DNA Methylation Biomarkers in Serum for Gastric Cancer Screening

Ting Ye, Yingxuan Chen* and Jingyuan Fang

Department of Gastroenterology, Shanghai Jiao-Tong University School of Medicine, Ren-Ji Hospital, Shanghai Institute of Digestive Diseases, Shanghai 200001, P.R., China

Abstract: Epigenetic alterations occur throughout the development and carcinogenesis of gastric cancer. Therefore, the detection of methylated DNA markers is predicted to be able to detect gastric cancer earlier, and improve the risk assessment, surveillance, relapse, and prognosis of patients with gastric cancer. Detection of free methylated DNA also represents a promising approach for developing serum-based detection methods for the non-invasive monitoring of gastric cancer progression.

Keywords: DNA methylation, biomarker, serum, gastric cancer, proto-oncogene, tumor suppressor gene.

1. INTRODUCTION

Gastric cancer is one of the most common causes of death worldwide. Despite advances in the management of this disease, patient prognosis and survival remain poor due to detection of the primary tumor in its later stages. Although endoscopy is considered to be the most sensitive screening tool for detecting gastric cancer, its use is limited due to its considerable cost and risk, as well as patients' reluctance in undergoing this screening method. Therefore, a reliable, non-invasive test (preferably a blood test) would facilitate the screening and diagnosis of gastric cancer. Of the many new molecular approaches available for the early detection of gastric cancer, a serum assay of epigenetic events appears to be one of the most promising methods being developed. With an increase in the number and type of aberrant epigenetic events identified in the earliest tumor stages through the most advanced tumor stages, the potential for identifying tumor markers relevant for risk assessment, patient prognosis, therapeutic strategies, and patient follow-up have improved.

2. ALTERED PATTERNS OF DNA METHYLATION IN GASTRIC CANCER

Aberrant DNA methylation is recognized as the most important epigenetic change involved in the malignant transformation of gastric cancer. It affects global hypermethylation patterns, hypomethylation of proto-oncogenes, and hypermethylation of tumor suppressor genes (TSGs). Hypomethylation events can also influence chromosome stability, while aberrant methylation in the promoter regions of specific genes can affect the transcriptional regulation of various genes involved in the induction and promotion of cancer. In gastric cancer, altered methylation patterns have been detected for proto-oncogenes (*c-myc, c-Ha-ras*), TSGs (*APC, RASSF1A*) [1,2], cell cycle regulator genes (*p16, COX2*) [3,4], DNA repair genes (*hMLH1, MGMT*) [5], tissue invasion-related genes (*CDH1, TIMP-3, DAP-Kinase, THBS1*) [6-9], and metabolic enzymes (*GSTP1*) [10]. Aberrant methylation of these genes have also been found to occur more frequently in gastric carcinoma tissues than in adjacent, non-tumor samples [11], indicating that methylation events may play a role in the various stages of gastric cancer. For example, methylation of *p16*, *APC*, *CDH1*, and *MGMT* have been associated with the early stages of gastric cancer, while markers such as *hMLH1*, *COX-2*, and *p16* have been found to be methylated in the later stages of tumor progression. Advanced cases of gastric cancer have been associated with the methylation of *hMLH1*, *RASSF1A*, and *GSTP1* [3].

Recently, several novel genes were found to be frequently hypermethylated in cases of gastric cancer, including the TSGs, *BTG4* [12] and *ZIC1* [13]. Hypermethylation of the *BRCA1* and *PTCH1a* promoters were also associated with the early-onset of gastric carcinogenesis [14,15]. Similarly, methylation of *CHFR* and vimentin have been found to be significantly associated with the differentiation of gastric cancer [16,17]. A comparative analysis of DNA methylation patterns detected in primary vs. metastatic gastric carcinomas showed that *FLNC* was more frequently methylated in the latter than the former [18]. Promoter hypomethylation of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-related gene, *DR4*, was also associated with invasive gastric cancer, and may represent a valuable target gene for an anti-tumor therapy approach [19]. The number of genes aberrantly methylated in gastric cancer is growing rapidly. Moreover, with different methylation patterns associated with each stage of gastric cancer, and various gene functions affected, the identification of additional biomarkers will help to further refine the diagnosis of gastric cancer.

3. THE POSSIBLE USE OF METHYLATED DNA MARKERS IN SERUM FOR GASTRIC CANCER

More than three decades ago it was reported that levels of free DNA were higher in the serum of cancer patients than in healthy individuals [20]. The free DNA present was hypothesized to have been released from apoptotic and necrotic cancer cells [21]. In recent years, multiple genetic and epigenetic alterations have been detected in serum DNA in asso-

*Address correspondence to this author at the Department of Gastroenterology, Shanghai Jiao-Tong University School of Medicine, Shanghai 200001, P.R., China; Tel: (+86)-21-63200874; Fax: (+86)-21-63266027;
E-mail: yingxuanchen71@yahoo.com.cn

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ciation with the presence of lung tumors, neuroblastoma, head and neck, gastric, and colorectal cancers [22-28]. Since these alterations can have an important role in the development and progression of primary tumors, it has been suggested that a molecular analysis of changes in serum DNA levels in cancer patients may represent a useful, non-invasive approach to diagnose different types of cancer. Compared to genetic alterations, aberrant DNA methylation is the primary type of epigenetic change exhibited, with global hypomethylation and regional hypermethylation observed. In addition, higher serum DNA levels in relation to the tissue and tumor type involved may provide additional information. For hypermethylation of CpG islands in promoter regions, the resulting transcriptional silencing can lead to gene inactivation, and this is an important aspect of tumorigenesis. In contrast, hypermethylation is rarely observed in healthy individuals, with the exception of a few hypermethylation events that naturally occur with age, or in response to an environmental effect. Gastric cancer has previously been established as a major epigenetic disease. In addition, the inactivation of many TSGs, such as *E-cadherin*, *p16*, *hMHL1*, and *Runx3*, are frequently caused by DNA hypermethylation, rather than by gene mutations, in cases of gastric cancer [29]. Therefore, detection of promoter hypermethylation of TSGs in patients' serum may represent a promising biomarker for gastric cancers.

In serum samples from gastric cancer patients, the rates of methylation for *DAP-kinase*, *E-cadherin*, *GSTP1*, *p15*, and *p16* were 48.1%, 57.4%, 14.8%, 55.6%, and 51.9%, respectively. Detection of aberrant methylation also corresponded with methylation present in the corresponding tumor samples. For example, more than 65% of patients with aberrant methylation detected in the primary tumor also had hypermethylation of DNA detected in their serum. Furthermore, in general, more than 60% of serum samples from cancer patients were associated wtih aberrant methylation as a result of epigenetic alterations [27]. Promoter hypermethylation of *p16*, *E-cadherin*, and *RARβ* in serum samples from gastric cancer patients has been well-studied, with detection rates of 37%, 44%, 51%, and 48%, respectively, observed [30-33]. In addition, this panel was shown to be applicable to the early detection of tumor recurrence as well [34]. Among the many genes altered by methylation in gastric cancer, *p16* promoter methylation has been identified as an early event in carcinogenesis [3]. For example, a recent study reported that methylation of the *p16* promoter was detected in the serum of 60.9% of patients with tumor methylation, which accounted for 26.9% of the patients analyzed [35]. Another study reported a detection rate of 55% for the methylation of *APC*, *E-cadherin*, *hMLH1*, and *TIMP3*, in serum samples from patients with gastric cancer [36]. More recently, the hypermethylation of *RASSF1A*, *Runx3*, and *Reprimo* have also been detected in the serum or plasma of gastric cancer patients [28,37,38]. In all of the studies described, aberrant methylation was not detected in any of the control serum samples, indicating that the specificity of the assays performed was high. However, it is difficult to identify which gene or panel of genes are the best set of markers to use for the diagnosis of gastric cancer. Further screening studies will be needed to determine that aspect.

In addition to the detection of gastric cancer, aberrant methylation of various genes has also been shown to detect pre-malignant gastric lesions. These targets include *p15*, *p16*, and *E-cadherin* [39]. Moreover, methylated *SFRP2* has recently been detected in 66.7% of serum samples from gastric cancer patients in association with cases of intestinal metaplasia (IM), a pre-neoplastic gastric lesion [40]. Therefore, DNA methylation of these genes assayed in patient serum samples may also represent possible markers for risk assessment of gastric cancer cases. Similarly, serum analysis could also be used in assessments of cancer prognosis, to monitor disease recurrence following surgical resection, or to evaluate the efficacy of chemotherapy. For example, methylation of *DAP-kinase* has been shown to correlate with nodal metastasis, advanced cancer stages, and a reduced event-free survival rate [8]. The *MAL*, *PCDH10*, and *COX2* genes have also been shown to be associated with the survival rate of patients with gastric cancer [4,41,42]. Moreover, the loss of function of genes involved in cell cycle and apoptosis regulation, as well as intercellular interactions, can also contribute to cancer cell growth and metastatic potential.

Hypermethylation of *E-cadherin* has been significantly associated with shorter disease-free survival (DFS) rates in node-positive diffuse gastric cancer cases [43], and with early recurrence in patients with gastric stromal tumors [44]. In addition, the detection of both methylated *APC* and *E-cadherin* in the serum of patients with gastric cancer has been shown to identify patients with a negative prognosis [36]. Using the quantitative methylation-specific PCR (MSP) assay, the concentrations of methylated *APC*, *hMLH1*, and *TIMP3* were also found to be at higher levels in patients with advanced stages of gastric cancer [36]. In contrast, hypermethylation of the *p16* promoter was less frequently detected in well-differentiated tumors [35]. Taken together, these studies indicate that an analysis of DNA methylation patterns in patient serum samples could provide markers for the prediction of patient prognosis in cases of gastric cancer. However, results from other studies did not find an association between DNA hypermethylation and advanced malignant tumor behavior or metastasis [27]. Therefore, clarifying the mechanism(s) responsible for the presence of free tumor DNA in patient serum may elucidate the relationship between markers and tumor prognosis, and would also allow the effectiveness of these prognostic markers to be evaluated.

Another aspect to consider is the possible application of hypermethylated markers to assess a patient's response to chemotherapy. For example, DNA repair genes such as O^6-methylguanine methyltransferase (*MGMT*) and *MLH1* have been evaluated in a recent study [5]. For *MGMT*, following hypomethylation, a subsequent increase in gene expression was observed, which corresponded with a decreased response to alkylating agents. This response reflects an enhanced repair of drug-induced DNA damage. In contrast, hypermethylation of *MLH1* reduces its expression thereby leading to an increased resistance to DNA-damaging agents [5]. While these two targets may represent potential markers for assessing a patient's response to chemotherapy, their detection in patient serum in cases of gastric cancer has not been well-characterized. Therefore, additional long-term

Table 1. Methylated Genes Detected in the Serum of Patients with Different Stages of Gastric Cancer

Detection/Application	Gene name
Early tumor stages	p16, APC, CDH1, MGMT [3], BRCA1 [14], PTCH1 [15], SFRP2 [40]
Differentiation	CHFR, Vimentin [16-17]
Invasion and metastasis	FLNC [18], DR4 [19], DAP-kinase [8]
Advanced tumor stages	hMLH1, RASSF1A, GSTP1 [3], APC, TIMP3 [36], DAP-kinase [8]
Survival prediction	DAP-kinase [8], MAL [41], PCDH10 [42], COX-2 [4], E-cadherin [43]
Recurrence prediction	p16, E-cadherin [36], RARβ [34]
Chemotherapy prediction	MGMT, MLH1 [5]

studies are needed to determine the role of these markers in evaluating gastric cancer therapy.

4. PROBLEMS AND FUTURE PERSPECTIVES

In summary, as shown in Table **1**, methylated DNA detected in patients' serum represents a useful set of biomarkers for the diagnosis, prognosis, and risk assessment of patients with gastric cancer. Accordingly, this information can also help select the most appropriate therapeutic strategy and improve patient monitoring during follow-up. There are many advantages to detecting aberrant DNA methylation events in patient serum. First, compared with conventional serum tumor markers, such as carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9, methylation markers are associated with higher levels of sensitivity and specificity [30,31]. The detection rate using MSP assays has been shown to be better than that of RT-PCR in detecting CEA in blood [30,31], demonstrating that MSP assays can detect changes in methylation based on low levels of circulating tumor DNA [45]. Secondly, compared with mRNA and protein, DNA is a more stable molecule and its methylation status can be quantitated. Third, in contrast with mutations, the incidence of aberrant methylation is higher, with smaller, specific regions of the genome effected [46], thereby making it is easier to detect methylated targets.

Despite these advantages, there are still drawbacks to the clinical application of detecting methylated DNA in serum. One of the main drawbacks is false positives, which may be associated with patient age, environmental factors, or the analysis method employed. Another drawback is that the source of the circulating tumor DNA detected is not readily determined in the DNA methylation assay. Other studies have also shown that despite the high specificity associated with this method, its sensitivity is relatively low. Although there are many analysis techniques for the detection of cancer, they are all associated with their own shortcomings. In addition, no method provides equally high levels of sensitivity and specificity. While some groups have proposed that the detection of methylated DNA in patient serum, in addition to detection of traditional serum markers such as CEA, could improve patient diagnosis, correlation between the two sets of targets has not been observed [28]. Therefore, with so many assays able to detect and quantitate gene methylation, a standardized and simplified protocol needs to be developed and evaluated using external quality assurance programs for clinical application.

As a result of the human genome sequencing projects, the spectrum of possible epigenetic variants has expanded. Currently, the most promising DNA methylation biomarkers for tumors include: methylated *GSTP1* for the early diagnosis of prostate cancer, methylated *PITX2* for predicting the prognosis of lymph node-negative breast cancer patients, and methylated *MGMT* for predicting the benefits associated with the use of alkylating agents by patients with glioblastomas. Despite these markers being well-studied, however, they have not been applied in the clinic [47]. The aim of this study was to identify specific methylation makers in the serum of patients with gastric cancer. While a growing number of associated genes are being studied, extensive research is also needed to exclude age-related gene methylation events, to identify the most effective genes, and to determine the minimum panel of genes that would be clinically useful. Correspondingly, a recent report describes the detection of gene methylation events in gastric washes, where hypermethylation of *MINI25* was associated with rates of sensitivity and specificity of 90% and 96%, respectively [48]. Thus, another method for detecting the presence of gastric cancer has been identified. The overall goal, however, remains to identify specific DNA methylation events in the progression of gastric cancer that can be clearly identified, ideally from patient serum samples, and which will result in a signficant improvement in the survival rate of gastric cancer patients.

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