TOPIC HIGHLIGHT

2016 Gastric Cancer: Global view

New blood markers detection technology: A leap in the diagnosis of gastric cancer

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Abstract

Gastric cancer (GC) is still one of the malignant tumors with high morbidity and mortality in the world, with a 5-year survival rate of less than 30%. GC is often either asymptomatic or causes only nonspecific symptoms in its early stages, whereas when the symptoms manifest, the cancer has usually reached an advanced stage, which is one of the main causes of its relatively poor prognosis. Hence, the main focus of GC research has been on discovering new tools and technology to predict, screen and diagnose GC at an early stage which would prompt early treatment. With the tremendous advances in the OMICS technology, serum proteomics has been in the limelight of cancer research over the last few decades and has steered the development of several methods helping to understand the mechanisms underlying gastric carcinogenesis, resulting in the identification of a large number of molecular targets such as circulating tumor cells (CTCs), cell free DNA (cfDNA) and cell tumor DNA (ctDNA) and their sub-molecular components such as miRNA, that show great promise as GC biomarkers. In this review, we are underlying the recent breakthroughs about new blood markers technology for GC while scrutinizing the potential clinical use of CTCs, cfDNA, ctDNA and the role of the methylation of their sub-molecular components in the pathogenesis, diagnosis and management of GC.

Key words: Gastric cancer; Circulating tumor cells; Circulating tumor DNA; Proteomics; Biomarkers; Methylation

Core tip: Gastric cancer (GC)'s poor prognosis has partly been a result of its late diagnosis due to its asymptomatic and nonspecific symptoms in its early

stages. Tremendous advances in the OMICS technology have allowed the development of several methods helping to understand the mechanisms underlying gastric carcinogenesis, resulting in the identification of a large number of molecular targets such as circulating tumor cells, cell free DNA, cell tumor DNA and their sub-molecular components such as miRNA that show great promise as GC biomarkers. In this review, we are underlying the recent breakthroughs about new blood markers technology for GC while scrutinizing their potential clinical use in the pathogenesis, diagnosis and management of GC.

INTRODUCTION

Gastric cancer (GC), is still one of the world's most malignant tumors with high morbidity and mortality with 1 new million cases and about 800000 deaths per annum. According to the literature of GC, the 5-year survival rate is less than 30%[1]. In recent years, the advances made in the GC detection methods and treatment have definitely influenced the clinical outcome of patients but still, due to late diagnosis, the main clinical issue that requires attention pertains to the high prevalence of metastasis and recurrence. In the quest for tools for early diagnosis, scientists and physicians have undertaken much research about blood tumor markers which could detect GC, prompting early intervention.

Clinically, the detection of the physiological indicators or parameters of the serum has been always an auxiliary for the diagnosis of cancer[2]. Over the years, there have been major breakthroughs in the field of serum proteomics where more attention has been paid to quantitative or qualitative changes undergone by some important regulatory proteins secreted into the serum under many physiological and pathological conditions[3]. However, the positive rate of the currently clinically popular gastrointestinal tumor biomarkers CEA, CA19-9 and CA72-4 has been found to be lower than 40% in GC patients and lower than 20% in early GC patients, hereby making them insufficient for the diagnostic screening[4,5]. Hence, due to the lack of the specificity or sensitivity of such markers, researchers have been on a constant search to discover the most convenient tool to diagnose GC.

Over the last few decades, there have been major breakthroughs in the field of serum proteomics where, in addition to the conservative methods of cancer detection, there was a new leap in blood markers detection technology. In addition to the continued investigation about novel and unbiased GC serum biomarkers by different and innovative proteomics techniques, new concepts such as circulating tumor cells (CTC) and circulating tumor DNA (ctDNA) have been introduced[6-8]. Previous studies state that during the early stages of tumor formation, tumor cells and tumor DNA are released in the peripheral blood. The tumor cells and related DNA shed off in the circulatory system during tumor evolution have been followed closely over the last few years and it was found out that the qualitative and quantitative analysis of CTC and ctDNA was related to the diagnosis, evolution, follow-up and prognosis of tumors. As an alternative to primary tumor "liquid biopsy specimen"[7,9,10], CTC has already been approved by the FDA as a prognostic evaluation factor for metastatic breast, prostate and colorectal cancers[11]. Hence, this new leap in blood markers detection technology has provided a new platform for the early detection and diagnosis for GC while providing a more efficient tool for the evaluation of treatment efficacy and prediction of recurrence and metastasis.

Serum proteomics

In 1994, the concept of the proteome was brought to light by Wilkins and Williams from Macquarie University and after that, proteomics and related techniques underwent rapid development, allowing the large-scale screening of tumor biomarkers. Serum proteomics can be used to perform differential analysis of serum proteins between cancer patients and healthy controls and in so doing, differential specific and unbiased proteins could hence be adopted as tumor biomarkers for the early diagnosis of cancer. So far, several serum tumor biomarkers have been identified for GC but due to their poor specificity and sensitivity, they have proven to be insufficient for the reliable diagnosis of GC. Thus, there is the urgent need to find more reliable serum tumor biomarkers for earlier and reliable diagnosis of GC. In a study made by our research team, the role of new modern and advanced proteomic techniques, such as Surface-enhanced Laser Desorption Ionization (SELDI) and High Performance Liquid Chromatography, in the quest for new GC biomarkers was assessed: numerous novel serum tumor biomarkers such as Amyloid-related Serum Protein, plasminogen and C9c have been discovered through serological proteomics strategies[3,12] and these techniques have been well adopted throughout the globe in the fight against GC.

Proteomic-based techniques such as 2-DE (two-dimensional electrophoresis), iTRAQ (isobaric tags for relative and absolute quantitation), ICAT (isotope-coded affinity tag), protein chip array and liquid chromatography, have been used to identify and quantify proteins that can be used as biomarkers in bodily fluids and tissues in GC[3,12,13]. To date, the most common fluid biomarkers available for GC include CEA,

CA 19-9, CA 72-4, CYFRA 21-1, TPA, TPS, E-cadherin, pepsinogen, cytokines and the β-subunit of HCG.

As reported, the majority of tumor biomarkers in GC diagnosis are glycoproteins[14], with the most common being mucin-5AC, IgG, mucin-1, *IGHM, LRG1*, haptoglobin (HP), albumin, TF, kininogen-1, alpha-1-acid glycoprotein, ceruloplasmin (*CP*), *A1BG*, vitamin D binding protein (*GC*), alpha-1-antitrypsin (*SERPINA1*), antithrombin (*SERPINC1*), angiotensin, *CFB*, serpin peptidase inhibitor, Clade A (*SERPINA3*), alpha-2-HS-glycoprotein, Zn-alpha-2-glycoprotein, *CLU, ITIH2*, complement factor H, interalpha-trypsin inhibitor *HCRP, SERPING1* and C4A variant protein (*C4A*)[15-17]. These glycoproteins have been discovered and highly investigated over the last few decades but still, more research has to be done to evaluate their clinical value, their specificity and accuracy in predicting or evaluating GC.

Recently, Li *et al*[18] studied two multidrug-resistant cell lines and their parental drug-sensitive GC cell line to characterize the multiple drug resistance (MDR)-related cell surface glycoproteome: 56 cell membrane glycoproteins were successfully identified, 11 of which (*Mesothelin, EGFR, Integrin alpha-3, CD59, Folate receptor alpha, Peptidyl-prolyl cis-trans isomerase FKBP9, Laminin subunit alpha-5, Dihydropyridine receptor alpha 2, Multidrug resistance protein 1, Prostaglandin F2 receptor negative regulator and Golgi apparatus protein 1*) were found to be differentially expressed with the same trend in both the drug-resistant and sensitive cell lines. This report was the first concerning the relationship between glycoprotein alterations and MDR in gastric tumors and was also helpful for better interpreting the sophisticated mechanisms of MDR in GC, which, of course, still require further investigation and verification.

Given the current multiplicity of proteomic studies in GC, due to the vast amounts of data generated, it is important to maintain an up-to-date and searchable index of the lists of biomarkers obtained and evaluated from different research works. It is eventually essential that future research not only focus on identifying the disease-associated alterations in the proteins but also on determining the cellular functions of the proteins identified as well as the mechanistic networks in which they participate. The biomarkers identified experimentally should serve as entry points for investigating the mechanisms of carcinogenesis and tumor progression.

With the developments in proteomics and mass spectrometry, a large number of new methods and technology has been used in the serum proteomics research for GC. Since tumor markers lack specificity, particularly in the early stages of cancer, there has been a lot of research laying more emphasis on the different combinations of peptides or glucosides secreted into blood rather than protein markers and then GC diagnosis models have been established based on the statistical information. The SELDI method has been one of the most popular proteomics technologies over the last few years and, despite its high diagnostic rate, this method has poor repeatability and reliability, thereby hindering its reliability and clinical application. Hence, this research group has used the magnetic beads combined with mass spectrometry technology for the diagnosis of GC since the repeatability and clinical value of this method are relatively higher, mostly due to high capture efficiency of the magnetic beads for the specific proteins and peptides, hence improving the diagnostic accuracy and specificity for GC[3]. Our research team used serum proteomics to analyze serum samples from 20 GC patients including pTNM stages I to IV, where serum proteins were separated by 2-DE and identified by MALDI-TOF/TOF-MS; the results showed 3 proteins, complement C4-B precursor, complement factor I (CFI) precursor and haptoglobin precursor in the GC patients were significantly different between the normal control group while western blot showed that the detection of CFI precursor was under-expressed in GC with declining expression while advancing from pTNM stage I to IV GC and this implied that 2-DE-based serum proteome analysis could not only be useful in the screening of serum biomarkers for GC, but the CFI precursor could also help in the diagnosis and indicate disease progression in GC[12].

Li *et al*[19] used proteomic analysis of serum samples for identifying gastric precancerous lesions and GC by studying the serum samples from 25 patients with gastric precancerous lesions (chronic atrophic gastritis with mild to moderate dysplasia), 25 GC patients and 25 healthy controls using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS): the spectral peaks for the peptides with mass-to-charge (m/z) values of 1741 and 4210 were the most significantly different among the three groups and the sensitivity of this diagnostic model for detecting healthy controls, patients with gastric precancerous lesions and patients with GC was found to be 80.0% (12/15), 66.7% (10/15) and 66.7% (10/15) respectively, while the specificity was 66.7% (20/30), 73.3% (22/30) and 73.3% (22/30), respectively. Lu *et al*[20] analyzed 125 serum samples from GC patients and normal healthy control subjects using surface-enhanced laser desorption/ionization technology and the results of the surface-enhanced laser desorption/ionization model were compared with the biomarkers CEA and CA199 in a subset of samples using a micro-particle enzyme immunoassay: the results showed that 5 distinct protein peaks at 2046, 3179, 1817, 1725 and 1929 m/z were automatically chosen as components of the best biomarker pattern for diagnosis of GC. Lu *et al*[20] also identified a single protein peak at 4665 m/z, which could distinguish between stage I/II and stage III/IV GC with a specificity and sensitivity of 91.6% (11/12) and 95.4% (21/22), respectively. When this

biomarker was validated in the second set of samples, the specificity and sensitivity were 91.7% (11/12) and 86.3% (19/22), respectively, suggesting that the serum surface-enhanced laser desorption/ionization protein profiling could distinguish the GC patients and in particular stage Ⅰ/Ⅱ patients, from normal subjects with relatively high sensitivity and specificity. Even though more clinical evidence with a bigger data pool is required, Surface-enhanced Laser Desorption/ionization-Time-of-flight-Mass Spectrometry holds potential as a screening tool for GC.

Circulating tumor cells

Circulating tumor cells (CTCs) are cells shed into the vasculature from a primary tumor that circulate in the bloodstream[21]. CTCs were observed for the first time in 1869 by Thomas Ashworth[11] who postulated that "cells identical with those of the cancer itself being seen in the blood might tend to throw some light upon the mechanisms behind cancer metastasis". The importance of CTCs in modern cancer research began in the mid 1990's with the demonstration that CTCs exist early in the course of the disease. Most of the tumor cells shed off into the circulation system have a short lifespan and only a handful of highly active tumor cells with high metastatic potential survive in the circulatory system to eventually aggregate forming clusters, hence tumor micro-thrombi. Thus, the detection of CTCs in the peripheral blood system would be an indication of possible occurrence of metastasis. CTCs have emerged as a reliable source of tumor cells while their concentration was found to have prognostic implications. CTC capture offers real-time access to cancer tissue without the need of an invasive biopsy, while their phenotypic and molecular interrogation can provide insight into the biological changes of the tumor that occur during treatment[22,23].

CTC detection and GC

The CTC detection process mainly includes separation, enrichment and identification, and since CTC in the peripheral blood of lower density, CTCs and blood cells should first be separated and enriched in order to improve the efficiency of the detection mechanism. There are mainly two kinds of separation methods: the first method comprises separating the CTCs according to the morphological differences existing between the tumor and blood cell structure and conducting relevant immunohistochemistry or immunofluorescence analysis. However, this method lacks specificity and is prone to false positives. Hence, the second method, comprising separation using immunological analysis, is more favorable.

Most tumors of epithelial origin express the epithelial cell adhesion molecule (EpCAM) and cyto-keratin (CK) antigen, while cells originating from bone marrow cells in the blood express molecules such as CD45 molecule[24]. The CELLSEARCH® Circulating Tumor Cell Kit, a product of Johnson (Veridex), is currently the world's first and only FDA approved CTC detection kit intended for the enumeration of CTC of epithelial origin (CD45-, EpCAM+, and cytokeratins 8, 18+, and/or 19+) in whole blood. This method is based on the use of iron nano-particles coated with a polymer layer carrying biotin analogues and conjugated with anti EpCAM antibodies for capturing CTCs. CTCs are hereby defined as cells tested as CK-PE+, DAPI+ and CD45-[25,26]. Clinical research has already proven than the CTC count can effectively predict prognosis, progression free survival and overall survival (OS) for breast, prostate and colorectal cancers with good specificity (99.7%) and high repetition rate (99.4%)[27]. Previous research work has also stated that the CTC count is as good as a diagnostic indicator as imageological indexes, further emphasizing that the CTC count can predict cancers at an early stage[28].

Nevertheless, in addition to the CTC count, recent researchers have shown interest in the molecular changes occurring in CTCs: Cui *et al*[29] found out that *piR-651* and *piR-823* are significantly lower in the peripheral blood of GC patients when compared to normal controls; Zhou *et al*[30] found that the miR-421 level in the CTC obtained from peripheral blood of GC patients was significantly higher than that of their control group and their further investigation discovered that the transfection of *miR-421* inhibitors could significantly inhibit tumor growth *in vivo*. Zhang *et al*[31] analyzed the miRNA expression profile in 65 GC patients, 29 patients with recurrence and 36 patients without recurrence: the results indicated that the combination of *miR-375* and *miR-142-5p* could predict recurrence risk for GC patients. Furthermore, frequently recurring high levels of *miR-335* and poor overall survival correlated significantly with high levels of individual miRNAs in patients with GC[32]. Although previous studies suggested the use of numerous potential miRNAs as biomarkers in the diagnosis and prognosis of GC, the values of these miRNAs as biomarkers need to be further confirmed in human GC patients[33]. The changes at the level of miRNA in GC CTCs can prove to be ground-breaking and pivotal for GC research but the technology and expertise required for manipulating and fully observing the behavior of miRNA are still crude and for future clinical use, this asset should be further developed and adapted for more clinical availability.

The molecular changes found in CTCs obtained from the peripheral blood of GC patients have grasped the attention of more and more researchers recently; Cao *et al*[34] studied the expression of *Survivin* in the peripheral blood CTC of 98 GC patients and stated that just like the conventional pathological biomarkers, *Survivin* mRNA was an independent predictive factor for disease free survival and that high *Survivin* mRNA expression after radical tumor resection was an

indicator of tumor recurrence. Arigami *et al*[35] used quantitative RT-PCR for the evaluation of *B7-H3* mRNA expression in 4 GC cell lines, 95 GC cases and 21 cases of healthy human peripheral blood: the results of the investigation confirmed that *B7-H3* mRNA was expressed in all 4 GC cell lines while its expression in the peripheral blood of the GC patients was significantly higher than in the healthy control group. They further discovered that the 5-year survival rate of GC patients with high *B7-H3* expression was significantly lower than in patients with low expression. Other researchers[36,37] collected blood samples from 53 preoperative GC cases and 20 healthy volunteers and investigated the expressions of *miR-21, miR-106a and miR-17* using RT-PCR: the results showed that the microRNA levels in the GC patients were significantly higher than the control group and the level of micro-RNA was related to the TNM staging, tumor size and histological classification[38]. Wang *et al*[39] used RT-PCR to monitor the mRNA expression of *MAGE-1* and *MAGE-3* in tumor cells and peripheral blood of GC patients: their results showed that among the 40 patients, the positive rates of *MAGE-1* and *MAGE-3 mRNA* were, respectively, 47.5% and 25% in the peripheral blood samples and 62.5% and 30% in the tumor tissue samples. They furthermore discovered that when *MAGE* mRNA was not expressed in the tumor tissues, it was also negative in the peripheral blood while the control comprising of 20 healthy subjects tested negative for *MAGE* mRNA expression, suggesting that *MAGE* could be a specific tumor marker in the detection of CTCs.

Nevertheless, the method of collection of CTCs markers such as CELLSEARCH also have their short-comings; that is these methods are unable to capture and analyze cells lacking tumor marker expression, cells with low differentiation or tumor stem cells or EMT related cells in the blood. Therefore, there are other CTCs acquisition methods such as the ISET method (Isolation by Size of Epithelial Tumor cells) where the tumor cells are isolated and individually filtrated from the blood cells as a result of the different sizes[40] or the CanPatrol technique based on the nano-filtration of RNA in situ hybridization, methods which have been actively contributing to the research of serological diagnosis technology for GC, hence providing a new direction for development.

ctDNA and circulating cell-free DNA
Circulating cell-free DNA (cfDNA) is defined as extracellular DNA occurring in blood. cfDNA can be detected in the plasma or serum samples of not only patients suffering from cancer or other destructive diseases but also in healthy individuals. The tumor cells in the CTC and metastatic legions release circulating ctDNA in the peripheral blood after necrosis and apoptosis. In tumor patients, the ctDNA forms only part of cfDNA, other non-tumor cells also release

cfDNA in the peripheral blood after necrosis and apoptosis. Previous research found that the level of ctDNA in the circulation of tumor patients is usually higher than that of healthy individuals and that cfDNA displayed the same biological characteristics as tumor tissues, suggesting that the cfDNA in tumor patients mostly comes from ctDNA while healthy human cfDNA is mostly derived from blood cells.

The similar and specific alterations found in DNA from both the tumor tissue and cfDNA proved the tumoral origin of the cfDNA in cancer patients. These tumor-specific changes of cfDNA included changes in molecular size, oncogene and tumor suppressor gene mutations, microsatellite alterations and hyper-methylation of several genes. The detection of these characteristic alterations in the background of "normal" cfDNA molecules in principle offers a higher diagnostic specificity in comparison with only quantitative measurements of total cfDNA alone. Qualitative and quantitative analysis of circulating cfDNA is an emerging non-invasive blood biomarker utilized to assess tumor progression and to evaluate prognosis, diagnosis and response to treatment. However, detection methods with high analytical sensitivity are an essential pre-condition for detecting these specific alterations because the proportion of cfDNA in tumor-specific alterations could be very small and varied[41].

ctDNA/cfDNA and GC
Recent literature has shown that there is a certain concentration of cfDNA in the plasma of healthy persons, mostly released during blood cells apoptosis. Nevertheless, it has also been reported that in patients with advanced cancer, the cfDNA released during the necrosis or apoptosis of the tumor cells and tumor adjacent tissues can cause a significant increase in the plasma cfDNA level[42]. In a study conducted by Diehl *et al*[43], it was found that per 100g tumor of colorectal cancer, 3.3% of the tumor DNA was shed into the circulatory system on a daily basis. Therefore, the determination of cfDNA in the peripheral blood of cancer patients can be used as a potential tumor marker.

Huang *et al*[44], Frattini *et al*[45] and Sozzi *et al*[46], respectively, monitored the cfDNA levels in breast, colon and lung cancers: their results showed that the level of cfDNA significantly decreased after surgery and therefore, a decrease in the cfDNA level after surgery would imply positive outcome of the surgery and postoperative treatment while an increase in the cfDNA level would indicate poor outcome or signs of disease progression such as metastasis. Therefore, cfDNA may be used as an efficient marker in the monitoring, prediction and evaluation of tumor therapy. However, the value of cfDNA in the early diagnosis of cancer seems to be limited and the main reason is the lack of highly sensitive, reliable and specific methods of cfDNA extraction and quantitation.

At present, there have been some small sample studies on the status of cfDNA on the different outcomes of GC: Kim *et al*[47] studied the plasma cfDNA levels of 30 GC patients and 34 healthy controls, where the GC patients samples were retrieved before surgery and 24 h after surgery; the results showed that the average levels of cfDNA in the healthy control group, patients with early GC, and with advanced GC were 79.78 ± 8.12 ng/mL, 106.88 ± 12.40 ng/mL, and 120.23 ± 10.08 ng/mL, respectively while the levels of cfDNA in the 24-h-after-surgery group decreased significantly compared to the levels of cfDNA in the preoperation group. In another study, Park *et al*[48] found that in their 54 GC patients and 59 age-matched healthy controls, the mean levels of plasma cfDNA were 2.4-fold higher in the patient group compared with the control group, indicating that plasma cfDNA levels may be useful for predicting patients with GC.

ctDNA, as an emerging candidate biomarker for malignancies, has also been thoroughly tackled over the last few years. Hawakawa *et al*[49] investigated the possible application of ctDNA in the monitoring of disease status of GC by performing targeted deep sequencing of plasma cell-free DNA by massively parallel sequencing in patients with tumors harboring TP53 mutations and they found out that 3/10 patients with TP53 mutations in primary tumors showed detectable TP53 mutation levels in preoperative cfDNA to conclude that ctDNA could serve as a useful biomarker to monitor GC progression and residual disease.

Hence, in light of the research results, it is clear that changes in the levels of cfDNA/ctDNA can prove to be reliable biomarkers in the detection of early stages of GC and as a valuable tool for monitoring, estimating and evaluation curative resection, therapeutic effect of preoperative and postoperative treatment and disease prognosis.

cfDNA methylation and GC

The methylation of the tumor suppressor gene promoter is an important mechanism for the inactivation of the tumor suppressor gene and this mechanism plays a very important role in the occurrence and progression of a variety of tumors. The abnormal gene methylation has already been detected in the plasma of head and neck, nasopharyngeal, breast, esophageal, gastric and colorectal cancer patients. P14 belongs to INK4a/ARP of the tumor suppressor gene and during the investigation of the GC cell line[41,50], 5 out of 7 cell lines were found to lack the corresponding gene mRNA and further analysis discovered that the methylation of the gene promoter was an important regulation mechanism for the silencing of the p14 gene in GC. Analysis of GC specimens found that the rate of methylation of the p14 promoter in invasive GC was 45.5%[51].

On the other hand, p15, as a CDKIs inhibitor, is also a tumor suppressor gene. The deletion or mutation of p15 is rare in GC, but the methylation of CpG region promotes the abnormal transcription of mRNA, leading to the inactivation of p15, causing GC. A study conducted by Leung *et al*[52] showed that methylation of the p15 promoter was present in the tumor tissues of 68% of GC patients and in the plasma of 81% of GC patients. In the plasma of the GC patients, the detection rate of abnormal methylation of O6-methylguanine DNA methyltransferase (MGMT), p15 and mismatch repair gene 1 was 59%, 40% and 41%, respectively, while in the plasma of healthy controls, the detection rate of the abnormal methylation of the corresponding genes was only 34%, 13% and 8%.

Kolesnikova *et al*[53] and Tani *et al*[54] used the methylation specific polymerase chain reaction (MSP) method to detect the methylation status of MGMT, p15, and Hm-LH1 with a normal control of 22 healthy volunteers: the results showed that the detection rate of MGMT, p15 and Hm-LH1 methylation in the plasma of 20 GC patients was 50%, 70% and 25%, respectively, while the methylation detection rate in GC patients with stages Ⅲ, Ⅳ and distant metastasis GC was 90%, 90% and 60%, respectively. No abnormal corresponding methylation was detected in the healthy controls.

Leung *et al*[55,56] used methylation-specific PCR to investigate the methylation status of p15 and p16 in GC cell lines and tumor tissues of 26 GC cases: p15 methylation disorder was detected in 4 of the GC cell lines, p16 methylation disorder was detected in 3 of the cell lines while in the 26 GC cases, there was a prevalence of 73.1% for p15 hyper-methylation and 64.5% for pl6 hyper-methylation. This result implicated that the inactivation of the multiple tumor suppressor gene p15 and p16 genes was associated with the occurrence of GC.

The down-regulation of mRNA expression is caused by DNA methylation disorder. It is considered that DNA methylation plays a role in the inactivation of p16 gene. Shim *et al*[57] used MSP to investigate the level of p16 in GC; they found out that 42% of the GC patients had hyper-methylation and among the 22 positive cases, 19 cases had complete p16 immuno-activity deactivation while only 2 cases tested negative for methylation. The correlation of the immune activity and hyper-methylation of p16 shows that methylation is an important mechanism for the deactivation of p16 in GC. Song *et al*[58] carried out an analysis of 9 GC cell lines and found out that the inactivation of p16 expression was accompanied by the hyper-methylation of the promoter: in 28 of the GC patients, 6 cases presented with the absence of p16 expression while in hyper-methylation of the promoter region was noted in 5 cases. *In vitro* studies suggest that 22% of GC patients did not express the p16 protein due to the methylation of the p16 while there was

Table 1 Novel biomarkers in the breakthrough of gastric cancer diagnosis

Biomarkers/proteomics techniques	Methods and Investigation	Ref.
MALDI-TOF-MS	Spectral peaks for the peptides with (m/z) values of 1741 and 4210 were the most significantly different between precancerous lesions, GC patients and healthy controls and the sensitivity and specificity of this diagnostic model was found to be clinically significant	Li *et al*[19]
SELDI	Compared to conventional biomarkers CEA, in a micro-particle enzyme immunoassay, 5 distinct protein peaks at 2046, 3179, 1817, 1725 and 1929 m/z were automatically chosen as components of the best biomarker pattern for diagnosis of GC, with a single protein peak at 4665 m/z, which could distinguish between stage Ⅰ/Ⅱ and stage Ⅲ/Ⅳ gastric cancer with high specificity and sensitivity	Lu *et al*[20]
CTCs	Clinical researches have already proven than the CTC count can effectively predict prognosis, progression free survival (PFS) and overall survival (OS) for breast, prostate and colorectal cancers with good specificity and high repetition rate	Dawson *et al*[27]
piR-651 and piR-823	piR-651 and piR-823 are significantly lower in the peripheral blood of gastric cancer patients when compared to normal control	Cui *et al*[29]
miR-421	miR-421 level in CTC obtained from peripheral blood of GC patients was significantly higher than that of their control group and the transfection of miR-421 inhibitors could significantly inhibit tumor growth *in vivo*	Zhou *et al*[30]
miR-375 and miR-142-5p	miRNA expression profile in gastric cancer patients indicated that the combination of miR-375 and miR-142-5p could predict recurrence risk for gastric cancer patients	Zhang *et al*[31]
Survivin mRNA	Survivin mRNA is an independent prediction factor for disease free survival, whereby a high Survivin mRNA expression after radical tumor resection was an indicator of tumor recurrence	Cao *et al*[34]
B7-H3 mRNA	Quantitative RT-PCR confirmed that B7-H3 mRNA was expressed in all 4 GC cell lines and that the 5-yr survival rate of GC patients with high B7-H3 expression was significantly lower than the ones with low expression	Arigami *et al*[35]
miR-21, miR-106a and miR-17	miR-21, miR-106a and miR-17 expression patterns showed that the microRNA levels in the GC patients were significantly higher than the control group and the level of microRNA was related to the TNM staging, tumor size and histological classification[38]	Zheng *et al*[36]
MAGE mRNA	MAGE-1 and MAGE-3 mRNA is expressed in tumor cells and peripheral blood of GC patients while they are not expressed in the healthy controls; suggesting that MAGE could be a specific tumor marker in the detection of CTCs	Wang *et al*[39]
cfDNA	cfDNA significantly decreased after surgery and therefore, a decrease in the cfDNA level after surgery would imply positive outcome of the surgery and postoperative treatment while an increase in the cfDNA level would indicate poor outcome or signs of disease progression such as metastasis	Huang *et al*[44], Frattini *et al*[45], Sozzi *et al*[46]
cfDNA	As compared to GC patients and advanced GC patients, the level of cfDNA in normal subjects was lower; whereby the level of cfDNA in the 24-h-after-surgery group decreased significantly compared to the pre-operation group	Kim *et al*[47]
cfDNA	The mean level of plasma cfDNA in GC was 2.4-fold higher in the patient group compared with the control group, indicating that plasma cfDNA levels may be useful for predicting patients with gastric cancer	Park *et al*[48]
TP53 mutations	Targeted deep sequencing of plasma cell-free DNA by massively parallel sequencing was performed in patients with tumors harboring TP53 mutations and found detectable TP53 mutation levels in preoperative cfDNA to conclude that ctDNA could serve as a useful biomarker to monitor gastric cancer progression and residual disease	Hawakawa *et al*[49]
p14 promoter methylation	Analysis of GC specimen found that the rate of methylation of the p14 promoter in invasive GC was 45.5%	Leung *et al*[52]
p15 promoter methylation	Methylation of the p15 promoter was present in GC patients whereby there was a higher detection rate of abnormal methylation of MGMT, p15 and mismatch repair gene as compared to healthy controls	Leung *et al*[52]
MGMT, p15, and Hm-LH1 Methylation	The methylation specific polymerase chain reaction (MSP) method was used to detect the methylation status of MGMT, p15, and Hm-LH1 and found that there was a high detection rate of MGMT, p15 and Hm-LH1 methylation in the plasma of GC, with an even higher methylation detection rate in stages Ⅲ, Ⅳ and distant metastasis GC while there was no abnormal corresponding methylation in the healthy controls	Kolesnikova *et al*[53] Tani *et al*[54]
p15 and p16 Methylation	methylation-specific PCR was used to investigate the methylation status of p15 and p16 in GC cell lines and tumor tissues and the findings implicated that the inactivation of the multiple tumor suppressor gene p15 and p16 genes was associated with the occurrence of GC	Leung *et al*[55,56]
p16 hyper-methylation	MSP was used to investigate the level of p16 in GC and they found out that the correlation of the immune-activity and hyper-methylation of p16 shows that methylation is an important mechanism for the deactivation of p16 in GC and that the inactivation of p16 expression was accompanied by the hyper-methylation of the promoter	Shim *et al*[57] Song *et al*[58]
Runt related transcription factor 3 (Runx3) gene methylation	The pre-operative and post-operative Runt related transcription factor 3 (Runx3) gene methylation level and the results showed that the Runx3 methylation index was related to the tumor grade, tissue type, lymphatic invasion and metastasis and its sensitivity was higher than CEA. On the other hand, the Runx3 methylation level in post-operative serum significantly	Sakakura *et al*[63]

MALDI-TOF-MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SELDI: Surface-enhanced Laser Desorption Ionization; CTCs: Circulating tumor cells; cfDNA: Cell free DNA; ctDNA: Cell tumor DNA; MGMT: O6-methylguanine DNA methyltransferase; GC: Gastric cancer.

no methylation of the promoter of the latter gene in gastritis patients. 48% of GC and 59% of precancerous tissues presented with the p16 promoter methylation phenomenon, which indicated that the methylation of the promoter of this gene starts in the early stages of GC and its frequency was relatively high, further classifying it as a potential biomarker for the prediction of GC[59-62].

Sakakura *et al*[63] investigated the pre-operative and post-operative Runt related transcription factor 3 (Runx3) gene methylation level in 65 GC cases and found out that Runx3 methylation was detected in the pre-operative serum of 29% (19/65) GC patients and that the Runx3 methylation index was related to the tumor grade, tissue type, lymphatic invasion and metastasis and its sensitivity was higher than CEA. On the other hand, the Runx3 methylation level in post-operative serum significantly. Therefore, it can be seen that the detection of abnormal methylation of DNA can also be used as a tumor marker for cfDNA.

Other research work pertaining to the APC gene in GC[64] revealed no APC genes mutations while using MSP to analyze GC sample and GC cell lines showed that 82.5% of primary GC, GC adjacent mucosa of 97.5% of the patients and 10 GC cell lines had high APC 1A promoter methylation while the 1B promoter had not undergone methylation[65-69]. Due to methylation, in 10 GC cell lines, the 1A exon did not express APC while the IB exon expressed APC, suggesting that the 1A APC promoter methylation could be used as a biomarker for early stage GC.

CONCLUSION

The tremendous advances in technology have allowed the development of several methods to understand the mechanisms underlying gastric carcinogenesis, resulting in the identification of a large number of molecular targets such as CTC, ctDNA, serum markers that can be used as biomarkers with diagnostic and prognostic potential. Several of these (especially HER-2 amplification, miR-19a/b, miR-160a and p16 hyper-methylation) can also be used for the early indication of carcinogenic activity, prediction of therapeutic response or prognosis. However, most of the bio-markers being unraveled over time tend to show very low sensitivity when tested on a large population. Furthermore, many of these biomarkers, especially genetic markers such as miRNA, ctDNA and so on, have been tested in very restricted parameters since GC, as with other types of tumor, is highly influenced by ethnic and environmental factors, therefore making it even harder to find specific and unbiased markers for the disease. Therefore, the simplest approach at present is to validate the discovered markers in the target population and to use several biomarkers for each patient. The use of proteomic approach is very pragmatic whereby more emphasis is laid on protein expression, which is independent of the cause (genetic or epigenetic) of any altered pattern. However, there are some limitations to that approach, such as the availability of studies in only a few populations and the cost of the analysis, which remains very high.

There has been a lot of progress over the years in the mechanisms behind the correlation between methylation and GC, but so far there has been no concrete clinical use of GC gene methylation in the early diagnosis and intervention of GC. Currently methylation of circulating DNA is detected in whole blood, but a higher positive rate could be achieved with the enrichment and separation of CTC. While investigating possible biomarkers, various molecular features of GC have been unfolded, giving researchers more ground for research since the characteristics and molecular background of GC have been so vague and indistinctive.

Nevertheless, the detection and analysis of CTC and circulating GC cell DNA (ctDNA) and its methylation, have shown great promise for the early detection, evaluation of treatment efficacy and outcome in GC. The molecular changes in CTC and ctDNA could be the possible molecular targets as not only more sensitive and specific GC blood indicators, but also as targets for individualized treatment, hence opening up a new field for clinical application in oncology as Table 1. Hence, new and reliable proteomics techniques are encouraged in order to identify novel and unbiased serum biomarkers which not only would give us more perspective on the nature of GC, but would give us a better interface while dealing with GC patients.

With the development of technology, a large number of modern techniques of research can be applied in the serological diagnosis of tumor. Yet, the main important features to the serological diagnosis model should not only be high sensitivity and specificity, but also high maneuverability, reliability and repeatability. In order to investigate the feasibility of the new diagnostic technologies in the clinical diagnosis of tumors or GC, more clinical data needs to be processed to design a reasonable and repeatable validation model.

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