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Circulating cell-free DNAs and miRNAs as promising non-invasive biomarkers for early detection of gastric cancer

Minireview

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Early diagnosis of gastric cancer is critical to decrease the mortality of this globally fatal disease. Currently, endoscopic biopsy is the gold standard for diagnosis of gastric cancer. However, invasiveness and high expense limit its application. Hence, non-invasive and cost-effective biomarkers for early detection and subsequent management are crucial steps to monitor gastric cancer. Recent studies suggest that circulating cell-free nucleic acids, including circulating tumor DNAs and microRNAs (miRNAs), are promising for various applications. Development of such blood-based biomarkers is expected to facilitate detection, predict prognosis, monitor chemotherapeutic response and manage recurrence of human cancers. In this review, the characteristics of circulating cell-free DNAs and miRNAs will be elucidated, including their origin and dysregulation. Mutations and hypermethylation of circulating DNAs, abnormal alternations of expression of circulating miRNAs will be revealed as aberrant changes indicating gastric cancer. The roles of circulating DNAs and miRNAs for early detection of gastric cancer will be focused on, as well as the challenges of developing circulating nucleic acids as biomarkers.

Key words: circulating DNA, circulating miRNA, biomarker, gastric cancer, early detection

Epidemiology and diagnosis of gastric cancer

Gastric cancer is the fourth commonest cancer and the second leading cause of cancer associated death worldwide [1, 2]. More than 70% of gastric cancer cases occur in eastern Asia [2]. This is mainly attributed to the high frequency of *Helicobacter Pylori* (*H. Pylori*) infection, salty and preserved dietary, as well as large number of smoking population in eastern Asians [3]. Currently, complete surgical resection is the most effective therapy for patients with early gastric cancer, offering an excellent survival rate of more than 90% [4]. However, the majority of patients with gastric cancer are diagnosed at an advanced stage, resulting in the average 5-year survival rate around 20–25% [5]. The dramatic difference indicates the critical influence of early detection of gastric cancer on its prognosis. However, the symptoms of gastric cancer at the early stage, such as uncomfortable in the upper abdomen, belching, early satiety, etc. are atypical and subjective. As there is no standard assay for screening or early detection of gastric cancer, most of the patients go for medical examination until they are at an advanced stage [6, 7]. This situation makes it urgent to develop a method for early detection of gastric cancer.

As *H. Pylori* infection is the strongest known risk factor for gastric cancer, people with persistent *H. Pylori* infection, especially those with chronic atrophic gastritis are highly recommended to take surveillance by regular examination [8]. Currently, the most reliable diagnosis of gastric cancer is biopsy [9]. The observation of gastric mucosa under endoscopy and the histological evaluation of gastric tissue taken from endoscopy make the final decision of gastric cancer [10]. However, biopsy requires experts with intensive training to operate endoscopy and pathologists with experience to examine the mucosa, as well as the purchase and maintenance of the equipment. These conditions may not be available in some developing countries or remote regions. Besides, biopsy is not applicable for some patients refusing such invasive ex-

amination [11]. Moreover, biopsy is taken just once, making it not practical for repeated sampling for cancer monitoring. Therefore, a non-invasive, cost-effective and patient-friendly application for early detection of gastric cancer and the follow up tracing of therapy is necessary and urgent.

Current blood-based biomarkers for detection of gastric cancer

Biomarkers refer to molecules or substances found in blood, other bodily fluids or tissues that reflect a particular biological or pathological state. Blood-based biomarkers are ideal application for cancer diagnostics. Biomarkers from blood provide a non-invasive, cost-effective and patient-friendly assay for detection of gastric cancer. It is promising to revolutionize gastric cancer diagnostics. Carcinoembryonic antigen (CEA), carbohydrate antigen 19–9 (CA 19–9) and carbohydrate antigen 72–4 (CA 72–4) [12, 13], or pepsinogen I /II ratio (PGI/II) [14, 15] they all are common blood-based biomarkers for risk screening of gastric cancer. However, the sensitivity and specificity of these biomarkers are just around 30-50% [16, 17], much less than satisfactory for accurate detection of gastric cancer. Besides the biochemical biomarkers, it has been proposed that circulating tumor cells (CTCs) are alterative biomarkers for cancer detection and monitoring [18, 19]. But this requires physically separating multiple kinds of cells in blood to accumulate circulating tumor cells. This is difficult as around 1-10 CTCs together with several million blood cells could be found in 1 ml of whole blood [20]. And the epithelial markers to characterize CTCs are downregulated during tumor cell dissemination in certain tumor types. Until now, there are no standard criteria to differentiate tumor cells from other kinds of cells in blood [21, 22]. Hence, a brand new biomarker with high sensitivity and specificity, as well as non-invasiveness is urgent in need.

Circulating nucleic acids for cancer detection and management

In recent years, a new kind of biomarkers, circulating cell-free nucleic acids (cfNAs), attracts lots of attention. Circulating cell-free tumor-associated nucleic acids are thought to be released into the blood through apoptosis, necrosis or secretion from cancer cells in the tumor microenvironment [23, 24]. Macrophages may also play an important role in generating circulating cell-free nucleic acids [25]. The amount of circulating cfNAs is influenced by the tumor size and the rate of their clearance [26-28]. As the quantification of cfDNA concentrations overlap between healthy individuals and patients with benign and malignant disease [29, 30], it is necessary to assess quantitative cfNAs in large cohorts of patients with well-defined clinical parameters. But the remaining high level of cfDNA of the same patient might indicate the presence of residual tumor cells or micro-metastasis in the tumor microenvironment [31].

On the other hand, it has been reported that circulating cfDNAs contain the aberrant genetic or epigenetic alterations that represent their original cancer, even at the early stage of cancer development [32, 33]. This evidence provides the base of early detection of cancer using circulating cfDNAs. Circulating cfDNAs contain tumor mutations or abnormal DNA methylation that is unique to the patient [34, 35]. The amount of circulating tumor-derived miRNAs also provides the hints to tumor occurrence and progression [36]. This provides evidence to apply circulating cfNAs as "liquid biopsy" in clinic. This application is quite meaningful as it is convenient to provide sensitive and specific serial sampling during the course of disease, considering that avoiding repeated biopsy. Convenience of serial sampling also facilitates cancer management and monitoring, such as, therapy response prediction, disease prognostics and recurrence in patients [33, 37, 38]. For example, it has been demonstrated that detection of mutations in circulating tumor DNAs correlated with responses to chemotherapy and metastasis of breast cancer [39].

Circulating cell-free DNAs for early detection of gastric cancer

Circulating DNAs with gene mutations. High frequency of gene mutations contained in circulating cell-free DNAs (cfDNAs) provides evidence to assess the tumor occurrence and progression. A patient's tumor contains large chromosomal rearrangements that were unique to that patient, which could be picked out by polymerase chain reaction (PCR) [32]. The amount of blood-based tumor DNA with specific mutations could be tracked for cancer detection and could be applied to monitor the patient's response to treatments. Genes such as *KRAS* and *TP53* are with high frequency of mutations in various human cancers [40-42]. But these genes are not specific to gastric cancer. They may combine with other mutations more specific to gastric cancer for its early detection. Hence, identification of oncogenes or tumor suppressor genes with frequent mutations in gastric cancer is the premise of such application.

It has been reported that germ-line epithelial cadherin (*CDH1*) contains mutations in 25% to 40% of the hereditary diffuse gastric cancer (HDGC) [43, 44]. *CDH1* mutation is very important considering it harbors relative high frequency of gene mutations in gastric cancer, especially in the low-risk areas [45]. Recently, novel *CDH1* mutations have been identified in sporadic Chinese gastric cancer patients, providing new evidence for screening and early detection of gastric cancer in the high risk areas [46]. Besides *CDH1*, high frequency of mutations of the *PIK3CA* (phosphatidylinositol 3-kinase) gene in the helical domain and kinase domain has been reported in various human cancers, including gastric cancer [47]. The mutation frequency varies up to 25% in gastric adenocarcinoma [48, 49], higher than other genes examined [50]. Recently, it has been revealed that mutations in *ARID1A* (AT rich interactive domain 1A) are associated with

concurrent *PIK3CA* mutations and microsatellite instability in gastric adenocarcinoma [51]. This may provide more accurate detection by analysis of the concurrent mutations of *PIK3CA* and *ARID1A* in the circulation of gastric cancer candidates. Besides the genes aforementioned, mutations in genes such as *EGFR* [52, 53] and *PTEN* [54, 55] have also been reported in gastric cancer, providing potential circulating DNAs for detection of gastric cancer.

The leading challenge of mutation assays in cfDNA is the mutations might be diluted by wild-type DNA in the blood, making them as relative low frequency mutations occurring in gastric cancer. Moreover, not all of the tumors that carry DNA mutations can be detected in the blood. Though the detection of gene mutations aforementioned provides promising potential circulating cfDNAs for early diagnosis of gastric cancer, identification of oncogenes or tumor suppressor genes with high frequency of mutations specific to gastric cancer is undergoing. This is critical for its clinical application in blood-based detection of gastric cancer.

Circulating DNAs with hypermethylation. Besides mutations, aberrant alterations of methylation status in circulating cfDNAs released from gastric cancer cells are also promising biomarkers for risk assessment of gastric cancer. Promoter hypermethylation of tumor suppressor genes, such as *p16*, has been found to be more frequently responsible for the silence of them than mutations in sporadic gastric cancer [56]. Although epigenetic alterations are not unique for a single tumor type, there are particular tumor suppressor genes that are frequently methylated and downregulated in certain tumors [57-59]. Indeed, abnormal DNA methylation was detected in plasma samples of cancer patients. For example, it was revealed that detection of circulating methylated *RASSF1A* (Ras association domain family 1) by fluorescent-based methylation specific PCR (MS-PCR) in melanoma patients [60]. For digestive tract tumors, the development of detection of methylated *SEPT9* (septin9) in early colorectal cancer (CRC) by real-time PCR in plasma samples is undergoing [35, 61]. This demonstrates the potential utility of diagnostic screening of methylated tumor-related cfDNA in population to identify the tumor type.

For gastric cancer, it was shown that methylation of *TFPI2* (tissue factor pathway inhibitor-2) can be detected in the serum of gastric cancer patients by quantitative methylation specific-PCR (q-MSP) [62]. Another paper demonstrated that cumulated methylated *RUNX3* (runt-related transcription factor 3) in serum samples was associated with gastric cancer initiation, progression and disease-free survival [63]. In addition to these, other studies indicated that significantly different frequencies of methylation status in genes were observed in circulation between early gastric cancer and non-cancer subjects [64]. For example, high frequency of methylation was found in *RPRM* and *RNF180* in plasma samples [65, 66], as well as in *p15, DAPK* and *SFRP2* in serum samples of patients with gastric cancer [67, 68]. Such evidence indicates the potential application of circulating methylated cfDNAs for detection of gastric cancer (Table1).

Table1. Circulating methylated DNAs for detection of gastric cancer.

Genes	Serum/Plasma	Methylation Frequency	References
p16	Serum	14 out of 52 patients 0 out of 50 controls	[56]
TFPI2	Serum	7 out of 73 patients 0 out of 20 controls	[62]
RUNX3	Serum	143 out of 202 patients 2 out of 854 controls	[63]
RPRM	Plasma	41 out of 43 patients 3 out of 31 controls	[65]
RNF180	Plasma	18 out of 32 patients 0 out of 64 patients	[66]
p15	Serum	30 out of 54 patients 0 out of 30 controls	[67]
DAPK	Serum	26 out of 54 patients 0 out of 30 controls	[67]
SFRP2	Serum	12 out of 18 patients 0 out of 18 controls	[68]

The application of this approach depends on the identification of tumor suppressor genes (TSGs) that are frequently methylated and downregulated in early stage of gastric cancer, as well as the accurate technique of detection of DNA methylation status in serum or plasma samples. To identify such TSGs, previous studies indicated that promoter hypermethylation of *p16* and *DAPK* (Death-associated protein kinase) genes might play an important role in the pathogenesis of gastric epithelial dysplasia and early gastric adenocarcinoma [69, 70]. Moreover, there was a significant association between hypermethylation of *p16* and *MGMT* (O-6-methylguanine-DNA methyltransferase) and elevated serum CEA level [70]. This evidence provides genes with DNA hypermethylation specific to gastric cancer. And the hypermethylation in these genes may be combined with other blood-based biomarkers (such as CEA) for more accurate early detection of gastric cancer. Besides *p16* and *DAPK*, other genes with DNA hypermethylation in gastric cancer tissue samples was revealed, such as *TSLC1* (cell adhesion molecule 1) and *DCC* (Deleted in colorectal carcinoma) [71, 72]. These events are clinically promising, as they demonstrate that blood-based samples obtained in population or clinic can be applied for estimating cancer risk or early detection of gastric cancer.

Circulating miRNAs for early detection of gastric cancer

Other than circulating cfDNAs, circulating miRNAs is another group of promising biomarkers for early detection of cancer due to a strong link between the dysregulation of miRNAs and cancer initiation and progression [73-75]. The discovery of miRNAs in plasma or serum provides the potential of applying them as non-invasive biomarkers in cancer detection and as predictors of response to chemotherapy [76]. In addition to their high stability in circulation, the

characteristics of miRNAs such as tissue-specific signatures and the availability of many copies per cell indicate their potential advantages as biomarkers. It was firstly reported that upregulation of circulating miRNAs was an indicator for diffuse large B cell lymphoma [77]. Later, dysregulation of circulating miRNAs was revealed in other cancer types, including breast cancer, colorectal cancer and liver cancer [78-80].

For gastric cancer, dysregulation of miRNAs was demonstrated by miRNA expression profiling between non-tumor mucosa and cancer samples [81]. This evidence indicates that unique miRNAs are associated with development of gastric cancer, providing the possibility of circulating miRNAs for gastric cancer detection and monitoring. Actually, aberrant amount of miRNAs have been detected in both serum and plasma samples from gastric cancer patients [82]. Dysregulation of circulating miRNAs for detection of gastric cancer was first revealed in 2010. Two studies indicated that miRNA signatures in plasma or serum could be applied as novel biomarkers for diagnosis of gastric cancer [83, 84]. Since then, a number of studies showed that circulating miRNAs were promising biomarkers for early detection of gastric cancer. For example, it was revealed that elevated serum level of miR-378 can be applied as a biomarker for early detection of gastric cancer, with an AUC of 0.861 [85]. Another paper suggested that circulating miR-223 (P<0.001) and miR-21 (P<0.001) were significantly higher in the plasma of gastric cancer patients than in healthy controls, while miR-218 (P<0.001) was significantly lower. The ROC analysis of the ratio of (miR-223 X miR-21)/miR-218 indicated an AUC value of 0.9531, which could discriminate GC patients from healthy controls [86]. As there are two types of gastric cancer, intestinal type and diffuse type, with different biological behaviors [87], a recent study focusing on diffuse-type gastric cancer (DGC) demonstrated a combination of four upregulated miRNAs (miR-103, miR-107, miR-194 and miR-210) in serum as biomarkers for early detection of DGC using a mouse model. With cut-off levels released by ROC curve analyses, the overall sensitivity and specificity of the miRNAs were >80% and >90% separately [88]. Dysregulation of circulating miRNAs as biomarkers for detection of gastric cancer was summarized in Table2 [89-91].

The evidence of aforementioned suggests a promising application of detection of gastric cancer using blood-based samples. However, there are discrepancies of the upregulation of circulating miRNAs from the same tumor type in different studies [92]. This is mainly attributed to the relative small sample size (N<100) and lack of a well-established endogenous miRNA control to normalize miRNA amounts. Even frequently used reference miRNAs (such as RNU6 and 5S) are variable under different physiological conditions or patient and donor demographics [36]. It was released that several miRNAs could be used as internal controls in plasma or serum samples [93-95]. But the universal standards of internal control of miRNAs to provide a better normalization of miRNA in plasma or serum are still under development. This approach also strongly requires the internal control miRNAs to be well studied in relevant defined populations

Table2. Circulating miRNAs for detection of gastric cancer.

miRNAs	Cancer Type (Intestinal/Diffuse)	Serum/Plasma	Sensitivity/Specificity	References
miR-17-5p, miR-21, miR-106a, miR-106b, (let-7a)	Both Types	Plasma	85.5%/80.0% for miR106b/let7a (optical value)	[83]
miR-1, miR-20a, miR-27a, miR-34, miR-423-5p	Both Types	Serum	88%/77% of Set1 81%/71% of Set2 (Value of combined five miRNAs)	[84]
miR-378	Both Types	Serum	87.5%/70.7%	[85]
miR-223, miR-21, (miR-218)	Both Types	Plasma	84.29%/92.86% for (miR-223*miR21)/miR218	[86]
miR-103, miR-107, miR-194, miR-210	Diffuse Type (mouse model)	Serum	81.8%/95.7% for miR-103 90.9%/95.7% for miR-107 90.9%/95.7% for miR-194 72.7%/87.0% for miR-210	[88]
miR-451, miR-486	Both Types	Plasma	96%/100% for miR-451 86%/97% for miR-486	[89]
miR-221, miR-744, miR-376c	Both Types (Dysplasia samples)	Serum	82.4%/58.8% for GC 56.5%/47.8% for Dysplasia (Value of combined three miRNAs)	[90]
miR-199a-3p	Both Types	Plasma	80%/74%	[91]

( ) indicates downregulation of miRNAs in circulation for detection of gastric cancer, while other miRNAs are upregulated ones.

[92]. Another crucial aspect is the extraction of miRNAs from plasma or serum samples. It should minimize the variation of the amount of miRNAs from one sample to another, as they are of small size and protected by lipids and proteins [69].

Conclusions and future perspectives

Early detection of gastric cancer is essential to decrease the mortality of this global lethal disease. Histological evaluation of gastric mucosa obtained from endoscopy is the "golden standard" of diagnosis [9, 10]. But sometimes it is not easy for patients in certain conditions or in regions lack of the experts or equipment. Routine screening and clinical application require easily implementable tests for biomarker analyses. Some serum biomarkers such as CEA and CA19-9 are not sensitive or specific enough to satisfy early detection of gastric cancer [3]. Thus, it is urgent to search for other blood-based tumor biomarkers for gastric cancer diagnosis. In this review, it has been demonstrated the emerging roles of circulating cell-free nucleic acids (cfDNAs and miRNAs) as novel non-invasive biomarkers for early detection of gastric cancer. Circulating cfDNAs with certain mutations or hypermethylation, or dysregulation of circulating miRNAs may also be useful biomarkers to help the doctors decide whether to apply prompt endoscopy to the patients. For example, endoscopy should be applied for dyspeptic patients with high level of circulating miRNAs or mutated DNAs, especially in regions with a high risk of gastric cancer.

The main challenge in evaluating cfNAs is the standardization of assays, such as DNA or miRNA extraction, internal controls, assay conditions, sensitivity and specificity [28]. It also remains controversial whether plasma or serum is the optimal sampling specimen, considering that the coagulation process may affect the spectrum of extracellular miRNA in blood [62]. To summarize, advantages and limitations of various biomarkers mentioned above were listed in Table3. To overcome the limitations, improvements in methodologies are undergoing. For instance, the development of a direct DNA assay without extraction may overcome many of these problems [96]. New approaches in the assessment of cfDNA are being developed to validate gene mutations or methylation status in circulating cfDNAs, such as cfDNA next generation sequencing (NGS). NGS is a brand new technology for faster and lower-cost sequencing to determine DNA sequence [97]. The advantage of NGS is to perform sequencing of millions of fragments of DNA from a single sample simultaneously. Application of such massively parallel sequencing technology facilitates the discovery of mutations in the blood samples, with less time, cost and personnel [98]. This technology is also promising in application for gastric cancer [99]. For circulating miRNA, the universal standard of miRNAs for normalization of miRNA amount is also in need. Although problems remain to be solved, such minimally invasive blood-based cell-free DNA and miRNA are promising to complement or replace the existing cancer tissue and blood biomarkers in the future.

Table3. Advantages and limitations of biomarkers

Biomarkers	Advantages	Limitations
Biochemical Biomarkers (such as CEA and CA19-9)	Easy to detect, Cost-effective	Low sensitivity, Low specificity.
CTCs (Circulating Tumor Cells)	Specific for GC	Difficult to separate, Be diluted in tumor cell dissemination.
Circulating Mutated DNAs	Early stage of GC, High specificity	Influence by tumor size and the rate of clearance, Relatively low frequency, Standard of DNA extraction.
Circulating Methylated DNAs	Early stage of GC, High sensitivity, Easy to detect	Influence by tumor size and the rate of clearance, Selection of methylation of GC, Standard of DNA extraction.
Circulating miRNAs	Early stage of GC, Easy to detect, High sensitivity, High specificity	Standard of miRNA extraction, Selection of internal control, Use of Plasma or Serum.

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