TOPIC HIGHLIGHT

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Methylation-mediated gene silencing as biomarkers of gastric cancer: A review

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Abstract

Despite a decline in the overall incidence of gastric cancer (GC), the disease remains the second most common cause of cancer-related death worldwide and is thus a significant global health problem. The best means of improving the survival of GC patients is to screen for and treat early lesions. However, GC is often diagnosed at an advanced stage and is associated with a poor prognosis. Current diagnostic and therapeutic strategies have not been successful in decreasing the global burden of the disease; therefore, the identification of reliable biomarkers for an early diagnosis, predictive markers of recurrence and survival and markers of drug sensitivity and/or resistance is urgently needed. The initiation and progression of GC depends not only on genetic alterations but also epigenetic changes, such as DNA methylation and histone modification. Aberrant DNA methylation is the most well-defined epigenetic change in human cancers and is associated with inappropriate gene silencing. Therefore, an increasing number of genes methylated at the promoter region have been targeted as possible biomarkers for different purposes, including early detection, classification, the assessment of the tumor prognosis, the development of therapeutic strategies and patient follow-up. This review article summarizes the current understanding and recent evidence regarding DNA methylation markers in GC with a focus on the clinical potential of these markers.

Core tip: This article summarizes the current understanding and recent evidence regarding DNA methylation markers in gastric cancer (GC) and includes our previous works. Current diagnostic and therapeutic tools of GC have not been successful in decreasing the global burden of this disease; however, it is promising that the early diagnosis and careful selection of patient subsets prior to initiating chemotherapy is a key factor for improving the outcomes of patients with GC. Methylation biomarkers would be useful for different purposes, including early detection, classification, assessment of the tumor prognosis, the development of therapeutic strategies and patient follow-up.

INTRODUCTION

Although the incidence of gastric cancer (GC) has declined on a yearly basis, it remains the fourth most com-

Table 1 Commonly methylated genes in gastric cancer tissue compared with normal tissue

Gene	Frequency (%)	Assay	Function	Ref.
ITGA4	96.0	Q-MSP	Other	[19]
ZIC1	94.6	MSP	Transcriptional regulation	[20]
PRDM5	88.0	MSP	Cell cycle	[21]
PCDH10	82.0	MSP	Apoptosis	[22]
TFPI2	80.9	MSP	Other	[23]
RUNX3	75.2	Q-MSP	Transcriptional regulation	[24]
SPINT2	75.0	MSP	Cell growth/ differentiation	[25]
BTG4	73.7	MSP	Other	[26]
SFRP2	73.3	Q-MSP	Apoptosis	[27]
hMLH1	72.9	MSP	DNA repair	[28]
DKK-3	67.6	MSP	Wnt pathway	[29]
TCF4	67.0	Pyrosequencing	Cell cycle	[30]
GRIK2	66.6	Q-MSP	Cell adhesion/invasion/migration	[31]
RARβ	65.8	MSP	Retinoic acid pathway	[32]
CHFR	65.0	Q-MSP	Other	[33]
BNIP3	65.0	Q-MSP	Apoptosis	[33]
RASSF1A	61.8	MSP	Ras pathway	[14]
LRP1B	61.0	Q-MSP	Other	[34]
SFRP5	56.0	Q-MSP	Wnt pathway	[19]

MSP: Methylation-specific PCR; Q-MSP: Quantitative methylation-specific PCR.

mon cancer and the second leading cause of cancer-related death worldwide[1]. Recent progress in early diagnosis, surgical techniques and perioperative management have improved patient satisfaction and outcomes; however, GC remains a major clinical challenge due to its high prevalence, poor prognosis and limited treatment options. The case fatality rate of GC is higher than that of other common malignancies, such as colorectal, breast and prostate cancer[2]. Because the prognosis of patients with GC depends on the tumor stage at diagnosis and the selection of an appropriate treatment strategy, the identification of novel biomarkers for early diagnosis and predictive markers of drug sensitivity is urgently needed.

Accumulating evidence suggests that cancer is caused by both epigenetic and genetic abnormalities[3]. DNA methylation, a major epigenetic modification that is strongly involved in the control of gene expression, is an early landmark event in carcinogenesis[4]. DNA methylation is a covalent chemical modification catalyzed by enzymes of the DNA methyltransferase family that mediate the addition of a methyl (CH_3) group to the fifth carbon of the cytosine. DNA methylation usually occurs within the context of 5'-CG-3' (CpG dinucleotide) clustered regions known as CpG islands (CGIs), which are frequently found proximal to the promoters of housekeeping genes[5]. The occurrence of methylation within the CGI of a gene promoter is associated with a compact chromatin structure and is linked to the transcriptional silencing of the affiliated gene, particularly tumor suppressor genes[6]. Numerous studies have reported that many tumor suppressor genes, which play key roles in functions pertaining to cancer prevention (DNA repair, cell adhesion, cell cycle control and apoptosis), are silenced by the hypermethylation of their promoters during carcinogenesis[7]. Therefore, it is reasonable to hypothesize that the DNA methylation status of certain genes serves as a useful biomarker for predicting tumor behavior. Furthermore, DNA methylation biomarkers offer several advantages over genetic and serum markers[8,9]. First, the incidence of aberrant DNA methylation of specific CGIs is higher than that observed in genetic abnormalities[9,10]. Second, the aberrant DNA methylation observed in cancer can be sensitively detected using a simple technique, methylation-specific PCR. Third, aberrant DNA methylation appears to occur in early-stage tumors, causing the loss- and/or gain-of-function of key processes and signaling properties[4].

In this review, we summarize the current status and recent evidence regarding DNA methylation biomarkers in GC and assess the clinical potential of these biomarkers for use in risk assessment, early diagnosis and the evaluation of treatment and prognosis in patients with GC.

FREQUENTLY METHYLATED GENES IN GC

The methylation of gene promoters is now recognized to be one of the primary mechanisms used to inactivate tumor-related genes (particularly tumor suppressor genes) as well as genetic alterations. Since the first article by Fang *et al*[11] in 1996 describing the DNA hypomethylation of *c-myc* and *c-Ha-ras* in GC, numerous studies have been published on the involvement of DNA methylation in GC. To date, the aberrant DNA methylation of more than 100 genes, such as tumor suppressor genes (including *E-cadherin*, *RASSF1A*, *p16*, *GSTP1*, *SOCS1*, *SFRP1* and *PTEN*) has been reported in GC[12-17]. A recent meta-analysis[18] reviewed 143 case-control studies reporting the methylation frequency of 142 individual genes. Consequently, a total of 70 genes were found to be significantly hypermethylated in cancer tissue compared with those observed in normal tissue obtained from GC subjects. Table 1 shows a list of genes that are commonly methylated in GC compared with those in corresponding normal gastric tissue[14,19-34].

E-cadherin is one of the most important tumor suppressor genes in GC, and its inactivation is thought to contribute to tumor progression *via* subsequent increases in proliferation, invasion and metastasis[35-37]. *E-cadherin* is silenced by CpG methylation in various cancers, including GC and particularly undifferentiated GC, at the early stage[12,13]. However, *E-cadherin* methylation is frequently observed in both neoplastic and the corresponding non-neoplastic gastric mucosa. Age-related *E-cadherin* methylation is commonly present in the gastric mucosa starting from approximately 45 years of age[38]. However, *RASS-*

F1A, a member of the Ras association domain family, is a tumor suppressor gene that plays a critical role in cell cycle regulation, apoptosis and microtubule stability by regulating the Ras signaling pathway[39]. Mutations of *RASSF1A* are uncommon, whereas silencing by promoter methylation is frequent in cancers, including GC[40,41]. In contrast, such methylation occurs in only a small proportion of non-neoplastic gastric epithelia[42]. Moreover, *RASSF1A* methylation is closely associated with the TNM stage and a poor prognosis in GC patients[43]. Therefore, *RASSF1A* represents a potential diagnostic and therapeutic target in GC.

GENE METHYLATION AND CARCINOGENESIS

Helicobacter pylori infection

It has been reported that a widely known pathogen, *Helicobacter pylori* (*H. pylori*), is involved in the development of GC[44]. *H. pylori* is a Gram-negative spiral-shaped bacterium that is present in the stomach of approximately half of the world's population[45,46]. Numerous prospective studies have shown that *H. pylori* infection plays an essential role in gastric carcinogenesis[47], and the mechanisms underlying gastric carcinogenesis due to *H. pylori*-induced DNA methylation have been clarified. Maekita *et al*[48] collected tissue samples of gastric mucosa from 154 healthy volunteers and 72 patients with differentiated-type GC *via* endoscopy and evaluated the methylation levels in seven CGIs among eight lesions. The data indicated that *H. pylori* infection potently and temporarily induces the methylation of multiple CGIs to various degrees and that the methylation levels in specific CGIs in noncancerous gastric mucosa are associated with the risk of GC in *H. pylori*-negative individuals. Our previous study also demonstrated that *H. pylori* infection contributes to the loss of *RUNX3* expression *via* promoter methylation in GC[49]. In *RUNX3*-deficient mouse, some gastric epithelial cells differentiated into intestinal type cells, suggesting that the loss of *RUNX3* expression triggered precancerous intestinal metaplasia (IM), which possibly leads to cancer in the stomach[50]. This speculation is supported by independent evidence recently published by Lu *et al*[24]. Furthermore, regardless of the status of *H. pylori* infection, the number of methylated genes in IM was significantly higher than that found in chronic gastritis without IM[51]. However, recent studies have intensively investigated the role of miRNA methylation in GC. Ando *et al*[52] demonstrated that gastric mucosa infected with *H. pylori* exhibits significantly higher methylation levels of three miRNAs (miR-124a-1, miR-124a-2 and miR-124a-3) than that without *H. pylori* infection among healthy volunteers and that the noncancerous gastric mucosa of GC patients exhibits higher methylation levels than the gastric mucosa of healthy volunteers among *H. pylori*-negative individuals. Therefore, the methylation-induced silencing of miRNA genes, as well as protein-coding genes,

may contribute to the formation of field defects for GC. With respect to the correlation between the methylation level in the gastric mucosa and risk of GC, Nakajima *et al*[53] showed that the methylation levels in the gastric mucosa are significantly increased in patients with a single GC and even more prominently increased in patients with multiple GCs among *H. pylori*-negative individuals. In contrast, the methylation levels in *H. pylori*-positive individuals were increased to various degrees. Moreover, it has previously been shown that the development of inflammation triggered by *H. pylori* infection is pivotal for aberrant methylation and that the expression of inflammation-related genes, such as *IL-1β*, *Nos2* and *TNF* in the stomach is associated with the induction of DNA methylation[54,55]. IL-1β directly induces the promoter methylation of *E-cadherin*, an important extracellular matrix component involved in the maintenance of epithelial stability[56]. Namely, the *H. pylori*-induced methylation of *E-cadherin* promoter is mediated through IL-1β. Furthermore, IL-1β is an important mediator of *H. pylori*-induced *TGF-β1* methylation[57-59]. As previously described, basic and clinical studies have demonstrated that *H. pylori* infection is strongly correlated with aberrant methylation in GC. Meanwhile, it has become increasingly clear that the eradication of *H. pylori* significantly reduces gene methylation[60,61]. The removal of the aberrant DNA methylation induced by *H. pylori* in precancerous lesions would be a novel approach to preventing cancer. Niwa *et al*[62] showed that 5-aza-dC treatment prevents the development of *H. pylori*-induced GC using a Mongolian gerbil model. Therefore, the removal and/or suppression of *H. pylori*-induced aberrant DNA methylation may prevent *H. pylori*-associated cancers.

Epstein-barr virus

Epstein-barr virus (EBV) causes infectious mononucleosis on initial infection, and more than 90% of adult patients become EBV carriers[63]. It is well known that EBV is associated with several malignancies, such as Burkitt lymphoma[64] and nasopharyngeal carcinoma[65]. EBV-positive GC was first discovered in 1990[66], and the incidence of EBV-positive GC ranges from 7% to 15% regardless of race or region[67,68]. EBV infection occurs at an early stage of carcinogenesis and plays an important role in cancer development. Furthermore, previous studies have reported that aberrant promoter methylation is more frequently involved in EBV-positive GC than EBV-negative GC[69-72]. DNA methylation of the promoter region in the *APC*, *p16*, *MINT1*, *TP73* and *HOXA10* genes[19,69,73] has been specifically observed in patients with EBV-positive GC. Moreover, one recent study demonstrated that the frequency of the methylation of seven genes (*TP73*, *BLU*, *FSD1*, *BCL7A*, *MARK1*, *SCRN1* and *NKX3.1*) is significantly higher in EBV-positive GC patients than in EBV-negative GC patients[74]. Regarding the molecular mechanisms underlying host DNA methylation during the early stages of EBV infection in gastric epithelium, human DNA methyltransferases

(DNMTs) have received attention as methylation drivers during EBV infection. It has been reported that LMP2A induces the phosphorylation of STAT3, which activates DNMT1 transcription and causes a loss of the PTEN expression *via* the methylation of the *PTEN* promoter in EBV-associated GC[75]. Zhao *et al*[76] also showed that the induction of promoter methylation by EBV is regulated by the upregulation of DNMT3b by LMP2A. Furthermore, a recent large-scale analysis performed by Matsusaka *et al*[77] assessed the DNA methylation profiling of GC using the Infinium Human Methylation27 BeadChip (Infinium, Illumina, San Diego, CA, United States). The authors classified GC into three epigenotypes (EBV⁻/low methylation, EBV⁻/high methylation and EBV⁺/high methylation) according to the pattern of DNA methylation. EBV-positive GCs exhibited distinct and markedly high levels of methylation, while the *CXXC4*, *TIMP2* and *PLXND1* genes were specifically methylated in the EBV-positive epigenotype. *MLH1* was preferentially methylated in the EBV⁻/high methylation epigenotype; however, no methylation was detected in the EBV⁺/high methylation epigenotype. Namely, the authors identified the specific genotype associated with EBV infection and proved that the epigenetic alteration observed in EBV-positive GC is directly caused by EBV infection.

DNA METHYLATION MARKERS FOR DIAGNOSIS

Early detection
Less than 25% of GC cases are diagnosed at an early stage, and the 5-year survival rate is approximately 20%-25% worldwide[78]. However, the survival rate improves to over 60% if the disease is detected at an early stage[78], emphasizing the importance of making an early diagnosis of GC. Bodily fluids, particularly blood, contain various molecules that originate from other tissues and organs. These molecules can signal the presence of cancer and therefore are potential cancer biomarkers. First-generation tumor markers, such as CEA, CA19-9 and CA72-4, are associated with the development of GC; however, none of these markers are available for the purpose of screening and early detection of GC. Nucleic acids, which can be easily amplified using the PCR technique, represent obvious potential targets for biomarker development. The detection of circulating tumor DNA released from apoptotic or necrotic tumor cells or actively secreted from proliferating cells was first reported approximately three decades ago[79]. Circulating tumor DNA carries not only tumor-specific genetic information but also epigenetic marks, particularly DNA methylation. The use of DNA methylation tests in GC patients would facilitate the development of novel biomarkers for the early detection and diagnosis of GC.

Abbaszadegan *et al*[80] evaluated the methylation of *p16*, one of the most frequently methylated genes in GC, in cancer tissues and the corresponding serum obtained from GC patients. Aberrant methylation of the *p16* promoter was detected in 44.2% of the GC tissues (23/52), whereas all normal gastric mucosa samples (*n* = 50) were unmethylated. Furthermore, among the patients with *p16* methylation, 60.9% (14/23) also exhibited methylation in their corresponding serum samples. Therefore, the detection of DNA methylation in the serum is a potential biomarker for the early detection of GC. Kanyama *et al*[81] also demonstrated that the aberrant DNA methylation of *p16* was observed in six of 23 serum samples obtained from GC patients (26.1%), regardless of the tumor stage and/or clinicopathological features, thus suggesting that *p16* methylation is also a potential biomarker for the early detection of GC. Additionally, other methylation markers in the serum, such as *RUNX3*, *MGMT*, *DAPK*, *TFPI*, *RASSF1A* and *SOCS1*, were reported to be useful for the early detection of GC[82-87]. Leung *et al*[88] investigated multiple gene methylation in the serum DNA of GC patients, focusing on the incidence of promoter hypermethylation in 10 tumor-related genes (*APC*, *E-cadherin*, *GSTP1*, *hMLH1*, *MGMT*, *p15*, *p16*, *SOCS1*, *TIMP3* and *TGF-beta RII*). Among these 10 genes, *APC* (17%), *E-cadherin* (13%), *hMLH1* (41%) and *TIMP3* (17%) were significantly methylated in the GC patients compared with that observed in the healthy volunteers. Furthermore, methylation was detected in the serum of 33 of 60 (55%) patients in at least one of these four genes. These results suggest that the detection of DNA methylation in the serum has diagnostic value in GC patients.

Another attempt regarding the early detection of GC was made in which methylation markers in gastric washes were investigated. Because many mucosal cells can be found in stomach juice, the detection of molecular markers in gastric washes is a possible noninvasive approach to screening for GC. Oishi *et al*[89] identified *Sox17* methylation to be a candidate biomarker for detecting GC using a genome-wide DNA methylation analysis of gastric wash samples. Moreover, Watanabe *et al*[90] suggested that *MINT25* is a sensitive and specific marker for screening GC. The authors initially assessed 24 tissue samples and identified six methylated genes (*MINT25*, *RORA*, *GDNF*, *ADAM23*, *PRDM5* and *MLF1*). The methylation levels of these six genes significantly increased according to the progression from non-neoplastic gastric mucosa to normal adjacent tissue to dysplasia and finally to early GC. The power of these six genes was validated in 153 different populations using gastric washes. Consequently, *MINT25* methylation exhibited the best sensitivity (90%) and specificity (96%) in terms of tumor detection in the gastric washes.

Prediction of recurrence
The postoperative recurrence of GC usually occurs in the peritoneum, lymph nodes (LNs) or liver[91]. Peritoneal metastasis is the most frequent event in recurrent GCs[91], and 50%-60% of GC patients with serosal invasion following curative resection eventually develop peritoneal metastasis[92]. Peritoneal metastasis exhibits resistance to

various chemotherapeutic agents, causes massive ascites and occasionally results in intestinal obstruction. The average length of survival following the development of peritoneal metastasis is 4.9 mo[93]. Therefore, the ability to predict peritoneal metastasis is essential for selecting the treatment strategy before metastatic nodules in the peritoneum become large. We previously reported that DNA methylation is a possible marker for detecting cancer cells in the peritoneal washes (PW) of GC patients[33]. In that study, the DNA methylation of six genes, including *BNIP3*, *CHFR*, *CYP1B1*, *MINT25*, *SFRP2* and *RASSF2*, was analyzed in 107 PW specimens obtained from GC patients who underwent surgery. The patients were classified into the following three groups: group A (*n* = 42), patients with a depth of cancer invasion at the muscularis propria (MP) or less than the MP; group B (*n* = 45), a depth of cancer invasion beyond the MP; and group C (*n* = 20), histologically diagnosed peritoneal metastasis or cytologically detected cancer cells in the peritoneal cavity. The methylation status of the six genes significantly differed between the three groups (group A, 0%-5%; group B, 0%-15%; group C, 15%-45%; *P* < 0.01). Furthermore, three of nine patients in group B in whom methylation was detected in PW in at least one of these six genes experienced peritoneal recurrence after surgery. However, only one of 36 patients without gene methylation experienced peritoneal recurrence (*P* < 0.05). Yu *et al*[94] also showed that the presence of methylated *E-cadherin* in PW predicts a poor prognosis in GC patients. Conducting methylation analyses and cytological examinations of PW is therefore useful for predicting the risk of recurrence in patients with GC.

LN metastasis is one of the most important factors in predicting recurrence in patients with GC[95]. The presence of micrometastasis, which cannot be detected using conventional histological methods, is significantly correlated with the postoperative prognosis of GC patients[96-98]. The detection of micrometastasis in LNs is therefore very important for making an accurate classification of the cancer stage and selecting the appropriate treatment strategy. Several studies have reported RT-PCR analyses to detect a positive expression of CEA, CK19 or CK20 mRNA in 5.3%-23.8% of LNs that are free from histological metastasis in patients with GC[99-101]. However, accumulating evidence suggests that gene methylation, such as that of *E-cadherin*[102], *SULF1*[103], *TFPI2*[86], *NDRG2*[104] and *GPX3*[105], is a useful biomarker for detecting LN metastasis in GC patients. Yi Kim *et al*[102] investigated the expression of E-cadherin in resected specimens obtained from 60 patients with early GC and correlated the findings with the metastatic potential of the tumors. Their data suggested that the methylation of *E-cadherin* is involved in LN metastasis in patients with GC. Furthermore, Hur *et al*[103] examined the expression of SULF1 and SULF2 in a large cohort of 450 GC tissues. The GC tissues exhibited a conspicuously higher expression of SULF1 (*P* = 0.0002) regulated by the promoter hypomethylation compared with that observed in the normal mucosa. Additionally, SULF1 is

an independent prognostic (*P* = 0.0123) and LN metastasis predictive factor (*P* = 0.0003) in patients with GC. Our previous study[106] also evaluated the methylation of multiple genes to detect the micrometastasis of GC. Methylation analyses are useful for accurately staging tumors and selecting optimal candidates for chemotherapy.

GENE METHYLATION AS A PREDICTIVE BIOMARKER FOR DRUG SENSITIVITY

Five-fluorouracil

In addition to several chemotherapeutic agents, 5-fluorouracil (5-FU) is widely used as a key drug in chemotherapy for advanced GC. 5-FU is converted into fluorodeoxyuridine monophosphate, which inhibits the activity of thymidylate synthase (TS), an enzyme required for DNA synthesis[107]. Several enzymes involved in the metabolic pathway of 5-FU, including TS, dihydropyrimidine dehydrogenase, thymidine phosphorylase (TP) and orotate phosphoribosyltransferase, have been intensively examined as candidate biomarkers for the efficacy of 5-FU therapy. Our previous work also focused on the correlation between the response to 5-FU and the expression of the above enzymes[108]. However, the predictive power of these molecules with respect to the efficacy of 5-FU remains controversial[109]. Therefore, it is necessary to establish new predictive tools for selecting patients who will benefit from treatment with this conventional cytotoxic drug.

We previously demonstrated that the methylation of *p16* predicts survival benefits from 5-FU in patients with GC[110]. p16 proteins, a product of the *CDKN2/MTS1* gene, are G1/S-specific cell cycle inhibitors[111]. *p16* silencing induced *via* DNA methylation accelerates the cell cycle and increases the cell number in the S-phase. These conditions allow cancer cells to easily absorb 5-FU into S-phase cells, resulting in the enhancement of drug efficacy. Another *in vitro* study showed that *p16* dysfunction is correlated with increased sensitivity to 5-FU in patients with brain tumors[112]. Furthermore, Kato *et al*[113] showed that the methylation of two proapoptotic genes, *TMS1* (target of methylation-induced silencing; also called "*ASC*") and *DAPK* (death-associated protein kinase), is associated with resistance to 5-FU and a poor prognosis in GC patients. The expression of *ASCL2* (achaete scutelike 2), a cancer stem cell (CSC) marker in colon cancer[114], is also mediated *via* epigenetic mechanisms, and the upregulation of *ASCL2* by promoter demethylation in GC is associated with resistance to 5-FU[115]. CSCs are relatively resistant to commonly used cancer therapies, such as radiation and chemotherapy. Therefore, the development of reliable CTC biomarkers and CSC-targeted therapies is crucial for improving survival in cancer patients.

Multigene methylation has also been investigated as another approach for developing predictive biomarkers for a response to 5-FU. The prognostic relevance of the

CpG island methylator phenotype (CIMP) following the administration of standard therapy or a 5-FU-based chemotherapy regimen has been reported in different types of cancers, including GC[70,116-118]. Although no direct correlations between the CIMP status and chemosensitivity and/or resistance have thus far been demonstrated in patients with GC, the detection of the CIMP-positive phenotype can be used to independently predict better survival after 5-FU-based chemotherapy in patients with stage III colorectal cancer[116]. Moreover, the CIMP status in GC has been shown to be associated with microsatellite instability and clinicopathological features, such as tumor location, histology and the TNM stage[70,117]. Further investigations should clarify the role of the CIMP status in the chemosensitivity and/or resistance of GC.

Cisplatin

Cisplatin is a platinum-containing compound that is commonly used in various cancers, including GC. The use of cisplatin-based regimens, such as ECF (epirubicin/cisplatin/fluorouracil) and DCF (docetaxel/cisplatin/fluorouracil), has improved the survival of GC patients[119]. Cisplatin forms intrastrand and interstrand cross-links between purines, and these platinum-DNA adducts activate several signaling pathways, resulting in double-stranded DNA breaks and the impairment of replication and gene expression[120]. The initial approach to identifying biomarkers of cisplatin sensitivity focused on the genes involved in the drug's mechanisms of action. *ERCC1* (excision repair cross-complementation group 1) is a major component of the nucleotide excision repair (NER) complex that acts as the rate-limiting enzyme in the NER pathway[121]. De Dosso *et al*[122] showed that the overall survival of GC patients who receive cisplatin-based adjuvant chemotherapy following curative resection is longer among patients who exhibit a negative expression of ERCC1 proteins. A recent meta-analysis also indicated a significant association between polymorphisms of the NER genes (*ERCC1* and *ERCC2*) and the response to platinum-based chemotherapy[123]. In GC patients, a high expression of NER (*ERCC1*, *ERCC2*, *GSTP1*, *XRCC1*) and DNA damage proteins (TP53, GADD45A) is negatively associated with a response to cisplatin[124,125].

Despite the intensive investigation of genetic markers for platinum agents (as described above), little information regarding the epigenetic variability affecting the clinical outcomes of platinum-based therapy is available in the setting of GC. One recent *in vitro* study evaluated the methylation profiles of 20 GC cell lines and identified *BMP4* (bone morphogenetic protein 4) to be an epigenetically regulated gene that is highly expressed in cisplatin-resistant cells[126]. *BMP4* encodes a secreted protein belonging to the TGFβ superfamily. BMP4 binds to BMP type I / II receptors, thereby activating a signaling cascade that results in the phosphorylation of SMAD1/5/8. BMP4 induces the epithelial mesenchymal transition *via* the SMAD pathway in several cancers[127,128]

and may also be involved in the development of IM of the esophagus and stomach[129]. Other epigenetic markers for CDDP have been frequently assessed in patients with ovarian and lung cancers[130-132]. Stefansson *et al*[131] recently showed that *BRCA1* silencing induced by promoter hypermethylation predicts enhanced sensitivity to platinum in ovarian and breast cancer patients. Tumor suppressor BRCA1 proteins as well as BRCA2 proteins play major roles in DNA double-strand break repair *via* homologous recombination[133]. *BRCA1* and *BRCA2* are frequently mutated in patients with familial breast and ovarian cancer, and *BRCA1/2*-deficient cancers often exhibit a better response to DNA cross-linking agents, such as platinum[133]. Furthermore, Wang *et al*[134] demonstrated that the methylation of *14-3-3 sigma* predicts survival benefits from cisplatin plus gemcitabine treatment in lung cancer patients. *BRCA1* and *14-3-3 sigma* silencing induced by promoter methylation is also observed in GC[69,135]. Further attempts to identify novel methylation biomarkers of the efficacy of cisplatin therapy in GC patients are needed.

Taxane (paclitaxel and docetaxel)

Paclitaxel and docetaxel, both of which are microtubule-stabilizing agents that block cell division by interfering with the function of mitotic spindles by inhibiting microtubule dynamics[136,137], are commonly used in second-line chemotherapy regimens in GC patients who exhibit resistance to 5-FU-based chemotherapy in Japan[138]. Checkpoint with forkhead-associated domain and ring finger (CHFR) has been intensively investigated as a candidate biomarker of the response to microtubule inhibitors in various cancers, including GC[139-141]. CHFR functions as a mitotic checkpoint by detecting mitotic stress induced by microtubule inhibitors, such as paclitaxel, and, under such conditions, induces cell cycle arrest in the G2 phase to repair damaged DNA and is consequently resistant to taxane[142]. However, cells with a *CHFR* gene inactivated by aberrant hypermethylation cannot detect DNA damage and proceed to mitosis with subsequent cell death due to mitotic catastrophe, *i.e.*, these cells demonstrate a high sensitivity to taxane. We previously demonstrated that the methylation of *CHFR* is correlated with the response to treatment with paclitaxel in both GC cell lines and patients[143].

Stone *et al*[144] demonstrated that, in addition to *CHFR* methylation, *BCL-2* expression is associated with DNA methylation in human breast cancer and that *BCL-2* hypermethylation is a potential biomarker of sensitivity to antimitotic chemotherapy, including that with paclitaxel and docetaxel. The promoter hypermethylation of *RASSF1A* as well as *BCL-2* was also shown to be an important factor modulating the efficacy of docetaxel-based chemotherapy in patients with breast cancer[145]. Furthermore, a recent *in vitro* study indicated that *TGFβI* (transforming growth factor-beta-inducible gene-h3) is frequently methylated and associated with paclitaxel resistance in patients with ovarian cancer[146]. However,

Irinotecan

Irinotecan (CPT-11) is a semisynthetic derivative of camptothecin (CPT), which interferes with DNA replication and cell division through its potent interaction with the enzyme topoisomerase 1 (Topo 1)[147]. Both CPT-11 and CPT are Topo1 inhibitors. CPT-11 is primarily used in second-line or subsequent chemotherapy in the treatment of GC.

Several mechanisms have been proposed for resistance to CPT, such as variable levels of the enzymes involved in the conversion of CPT-11, reduced cellular accumulation due to active drug efflux, a reduced expression of *Topo 1*, alteration in the structure of Topo 1, alterations in the cellular response to CPT-Topo 1-DNA complex formation and the activation of NF-kappaB[148,149]. To identify the epigenetic predictive markers for the efficacy of CPT-11, we previously analyzed the gene methylation of *CHFR*, *p16*, *RUNX3*, *E-cadherin*, *MGMT*, *hMLH1*, *ABCG2*, *UGT1A1* and *BNIP3* in 27 colorectal cancer patients who were postoperatively treated with S-1 plus CPT-11 combined therapy. Among these candidate genes, we identified *BNIP3* gene methylation as a possible marker for predicting a poor response to S-1 plus CPT-11 therapy in patients with colorectal cancer[150]. Another study also demonstrated that metastatic colorectal cancer patients with *BNIP3* methylation exhibit resistance to first-line CPT-11 chemotherapy compared with those without such methylation[151]. Furthermore, two independent studies suggested that the methylation of the *UGT1A1* gene in colorectal cancer patients is an important mechanism of *UGT1A1* gene silencing and is associated with CPT-11 resistance[152,153]. However, no reliable markers of the sensitivity and/or resistance to CPT-11 have thus far been identified in GC, including *BNIP3* and *UGT1A1* methylation. Recently, Miyaki *et al*[154] focused on the DNA methylation profiles of colorectal cancer and GC and identified a novel gene, *DEXI* (glucocorticoid-induced protein coding gene), that is frequently methylated in colorectal cancer and GC patients using the MS-AFLP (methylation-sensitive amplified fragment-length polymorphism) technique[155]. *DEXI* methylation results in a poor response to CPT-11-based chemotherapy, suggesting that *DEXI* is a potent therapeutic target and an epigenetic biomarker for the selection of patients who will benefit from CPT-11-based chemotherapy.

Trastuzumab and bevacizumab

Two recent phase Ⅲ RCTs demonstrated the clinical efficacy and safety of antibody therapy in patients with advanced GC. The ToGA study investigated the additional benefits of trastuzumab in combination with chemotherapy as a first-line treatment of human epidermal growth factor receptor 2 (HER2)-positive advanced GC. The median overall survival was 13.8 mo in the trastuzumab plus chemotherapy group and 11.1 mo in the chemotherapy alone group (HR = 0.74; 95%CI: 0.60-0.91; P = 0.0046)[156]. The ToGA trial also demonstrated that patients who express a higher level of HER2 receive the greatest benefits from trastuzumab. Although the efficacy of trastuzumab appears to be predictable based on the expression level of HER2, the molecular mechanisms underlying trastuzumab resistance in GC patients are unknown. Preclinical studies have indicated that increased signaling *via* the PI3K/AKT pathway contributes to trastuzumab resistance in patients with breast cancer[157,158]. Moreover, several reports have shown that DNA methylation profiles can be used to predict the breast cancer subtype and support the prognosis determination and therapeutic stratification of patients with breast cancer[159-161]. Terada *et al*[162] evaluated the methylation levels in the promoter CGIs of 11 genes in 63 human breast cancer samples and concluded that frequent methylation exhibits a strong association with HER2 amplification in patients with breast cancer. Based on this evidence, increasing the understanding of epigenetic profiles in the setting of GC may enable physicians to classify the intrinsic subtypes of GC and predict the response to trastuzumab in GC patients.

The AVAGAST trial, which was designed to investigate the impact of an anti-vascular endothelial growth factor (VEGF) monoclonal antibody (bevacizumab) in combination with fluoropyrimidine-platinum as first-line therapy for advanced GC showed significant improvements in progression-free survival and the response rate compared with that observed in the placebo group (HR = 0.80, P = 0.0037; 46.0% *vs* 37.4%, P = 0.0315, respectively)[163]. Additional analyses of biomarkers[164], including VEGF-A, neuropilin-1 and VEGF receptors-1 and -2 (VEGFR-1 and VEGFR-2), showed that the baseline plasma VEGF-A levels and tumor neuropilin-1 expression are potential predictors of bevacizumab efficacy. The patients with a high plasma VEGF-A level exhibited a trend toward improved overall survival (HR = 0.72; 95%CI: 0.57-0.93) compared with the patients with a low VEGF-A level (HR = 1.01; 95%CI: 0.77-1.31; P = 0.07). Furthermore, the potential predictive power of the plasma VEGF-A level identified in the AVAGAST trial is supported by two other independent biomarker analyses performed in patients with metastatic breast cancer (the AVADO study)[165] and pancreatic cancer (the AViTA study)[166]. However, the levels of VEGFRs failed to predict the efficacy of bevacizumab in the AVAGAST study, although the VEGFR-2 expression in the AVADO study and the VEGFR-1 expression in the AViTA study were significantly associated with bevacizumab efficacy[167,168]. In a previous study, the methylation of the *VEGF* gene was not observed in most cancer cells, whereas the promoter hypermethylation of the *VEGFR-1* gene (*Flt1*) and *VEGFR-2* gene (*KDR*) were widely detected in various cancer cells, including those of GC[169]. A recent *in vitro* study by Kim *et al*[170] also suggested that the efficacy of various VEGF-target drugs, including anti-VEGF

monoclonal antibodies, is influenced by the epigenetic alteration of *VEGFRs*.

Among the several molecular targeted therapies for GC to date, only trastuzumab has succeeded in significantly increasing the overall survival in patients with HER2-overexpressing GC. Other agents, including bevacizumab, have failed to achieve efficacy in increasing survival rates over those obtained with standard chemotherapy. It is promising that the careful selection of patient subsets is a key factor for improving GC outcomes. Therefore, further investigations to identify reliable predictive biomarkers of drug efficacy, including methylation profiling, are needed.

DNA METHYLATION AND THE PROGNOSIS OF GC PATIENTS

The most common tool for predicting patient prognosis of GC is the TNM classification. However, some GCs exhibit different behavior from that predicted by the TNM classification; hence, the accumulation of further information and the identification of indicators, such as molecular biomarkers, is necessary to precisely predict the prognosis. Recently, numerous studies have investigated the molecular basis of GC, and an association between aberrant DNA methylation and the prognosis of GC patients has been reported[171-174]. A large number of genes, including *p16*, *E-cadherin*, *MGMT*, *RASSF1*, *RUNX3*, *etc.*, have been shown to be suppressed by CGI hypermethylation[175]. Among these genes, the promoter hypermethylation of *E-cadherin*[176] (http://link.springer.com/article/10.1007/s10120-012-0209-7/fulltext.html - CR32) and *MGMT*[177,178] (http://link.springer.com/article/10.1007/s10120-012-0209-7/fulltext.html - CR34) is associated with worse outcomes after surgery in GC patients. Additionally, Wanajo *et al*[172] identified the methylation of *CACNA2D3* (calcium channel voltage-dependent alpha 2/delta subunit 3) to be a strong indicator of poor prognosis among patients with advanced GC. Furthermore, Kim *et al*[179] suggested that the expression of *galectin-7* is critically regulated by DNA hypermethylation and may play a role as a prognostic marker of GC. Our recent work also demonstrated that trefoil factor 1 (*TFF1*) expression is silenced by DNA methylation and is associated with tumor invasion and poor survival in GC patients[180]. *TFF1* is considered to be a tumor suppressor gene in GC. We assessed the immunohistochemical expression of TFF1 in 182 GC patients and examined whether the level of TFF1 is associated with clinicopathological factors and/or patient survival. Consequently, a low expression of TFF1 was found to be independently associated with a poor survival in 108 GC patients treated with surgery alone. Furthermore, bisulfite sequencing demonstrated that *TFF1* expression was strongly mediated by DNA methylation in both the GC cells and tissues. Although most previous studies evaluated DNA methylation in GC tissues, epigenetic in-

formation obtained from blood samples may also be an important prognostic biomarker in GC patients. It has been reported that patients with hypermethylated insulin-like growth factor 2 (*IGF2*) in blood leukocyte DNA exhibit a significantly better survival rate than those with hypomethylated *IGF2*[181].

As described above, many previous studies of methylation in GC have focused on the prognostic significance of single methylated genes. However, the *LINE-1* methylation level is regarded to be a surrogate marker of global DNA methylation, and *LINE-1* hypomethylation is strongly associated with a poor outcome in several types of human cancers[182,183] (http://link.springer.com/article/10.1007/s10120-012-0209-7/fulltext.html - CR14). Shigaki *et al*[184] reported for the first time that the genome-wide DNA hypomethylation status measured according to the *LINE-1* level is independently associated with poor survival among patients with GC. Furthermore, regarding the prognostic value of CIMP status, it has been reported that CIMP-high is associated with better overall survival, although this parameter is not an independent prognostic factor in patients with resected GC[117]. In another study, patients with a CIMP-negative status demonstrated significantly worse survival than patients with a CIMP-high or CIMP-low status among 78 primary GC series in Japan[70]. In contrast, two investigators reported that hypermethylation at 14 CGI loci or more is closely associated with a poor clinical outcome and was found to be an independent prognostic factor in 196 cases of GC in South Korea[185,186]. Moreover, another report demonstrated no significant differences in survival between the CIMP-positive group and the CIMP-negative group[187]. The CIMP status prognostic value is therefore controversial in the setting of GC. A large-scale study is needed to validate the association between CIMP status and the prognosis of patients with GC.

Although DNA methylation at the fifth position of cytosine (5-mC) is a key epigenetic marker associated with the prognosis of GC, 5-mC is converted to 5-hydroxymethylcytosine (5-hmC) by the ten-eleven translocation family of DNA hydroxylases. In recent years, the effects of 5-hmC on the characteristics of cancer have been widely recognized. However, the significance of 5-hmC for the prognosis of human cancers, including GC, remains largely unknown. Yang *et al*[188] indicated that a decreased 5-hmC level is a strong and independent poor prognostic factor in patients with GC. The direct or consequential methylation of microRNAs may also influence the prognosis of GC[189]. Prompt and reliable methods for the analysis of the molecular basis of GC have recently been developed, and the impact of epigenetic modification on the prognosis of GC patients has been increasingly revealed. Increasing understanding of epigenetic molecular profiles will hopefully be successfully applied to predict and improve the prognosis of GC.
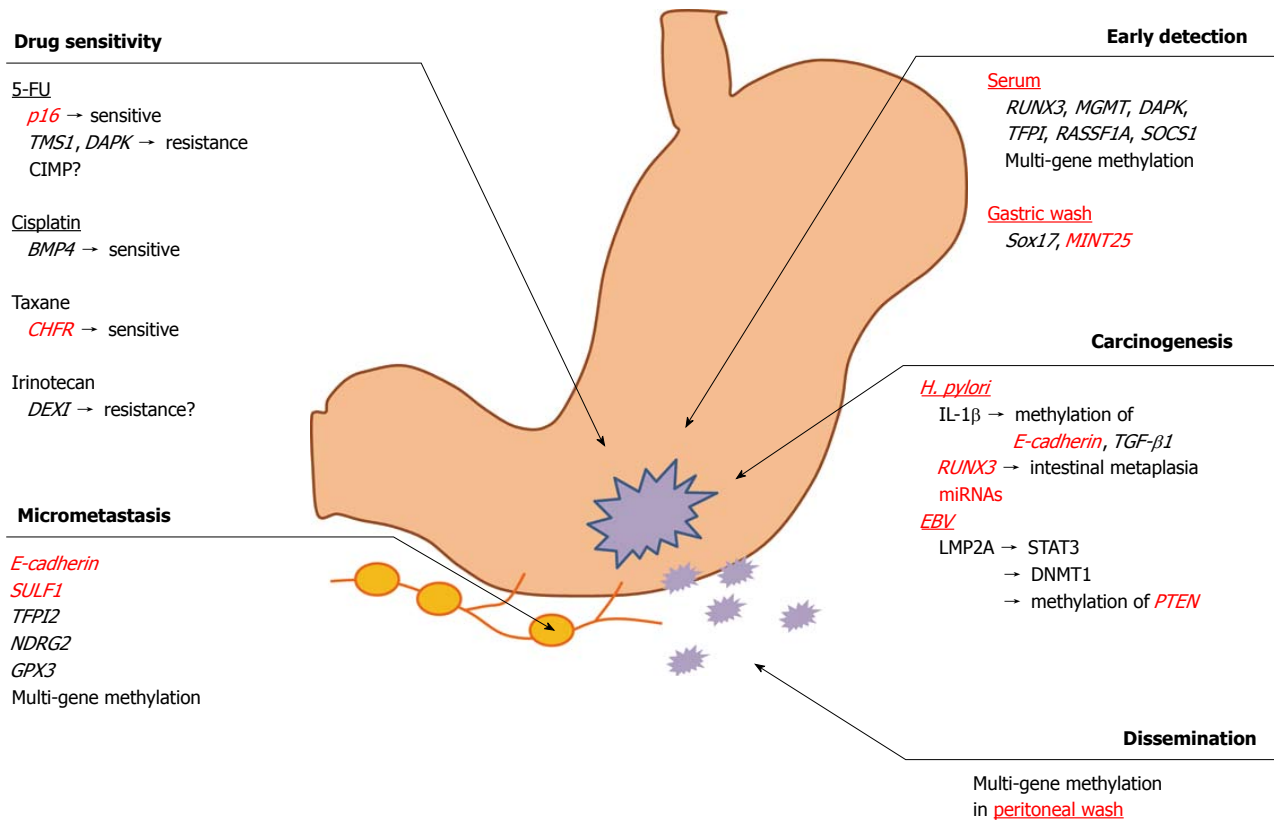
Figure 1 Candidate methylation biomarkers in gastric cancer. Summary of methylation biomarkers in gastric cancer. Numerous methylated genes are considered to be possible biomarkers for different purposes, such as risk assessment, early detection, the prediction of recurrence and prognosis and the development of therapeutic strategies. 5-FU: 5-fluorouracil; *H. pylori*: *Helicobacter pylori*; IL: Interleukin; EBV: Epstein-barr virus.

CONCLUSION

The field of cancer epigenetics has significantly expanded over the last decade, and technological advances have dramatically accelerated investigation in this area of cancer research. Aberrant DNA methylation results in the misregulation of cellular processes, such as proliferation, transformation and anti-apoptotic effects, all of which promote cancer progression. However, DNA methylation has great potential to provide valuable information for understanding the malignant behavior of GC (Figure 1). Further investigations of the DNA methylation status, which regulates cancer initiation, proliferation, invasion, metastasis and drug resistance, will aid in designing strategies for earlier detection and better therapeutic decision making in the setting of GC.

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