf* achieved a high sensitivity (95%) and specificity (92%). It was found that the methylation process is gene- and tumor stage-dependent during gastric carcinogenesis, some genes are highly methylated during dysplasia and early cancer phase compared with normal, but show lower methylation in advanced GC, similar with mechanism observed in ulcerative colitis-associated colon neoplasia[45].

But increased methylation process could have other causes as well, such as chronic inflammation of gastric mucosae, especially by *H. pylori* infection and aging. In order to test the effect of inflammation on methylation, the BarH-like 2 homeobox protein (*barhl2*) gene was chosen since is an *H. pylori*-independent biomarker. The *barhl2* methylation analysis of exosomal DNA (exoDNA) derived from gastric juice proven that the process is not influenced by atrophy of the gastric mucosa or *H. pylori* infection and could be used as a biomarker for detection of both early and advanced GC[54].

MIRNAS AS DIAGNOSTIC BIOMARKERS FOR GC

MiRNAs represent a class of small non-coding RNAs (19-25 nucleotides) involved by epigenetic mechanisms in many cellular processes, such as differentiation, proliferation, and apoptosis. These molecules, that seem to present specific expression
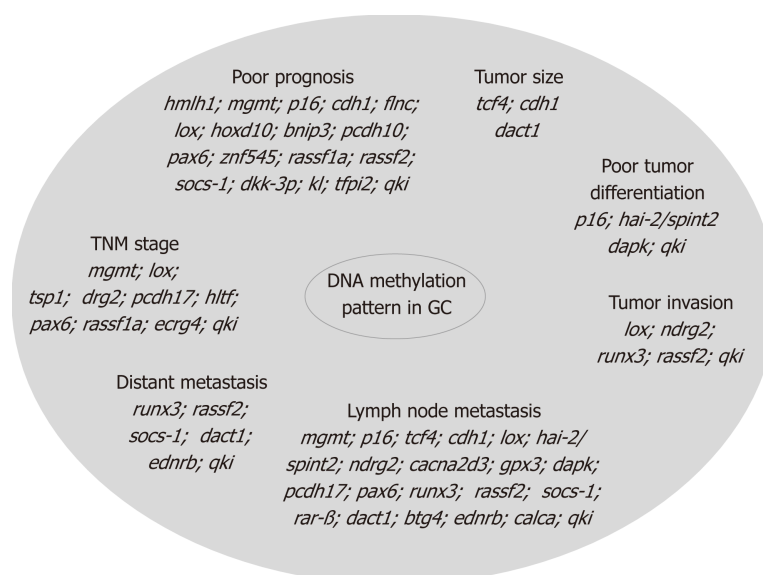
Figure 2 Methylation changes in gastric cancer. Epigenetic alteration by methylation occurs in specific genes involved in various processes such as cell cycle regulation, DNA repair, cell growth/differentiation, transcriptional regulation, cell adhesion/invasion/migration, apoptosis, angiogenesis, as well as in multidrug resistance genes, and in genes associated with Epstein-Barr virus-type tumors or *Helicobacter pylori* positive tumors. These gene alterations are correlated with tumor size, localization, differentiation, invasion, lymph node metastasis, distant metastasis, TNM stage, and prognosis. GC: Gastric cancer.

signatures in normal and tumor gastric tissue, can act as oncogenes and/or tumor suppressors depending on the role of the target mRNA/gene[55].

More studies suggested that miRNAs could be considered important potential biomarkers for gastric pathology as they are frequently found to be deregulated in gastric tissue in *H. pylori* infection, chronic gastritis, preneoplastic conditions such as atrophic gastritis and intestinal metaplasia, and also in early dysplasia and invasive cancer. Moreover, modifications of miRNA blood levels were also identified in GC patients supporting the development of new diagnostic and prognostic methods based on miRNA expression analysis[56].

A promising result was obtained by a study in which miRNA-21 levels, in serum and peripheral blood mononuclear cells, were found to be increased in GC patients with a positive prediction rate around 90%, while those of CA199 and CEA were around 50%. Moreover, circulating miR-21 levels can discriminate between stage I and stage IV of GC[57].

miR-376c was found to be up-regulated in tissue, plasma, and urine of GC patients, even from the early stage of the tumor. The increased expression of miR-376c was associated with the proliferation, migration and anchorage-independent growth of cancer cells, having as a direct target *arid4a* gene which is considerably down-regulated in tumor tissue[58].

Increased pre-operative circulating miR-196a and miR-196b levels were identified in GC patients compared to healthy controls, the expression level of these miRNAs being reduced after the surgical resection of the gastric tumor. Interestingly, higher circulating miR-196a/b levels were correlated with the metastatic potential of the tumor, advanced stages, and poorer survival. Moreover, the results of this study suggested that circulating miR-196a, miR-196b, and combined miR-196a and miR-196b can distinguish between GC patients and healthy controls with higher sensitivity and specificity compared to the CEA or CA19-9[59]. Another recent study analyzed circulating miRNA levels in GC patients and identified a four-miRNA panel (miR-501-3p, miR-143-3p, miR-451a, miR-146a) as possible noninvasive biomarkers for prediction and prognosis of lymph node metastasis (LNM). In addition, LNM patients with decreased levels of miR-451a and miR-146a presented worse overall survival[60]. A five-miRNA panel (miR-16, miR-25, miR-92a, miR-451, and miR-486-5p) was found to be differentially expressed in plasma of gastric non-cardia adenocarcinoma patients compared to healthy controls. This panel seems to be able to discriminate between early-stage of gastric non-cardia adenocarcinoma patients and cancer-free subjects[61]. Other panels containing up-regulated miRNAs (miR-200a-3p, miR-296-5p, miR-132-3p, miR-485-3p, and miR-22-5p)[62] and (miR10b-5p, miR132-3p, miR185-5p, miR195-5p, miR-20a3p, and miR296-5p)[63] were identified in serum of the GC patients compared to healthy controls. Based on the evidence that exosomes secreted by cancer

and normal cells can be released into the circulatory system, a recent study identified overexpression of circulating exosomal miR-19b and miR-106a in GC patients compared to healthy controls. These increased levels were correlated with lymphatic metastasis and advanced stages of GC[64].

miR-146, miR-375, and Let-7 were found to be downregulated while miR-19 and miR-21 presented an increased expression in plasma of the GC patients with *H. pylori* infection. The study also identified overexpression of the genes involved in IRAK4 signaling and a decreased expression of *pten* gene in the GC patients with *H. pylori* infection compared to the control group, suggesting the potential of these molecules as biomarkers for early diagnosis of GC[65]. There are also other molecular potential biomarkers for screening GC identified in gastric juice: miR-421, miR-21, miR-106a and miR-129[66].

Table 1 summarized several miRNAs presenting modified circulating expression in GC patients compared to healthy controls.

Even if these results need to be validated by independent groups or cohorts in prospective studies, circulating miRNAs could be considered a class of novel, non-invasive diagnostic biomarkers with sufficient diagnostic accuracy in detecting the early-stage GC.

LNCRNAS IN GC

LncRNAs are transcripts longer than 200 nucleotides with no or limited protein-coding potential. lncRNAs are implicated in the regulation of several biological processes like transcription and translation, cellular differentiation, gene expression, cell cycle, *etc*[71]. They are characterized by high stability while circulating in body fluids and their level in tumor tissue correlates with plasma levels. As such, lncRNAs can be used to distinguish tumor patients at early stages from healthy people, as well as to predict the prognostic, metastasis risks and recurrence after surgery[72,73].

In 2013, Cao *et al*[74] investigated the lncRNA expression in GC and detected 88 differentially expressed lncRNAs, 71 upregulated and 17 downregulated. Zhou *et al*[75] hypothesized that GC-related lncRNAs might be released into the circulation during tumor initiation and could be utilized to detect and monitor GC.

Highly upregulated in liver cancer (HULC) is a lncRNA implicated in the growth and tumorigenesis of human GC. *In vitro* overexpression of HULC in gastric cell lines stimulates proliferation and invasion, inhibits cell apoptosis and can induce autophagy patterns, while its silencing reverses the EMT phenotype[76]. Evaluated in plasma, HULC level is higher in preoperative patients compared with healthy control subjects[77].

Another candidate as a possible biomarker for early detection and prognosis prediction of GC is lncRNA PVT1 since the levels of PVT1 in gastric juice from gastric patients were significantly higher than those from normal subjects[78].

Zhou *et al*[75] proposed H19 (imprinted maternally expressed transcript) as a potential biomarker for diagnosis of GC, especially for early tumor screening. It stimulates cell proliferation and inhibits apoptosis[79]. H19 plasma level is significantly higher in GC patients compared with normal controls[75,80-82] and allows the discrimination of early stage GC[75]. On the other side, H19 plasma levels were significantly lower in postoperative samples than in preoperative ones[75,80]. Also, patients with smaller tumor sizes (< 5 cm) exhibit higher H19 level in their circulation compared with those with larger tumors (≥ 5 cm)[81].

Another abnormally expressed lncRNA in GC is long intergenic non-protein-coding RNA 152 (LINC00152), its plasma level being significantly elevated in GC patients compared with healthy controls[83,84] and presenting higher levels in postoperative plasma samples compared with preoperative ones[83]. This lncRNA allows differentiating GC patients from ones with benign gastric diseases and can be also detected in gastric juice[84]. Another lncRNA that can be detected in gastric juice is AA174084 characterized by higher levels in GC patients compared with healthy or other non-GC subjects. Its plasma level drops markedly in GC patients on day 15 post-surgery and is associated with invasion and lymphatic metastasis[85].

Hox transcript antisense intergenic RNA (HOTAIR) has been suggested to be implicated in GC tumorigenesis and progression[86]. It promotes cell proliferation and inhibits apoptosis[79]. HOTAIR plasma level is significantly higher in GC patients compared with healthy controls. Moreover, increased HOTAIR expression was associated with advanced tumor stages, higher grades, and metastasis[86]. Other up-regulated lncRNAs are human urothelial carcinoma associated 1 (UCA1), which is implicated in GC carcinogenesis and presents higher levels in GC patients[87], and ABHD11-AS, whose levels in gastric juice is significantly higher in GC patients, being

Table 1 Dysregulated circulating microRNAs reported in gastric cancer patients

MicroRNA	Biological function	Type of biomarker	Origin of specimen	Sensitivity/specificity	Study
miR-21	Upregulated; discriminates between stage I and stage IV of GC	Diagnostic, prognostic	Serum, PBMC	88.4%/79.6% (serum) 81.3%/73.4% (PBMC)	Wu *et al*[57]
miR-196a/b	Upregulated; correlated with metastatic potential of the tumor, advanced stages, and poorer survival	Diagnostic, prognostic	Plasma	69.5%/97.6%	Tsai *et al*[59]
miR-200c	Upregulated; predictor of progression and survival.	Diagnostic, prognostic	Blood	65.4%/100%	Valladares-ayerbes *et al*[67]
miR-940	Downregulated	Diagnostic	Plasma	81.25 %/98.57 %	Liu *et al*[68]
miR-551b-5p	Upregulated	Diagnostic	Serum	77.5%/80.0%	Jiang *et al*[69]
miR-19b, miR-106a	Upregulated; correlated with lymphatic metastasis and advanced stages	Diagnostic, prognostic	Circulating exosomes	95%/90%	Wang *et al*[64]
miR-501-3p, miR-143-3p, miR-451a, miR-146a	Differentially expressed; prediction and prognosis of lymph node metastasis	Prognostic	Serum	87.78%/63.33%	Jiang *et al*[60]
miR-16, miR-25, miR-92a, miR-451, miR-486-5p	Differentially expressed; discriminate between early-stage of GC and cancer-free subjects	Diagnostic	Plasma	72.9%/89.2%	Zhu *et al*[61]
miR-200a-3p, miR-296-5p, miR-132-3p, miR-485-3p, miR-22-5p	Upregulated	Diagnostic	Serum	N/S	Wang *et al*[62]
miR10b-5p, miR132-3p, miR185-5p, miR195-5p, miR-20a3p, miR296-5p	Upregulated	Diagnostic	Serum	N/S	Huang *et al*[63]
miR-17-5p, miR-21, miR-106a, miR-106b, let-7a	Differentially expressed	Diagnostic	Plasma	85.5%/80.0%	Tsujiura *et al*[70]
miR-146, miR-375, let-7, miR-19, miR-21	Differentially expressed in GC patients with *H. Pylori* infection	Diagnostic	Plasma	N/S	Ranjbar *et al*[65]

miR-: MicroRNA; GC: Gastric cancer; PBMC: Peripheral blood mononuclear cell; *H. pylori*: *Helicobacter pylori*; N/S: Not specified.

also associated with clinicopathological factors[88].

Yang *et al*[89] investigated the diagnostic value of gastric cancer associated transcript 2 (GACAT2) in GC. In the evaluated cohort, the plasma GACAT2 levels in GC patients were significantly higher compared with healthy individuals, as well as in the preoperative group compared with the postoperative one. In addition, the individual relative changes of GACAT2 expression following surgery were significantly associated with lymphatic metastasis, distal metastasis, and perineural invasion.

Also, some lncRNAs panels were investigated for GC diagnosis. Zhang *et al*[90] identified a panel of five novel plasma lncRNAs (TINCR, CCAT2, AOC4P, BANCR, and LINC00857) using genome-wide lncRNA screening analysis which could distinguish GC patients from healthy controls and can help monitor tumor dynamics, tumor, depth of invasion, lymphatic metastasis and more advanced tumor stages. Also, Dong *et al*[91] identified a three-lncRNA signature, CUDR, LSINCT-5, and PTENP1, that allows distinguishing healthy controls from early GC patients.

However, to introduce lncRNAs as plasma biomarkers, further studies and improvements of extraction, quantification, probe enrichment, and evaluation methods should be performed.

CIRCRNAS, A NEW CLASS OF GC BIOMARKERS

CircRNAs are a new class of non-coding RNAs that form a closed loop, without 5' and

3' ends[92]. CircRNAs were first identified in RNA viruses, but later with the progress of new molecular techniques like high-throughput RNA sequencing and microarray analysis, circRNAs were found in all eukaryotic organisms as stable and conserved sequences that control gene expression through interactions with miRNAs[93]. New emerging data have confirmed that circRNAs are involved in the occurrence of many diseases, and also are strongly associated with tumor growth and metastasis[94]. These findings underline the potential of circRNAs to act as novel biomarkers and therapeutic targets for various human tumors.

Several recent studies have analyzed the aberrant expression of circRNA in GC compared with adjacent normal tissue and presented various lists with upregulated and downregulated circRNA[95-98] (Table 2). The study performed by Huang *et al*[96] identified circRNA0026 (hsa_circ_0000026) as having significantly downregulated expression 2.8fold change in GC. Sui *et al*[95] found six differentially expressed circRNA in GC tissue and managed to validate through qRT-PCR three of them (hsa_circRNA_400071, hsa_circRNA_000543, and hsa_circRNA_001959) as having a consistent expression with the differentially expressed gene. Through analysis of circRNA and mRNA differential expression profiles in GC tissues, the authors managed to identify the target mRNA and their respective genes for selected circRNAs, like *cd44, cxxc5, myh9, malat1* and other genes with important implications in GC tumorigenesis and development.

One important finding related to circRNA in cancer was that they are not easily degraded by RNase and thus, are stably expressed in human cells, in plasma or in gastric juice[104,109]. These findings opened the way for plasma circRNA profiling studies, aiming to identify specific diagnostic and prognostic circRNA for GC patients. Li *et al*[108] performed circRNA microarray for three GC samples and plasma, to assess the differences of circRNA expression profiles. They found that 3 and 14 circRNA were upregulated and downregulated respectively, both in patients' plasma and tumor tissue. Further, they analyzed through RT-droplet digital PCR (RT-ddPCR) the circRNA levels in plasma for 121 GC patients. Two circRNA: hsa_circ_0001017 (30.85-folds change) and hsa_circ_0061276 (121.54-folds change) were selected for their non-invasion diagnostic values. Results showed that patients with low level of hsa_circ_0001017 or hsa_circ_0061276 in plasma had shorter overall survival than those with high levels. Moreover, patients whose plasma levels of the two circRNA recovered to normal after the operation had longer disease-free survival.

Due to their documented correlation between tissue and plasma level, stability and presence as cell-free RNA in plasma, circRNA may be valuable blood-based biomarkers for GC screening, diagnosis, and prognosis.

CIRCULATING TUMOR CELLS

GC diagnosis relies mostly on invasive procedures, which are rather expensive and may have sometimes serious adverse events[110]. In spite of being documented 150 years ago[111], only last years proved that analyzing circulating tumor cells (CTCs) in liquid biopsies, a blood-based diagnostic approach, as a substitute for tissue biopsies have emerged as real-time cancer development monitoring tool and management strategy[112].

CTCs are a very rare and heterogeneous population of cells circulating in peripheral blood, originating from either primary or metastatic tumors that express the antigenic or genetic characteristics of the specific tumor type[113]. CTCs were first described as expressing epithelial cell markers EpCAM, cytokeratin 8, 18, and 19 (CK8, CK18, CK19), and are CD45 negative[114]. Recently, EMT with potential overexpression of mesenchymal markers and decreased expression of epithelial cell markers or mesenchymal-epithelial transition (MET) that present mesenchymal and epithelial markers, were shown to characterize subpopulations of these cells[115,116]. Mesenchymal phenotypes have larger plasticity thus facilitating migration, invasion, and drug resistance[117]. Several studies revealed the presence of CTCs in circulating tumor microemboli (CTM), indicating poor prognosis and influencing disease progression[118].

This high heterogeneity of CTCs prompted researchers to develop different methodologies to enrich, isolate and/or enumerate them based on specific phenotypic or molecular characteristics. Basically, there are two general types of methods used in CTCs enrichment/isolation: biological and physical methods. Their combination is more likely to improve the efficiency of CTC detection. CellSearch™ platform (Veridex LLC, Huntingdon Valley, PA, United States) the only procedure approved for the enumeration and isolation of CTCs by the Food and Drug Administration (FDA) for clinical use, detect the adhesion molecule EpCAM, CK8, CK18 and CK19

Table 2 Various aberrantly expressed circular RNAs with the potential to serve as diagnostic and prognostic biomarkers for gastric cancer

circRNA name	Biological function	Type of biomarker	Origin of specimen	Sensitivity/specificity	Study
hsa_circ_002059	Downregulated in GC; correlated with TNM stage and metastasis	Diagnostic	Tissues; plasma	81%/62%	Li *et al*[94]
hsa_circ_0000096	Downregulated in GC; affects GC cell growth and migration	Diagnostic	Tissues	N/S	Li *et al*[99]
hsa_circ_0058246	Upregulated in GC; associated with poor clinical outcomes	Prognostic	Tissues	N/S	Fang *et al*[100]
hsa_circ_0000745	Downregulated in GC; correlated with tumor differentiation and tumor nodal metastasis	Prognostic	Tissues	85.5%/45%	Huang *et al*[101]
hsa_circ_00000181	Downregulated in GC; associated with TNM stage and metastasis	Prognostic	Plasma	99%/85.2%	Zhao *et al*[102]
hsa_circ_0047905, has-circRNA7690-15, hsa_circ_0138960	Substantially upregulated in GC; act as tumor promoters in the pathogenesis of GC	Diagnostic	Tissues	N/S	Lai *et al*[103]
hsa_circ_0014717	Downregulated in GC; stably expressed in gastric juice; associated with TNM stage and metastasis	Prognostic	Tissues	59.38%/81.25%	Shao *et al*[104]
hsa_circ_0001895	Downregulated in both GC tissue and gastric precancerous lesions	Diagnostic	Tissues	67.8%/85.7%	Shao *et al*[105]
has_circ_0000520	Downregulated in GC; associated with TNM stage and in GC plasma linked with CEA expression	Diagnostic	Tissues; plasma	53.57%/85.71% (tissue) 82.35%/84.44% (plasma)	Sun *et al*[106]
hsa_circ_0000190	Downregulated in GC; associated with TNM stage and metastasis	Diagnostic	Tissues; plasma	71.2%/75%	Chen *et al*[107]
hsa_circ_0001017 hsa_circ_0061276	Downregulated in GC; associated with shorter overall survival	Prognostic	Tissues; plasma	95.5%/95.7%	Li *et al*[108]

GC: Gastric cancer; CEA: Carcinoembryonic antigen; circRNA: Circular RNA; N/S: Not specified.

and exclude CD45 cells but may overlook CTCs with predominantly mesenchymal phenotype. Using cell size - and phenotype-based systems, as centrifugal microfluidic system based on fluid-assisted separation technique (FAST), or Cascaded Inertial Focusing Microfluidic device, coupled with detection of an extended panel of markers might identify a different subpopulation of CTCs with higher efficiency[119,120].

Exploiting a frequent genetic abnormality reported in GC tumors, the aneuploidy of chromosome 8, Li *et al*[121] created an integrated subtraction enrichment (SET) and immunostaining-fluorescence in situ hybridization (iFISH) platform claimed to be more sensitive than the CellSearch™ to detect and characterize CTCs in advanced GC patients. Multiple studies showed that SET-iFISH method to enumerate CTCs with chromosome 8 aneuploidy is efficient in monitoring GC patient treatment response[113]. Expression of different other markers as vimentin, twist, MUC1, HER2, *etc*. proved to be very useful to evaluate therapeutic response and prognosis in patients with GC. However, irrespective of the detection method employed, there is weak evidence that detection of CTCs has the potential for early biomarker detection in GC but all data are consistent in supporting its utility in assessing the tumor heterogeneity, monitoring treatment responses and real-time cancer management[113].

CIRCULATING TUMOR DNA

Circulating tumor DNA (ctDNA) analysis refined the liquid biopsy to the level of identification of tumor molecular traces circulating in the body fluids and may give deeper insight on the cancer heterogeneity, early biomarker detection, therapeutic target detection, real-time evaluation of treatment response and possible resistance and prognosis. Originating from primary tumor cells, CTCs and/or distant metastasis, ctDNA give a broad cross-section of the disease offering information on methylation status, genetic alterations as mutations, amplifications, rearrangements, copy number variation (CNV), the latter being more difficult to analyze due to the short length and possibly unequal distribution of the ctDNA fragments[111].

Generally, ctDNA represents only a fraction of the cell-free circulating DNA (cfDNA), which is increased considerably in late-stage disease[122]. However, there is evidence that ctDNA can be detected in the plasma of cancer patients even in the early stages of their disease[123,124]. In GC, Fang *et al*[125] found that ctDNA levels were correlated with vascular invasion and the highest ctDNA detectable levels were associated with peritoneal recurrence and a poor prognosis. Balgkouranidou *et al*[126] showed that *rassf1a* and *apc* promoter hypermethylation in cfDNA represents a frequent epigenetic event in patients with early operable GC demonstrating a prognostic capacity for these patients. Another study suggested that cfDNA can identify EBV-associated gastric carcinoma (EBVaGC) subtype and monitor tumor progression as well as treatment response in patients with EBVaGC[127].

Being a rare event, ctDNA requires highly sensitive and reproducible analytical methods for proper investigation. Multiplex mass spectrometric SNP genotyping technology, real-time quantitative PCR (qRT-PCR), digital droplet PCR (ddPCR) with improved nucleic acid quantification, next-generation sequencing (NGS) were already employed for ctDNA analysis in GC patients[125,128-130] proving the usefulness in personalized treatment decisions. A panel of more than 70 genes and genomic biomarkers for MSI and blood tumor mutational burden (bTMB) by Foundation Medicine, the FoundationACT® assay, was granted breakthrough device designation by the FDA[131] and might become the first FDA-approved liquid biopsy assay to incorporate multiple companion diagnostics (CDx) and multiple biomarkers.

CONCLUSION

GC remains an important cause of cancer death worldwide with a high mortality rate due to the fact that the majority of GC cases are diagnosed at an advanced stage when the prognosis is poor and the treatment options are limited. Unfortunately, the existing circulating biomarkers for GC diagnosis and prognosis display low sensitivity and specificity and the GC diagnosis is based only on the invasive procedures such as upper digestive endoscopy. Therefore, most current GC studies are focused on the identification and validation of non-invasive cancer biomarkers released from the tumor tissues into the body fluids, such as blood and stomach juice. Many of these biomarkers are not specific for the early stages, being detected in advanced stages of GC, and cannot be used for early GC detection. However, some of recently discovered circulating molecules (miRNAs, lncRNAs, circRNA) hold the promise for developing new strategies for early diagnosis of GC, being able to discriminate between the early stage of GC and healthy subjects, with a sensitivity more than 77.5%. In order to improve the sensitivity and enlarge the early stage biomarkers list, further studies should be performed to optimize laboratory techniques such as extraction, quantification, probe enrichment, and evaluation methods. Moreover, these results need to be validated by independent groups or cohorts in prospective studies.

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