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Invited critical review

Gene methylation in gastric cancer

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ABSTRACT

Gastric cancer is one of the most common malignancies and remains the second leading cause of cancer-related death worldwide. Over 70% of new cases and deaths occur in developing countries. In the early years of the molecular biology revolution, cancer research mainly focuses on genetic alterations, including gastric cancer. Epigenetic mechanisms are essential for normal development and maintenance of tissue-specific gene expression patterns in mammals. Disruption of epigenetic processes can lead to altered gene function and malignant cellular transformation. Recent advancements in the rapidly evolving field of cancer epigenetics have shown extensive reprogramming of every component of the epigenetic machinery in cancer, including DNA methylation, histone modifications, nucleosome positioning, noncoding RNAs, and microRNAs. Aberrant DNA methylation in the promoter regions of gene, which leads to inactivation of tumor suppressor and other cancer-related genes in cancer cells, is the most well-defined epigenetic hallmark in gastric cancer. The advantages of gene methylation as a target for detection and diagnosis of cancer in biopsy specimens and non-invasive body fluids such as serum and gastric washes have led to many studies of application in gastric cancer. This review focuses on the most common and important phenomenon of epigenetics, DNA methylation, in gastric cancer and illustrates the impact epigenetics has had on this field.

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Abbreviations: 5-hmC, 5-hydroxymethylcytosine; 5-mC, 5-methylcytosine; *ADAM23*, ADAM metallopeptidase domain 23; *ADAMTS9*, ADAM metallopeptidase with thrombospondin type 1 motif, 9; *ARID1A*, AT rich interactive domain 1A (SWI-like); AML, Acute myelocytic leukemia; *ATBF1*, AT motif-binding factor 1; *APC*, Adenomatosis polyposis coli; BS, Bisulfite sequencing; *BNIP3*, BCL2/adenovirus E1B 19kDa interacting protein 3; *BTG4*, B-cell translocation gene 4; *BMP-2*, Bone morphogenetic protein 2; *CACNA1G*, Calcium channel, voltage-dependent, T type, alpha 1G subunit; *CACNA2D3*, Calcium channel, voltage-dependent, alpha 2/delta subunit 3; *CD44*, CD44 molecule (Indian blood group); *CDH1*, Cadherin 1 or E-cadherin; CDK4, Cyclin-dependent kinase 4; CDK6, Cyclin-dependent kinase 6; *CDKN1C*, Cyclin-dependent kinase inhibitor 1C; *CDKN2A*, Cyclin-dependent kinase inhibitor 2A; *CDX2*, Caudal type homeobox 2; *CHD5*, Chromodomain helicase DNA binding protein 5; *CHFR*, Checkpoint with forkhead and ring finger domains, E3 ubiquitin protein ligase; CGI, CpG islands; *C-MET*, Met proto-oncogene (hepatocyte growth factor receptor); *CMTM3*, CKLF-like MARVEL transmembrane domain containing 3; *CRBP1*, Retinol binding protein 1, cellular; CNS, Central nervous system; *DACT1*, Dapper, antagonist of beta-catenin, homolog 1 (*Xenopus laevis*); *DAPK*, Death-associated protein kinase; *Dkk-3*, Dickkopf 3 homolog (*Xenopus laevis*); DNA, Deoxyribose Nucleic Acid; DNMT, DNA methylatransferases; EBV, Epstein–Barr Virus; *ECRG4*, Chromosome 2 open reading frame 40; *EDNRB*, Endothelin receptor type B; EGCG, Epigallocatechin gallate; *ERBB4*, V-erb-a erythroblastic leukemia viral oncogene homolog 4; FDA, Food and Drug Administration; *FLNc*, Filamin C; GC, Gastric cancer; *GDNF*, glial cell derived neurotrophic factor; GI endoscopy, gastrointestinal endoscopy; *GPX3*, Glutathione peroxidase 3 (plasma); *GRIK2*, Glutamate receptor, ionotropic, kainate 2; *GSTP1*, Glutathione S-transferase pi 1; *HACE1*, HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1; *HAI-2/SPINT2*, Serine peptidase inhibitor, Kunitz type, 2; *hDAB2IP*, DAB2 interacting protein; HGFA, hepatocyte growth factor activator; *HLTF*, Helicase-like transcription factor; *hMLH1*, mutL homolog 1; *HOXA1*, Homeobox A1; *HOXA10*, Homeobox A10; *HoxD10*, Homeobox D10; *H. pylori*, *Helicobacter pylori*; *HRASLS*, HRAS-like suppressor; IGF-1, Insulin-like growth factor 1 (somatomedin C); IGF-1R, Insulin-like growth factor I receptor; *IGFBP3*, Insulin-like growth factor binding protein 3; *IL-1β*, Interleukin 1, beta; *ITGA4*, Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor); *KL*, Klotho; *KRAS*, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; *LMP2A*, latent membrane protein; *LOX*, Lysyl oxidase; *LRP1B*, Low density lipoprotein receptor-related protein 1B; MAPK, RAS/RAF/MEK/ERK; MBPs, Methyl-CpG binding proteins; MDS, myelodysplastic syndromes; *MINT25*, Matrix metallopeptidase 24 (membrane-inserted); *MGMT*, O-6-methylguanine-DNA methyltransferase; *MLF1*, Myeloid leukemia factor 1; *MLL*, Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); *LL3*, Myeloid/lymphoid or mixed-lineage leukemia 3; MMR, DNA mismatch repair; MSI, Microsatellite instability; MSP, Methylation-specific PCR; *NDRG2*, NDRG family member 2; *Notch 1*, Notch 1; *NPR1*, Natriuretic peptide receptor A/guanylate cyclase A; *NR3C1*, Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor); *p15*, Cyclin-dependent kinase inhibitor 2B; *p16*, Cyclin-dependent kinase inhibitor 2A; *p21*, Cyclin-dependent kinase inhibitor 1A; *p27*, Cyclin-dependent kinase inhibitor 1B; *p53*, tumor protein p53; *p73*, tumor protein p73; *PCDH10*, Protocadherin 10; *PCDH17*, Protocadherin 17; PI3K/Akt, phosphoinositide 3-kinase (PI3K)/Akt; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; *PRDM5*, PR domain containing 5; *PTCH1*, Patched 1; Q-MSP, Quantitative methylation-specific PCR; *RARRES1*, Retinoic acid receptor responder (tazarotene induced) 1; *RARβ*, Retinoic acid receptor, beta; *RASSF1A*, Ras association (RalGDS/AF-6) domain family member 1; *RASSF2*, Ras association (RalGDS/AF-6) domain family member 2; *Rb*, retinoblastoma; *RBP1*, Retinol binding protein 1, cellular; *RKIP*, Phosphatidylethanolamine binding protein 1; *RORA*, RAR-related orphan receptor A; *ROS*, reactive oxygen species; *RUNX3*, Runt-related transcription factor 3; SAM, S-adenosylmethionine; *SFRP2*, Secreted frizzled-related protein 2; *SFRP5*, Secreted frizzled-related protein 5; *SHP1*, Protein tyrosine phosphatase, non-receptor type 6; *SOCS-1*, Suppressor of cytokine signaling 1; STAT3, signal transducer and *activator of transcription-3*; *SYK*, Spleen tyrosine kinase; *TCF4*, Transcription factor 4; TET, ten-eleven translocation; *TFPI2*, Tissue factor pathway inhibitor 2; TGF-β, transforming growth factor-β; *TIMP3*, TIMP metallopeptidase inhibitor 3; *TP73*, Tumor protein p73; *TSP1*, Thrombospondin 1; TNM, Tumor Node Metastasis; *ZIC1*, Zinc finger protein of the cerebellum 1; *ZNF545*, ZFP82 zinc finger protein.

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Contents

1. Introduction

Gastric cancer is highly prevalent in Asia, particularly China, and is one of the leading causes of cancer-related death worldwide [1]. There are two main types of gastric adenocarcinoma: intestinal and diffuse. The accepted paradigm for the pathogenesis of the intestinal-type is a multistep progression from chronic gastritis to gastric atrophy to intestinal metaplasia to dysplasia. The pathogenesis of diffuse-type gastric cancer is not fully understood. Although *Helicobacter pylori* (*H. pylori*) infection is probably a predisposing factor [2], there are no known histologic precursor lesions of this type of gastric cancer. Gastric cancer is largely resistant to radio/chemo-therapy, and the main treatment consists of performing a gastrectomy. Although the recent diagnostic and therapeutic advances have provided excellent survival for patients with early gastric cancer, gastric cancer is usually diagnosed at an advanced stage and the prognosis is still poor [3]. Thus, a better understanding of the pathogenesis and molecular events of gastric cancer may lead to new diagnostic, therapeutic and preventive strategies to this disease.

Gastric carcinogenesis involves gradual accumulation of various genetic and epigenetic alterations, leading to gain-of-function in oncogenes and loss-of-function in tumor suppressor genes. Genetic alterations, such as *p53*, *KRAS*, *PIK3CA*, *ARID1A*, *MLL3* and *MLL* mutations, as well as *PIK3CA*, *C-MET*, *ERBB4*, and *CD44* amplifications, are frequently found in gastric cancer, suggesting that they may be key tumorigenic events and may play a critical role in gastric tumorigenesis [4–9]. A growing body of evidence now suggests that, in addition to genetic alterations, epigenetic alterations, including DNA methylation of CpG islands, post-translational modifications of histones, microRNAs, noncoding RNAs, and nucleosome positioning, are also involved in the initiation and progression of gastric cancer [10–13]. Epigenetic events, most prominently manifested by stable and heritable changes in gene expression that are not due to any alteration in the primary DNA sequence, signify the fundamental molecular principles in which genetic information is organized and read [14].

In the past decades, it has become increasingly evident that altered epigenetic control of gene expression plays a substantial role in many different diseases, including malignancies [13]. Gene transcription depends strongly on chromatin structure: the open or loosely coiled conformation has a permissive effect on transcription, whereas the closed conformation represented by tightly packed protein–DNA complexes is transcriptionally inactive. DNA methylation is the first epigenetic mark shown to be critically involved in the tumorigenesis [15], which provides a stable gene silencing mechanism that plays an important role in regulating gene expression and chromatin architecture, in association with histone modifications and other chromatin associated protein. Unlike DNA methylation, histone modifications lead to either transcriptional activation or repression depending upon which residues are modified and the type of modifications present. For example, lysine acetylation correlates with transcriptional activation, whereas lysine methylation leads to transcriptional activation or repression [16–18]. In this review, we take a comprehensive look at the current understanding of aberrant DNA methylation as it is the most extensively studied deregulated epigenetic mechanism in gastric cancer.

2. Mechanism of gene silencing mediated by promoter methylation

DNA methylation is the most extensively studied epigenetic modification in which a methyl group is added to the fifth carbon position of cytosine residue in a CpG dinucleotide. Clusters of CpG dinucleotides in GC rich regions of the genome called “CpG islands (CGI)” frequently occur in the 5′-flanking promoter areas of genes. The process of cytosine methylation is catalyzed by DNA methylatransferases (DNMTs) [19]. Currently, there are three established DNMTs: DNMT1, DNMT3a and DNMT3b. DNMT1 is involved in maintaining methylation by methylating newly synthesized strands of DNA during DNA replication, whereas DNMT3a and DNMT3b are mainly involved in de novo methylation [20]. In general, increased methylation in the promoter region of a gene leads to reduced gene expression, whereas methylation in the transcribed region has a variable effect on gene expression [21,22]. Several direct mechanisms have been proposed to account for transcriptional repression by promoter methylation. The first mechanism involves direct interference with binding of specific transcription factors to their recognition sites in their promoters [23,24]. The second possibility is that one family of proteins that recognize methyl-CpG, known as

methyl-CpG binding proteins (MBPs), can elicit the repressive potential of methylated DNA [25].

3. Factors affecting gene methylation in gastric epithelia

Considering the deep and wide involvement of aberrant DNA methylation of CGI in human cancers, therefore, any insightful understanding of aberrant methylation and subsequent gene silencing, such as methylation inducing factors, is essential for cancer prediction, prevention, treatment and prognosis evaluation. Increasing evidences have revealed the potential of some environmental factors, such as chemical pollutants, dietary components and other exogenous factors, to modulate the establishment and maintenance of epigenetic modifications, thereby leading to long-lasting effects [26]. Similarly, environmental factors are critical to the development of gastric cancer. It is clear that the major etiologic risk factor for gastric cancer is *H. pylori* infection. Marshall and Warren are awarded the 2005 Nobel Prize in Medicine and Physiology in part for their discovery of its causative role in gastric cancer. Currently, some contributing factors have been identified in the aberrant methylation process in gastric epithelia, such as aging, diet, chronic inflammation and microbial infection [27].

3.1. Aging

Age is an important risk factor in the development of gastric cancer. Tumor-related genes are rarely methylated in nonneoplastic gastric epithelia from young people, which, in contrast, are frequently found in older people. The association between decreasing global DNA methylation and aging has been reported in corroborating studies involving both human and animal models [28–30]. Moreover, aberrant methylation of several tumor-related genes is significantly associated with the age in gastric cancer, such as *CDH1* and *DAPK* [31]. However, it is noteworthy that age-related methylation of tumor-suppressor genes applies mostly to exonic or far upstream regions within a promoter CGI, and that, even within the same promoter CGI, a small region covering the transcription start site is kept unmethylated [32,33]. Rapidly expanding knowledge related to life-long cellular–environmental interactions has been observed during the last decades. However, the molecular mechanisms by which the environment sensitizes cells and its effects on human health and aging are not totally understood. The functional relationship between epigenetic modifications and aging still remains largely unknown, although the relationship between specific epigenotypes and disease phenotypes has been thoroughly studied [34].

3.2. Diet and physical activity

Dietary factors, such as folate deficiency and choline deficiency, are known to induce genomic hypomethylation, through induction of deficiency of methyl donors, such as S-adenosylmethionine (SAM) [35]. Methionine is an essential amino acid found in poultry, fish, and dairy products while folate is an essential nutrient found in fruits and vegetables [36,37]. Moreover, it has been reported that the prevalence of promoter methylation of *CDX2* and *BMP-2* is significantly higher in gastric cancer derived from patients with a low green tea intake than those with a high intake. One possibility is that green tea contains several polyphenolic compounds inhibiting DNMT activity, such as 2-epigallocatechin-3-gallate (EGCG) [38]. It is well known from epidemiological studies that physical activity protects against malignancies, including gastric cancer [39,40], which is supported by a previous study that shows *CACNA2D3* methylation to be more frequently found in gastric cancer patients with no physical activity than in those with physical activity [38]. In fact, a large number of tumor-related genes are methylated in gastric epithelia, and it is therefore essential to pin down whether and which diet factors are relevant. Equally important is the thorough understanding of the underlying mechanisms in these cases so as to deter potential gastric carcinogenesis.

3.3. Chronic inflammation

Epidemiological studies have identified chronic infections and inflammation as major risk factors for various types of cancer. Several inflammatory mediators, such as TNF-α, IL-1β and reactive nitrogen species, are thought to be involved in the aberrant DNA methylation during tumorigenesis, including gastric cancer [27,41]. Although the link between cancer and inflammation is firstly proposed in the nineteenth century, the molecular mechanism has not yet been clearly understood [42].

3.4. H. pylori

The connection between *H. pylori* and gastric cancer is based on the epidemiologic data and animal models [43–45]. *H. pylori* infection may cause chronic inflammation, accumulation of reactive oxygen species (ROS), and oxidative DNA damage in the gastric mucosa [46]. In addition, it has been reported that *H. pylori* infection enhances aberrant DNA methylation in gastric mucosa, and that further contributes to gastric carcinogenesis through silencing tumor suppressor genes [47–50]. *H. pylori* eradication leads to a dramatic decrease of gene methylation, which also suggests that this process may delay or reverse *H. pylori*-mediated gastric tumorigenesis [51,52]. However, *H. pylori* infection cannot affect mRNA and protein expression of DNMTs [48,53]. To date, the mechanism of *H. pylori*-induced aberrant gene methylation in gastric carcinogenesis remains poorly understood.

3.5. Epstein–Barr Virus (EBV)

EBV is a ubiquitous human herpes virus that was first identified in Burkitt's lymphoma cells. *EBV* is the etiologic agent of infectious mononucleosis, and more than 90% of adults become *EB*V carriers. *EBV* may cause many malignancies, such as Burkitt lymphoma, nasopharyngeal carcinoma, Hodgkin lymphoma, peripheral natural killer/T-cell lymphoma, smooth muscle tumor, and gastric cancers [54]. Aberrant methylation of tumor suppressor genes, such as *CDH1*, *p15*, $p16^{INK4a}$ and *p73*, is frequently observed in *EBV*-associated gastric cancer, which is one of the most characteristic abnormalities in *EBV*-associated gastric cancer., whereas methylation is less frequently detected in surrounding non-neoplastic mucosa [55–58], suggesting that aberrant methylation may be a critical mechanism of *EBV*-related gastric tumorigenesis. However, the molecular mechanism underlying *EBV*-induced gene methylation is still not clear. One possible mechanism is that *EBV* upregulates LMP2A expression through the phosphorylation of STAT3, further inducing DNMT1 expression [59]. Therefore, LMP2A may play an essential role in the epigenetic abnormalities in host cells and in the development and maintenance of *EBV*-associated cancer.

4. Tumor-related gene methylation and their clinical significance in gastric tumorigenesis

Promoter methylation is now regarded as one of the major mechanisms to inactivate tumor-related genes, particularly tumor suppressor genes, along with genetic alterations, ultimately leading to gastric carcinogenesis. Until now, a large number of genes with different biological functions have been found to be methylated in gastric cancer (Table 1). Promoter methylation is an important hallmark of cancer cells, which plays a key role in the initiation and progression of tumor, including gastric cancer. Additionally, aberrant methylation of a number of genes is significantly associated with clinicopathological characteristics and clinical outcomes in gastric cancer (Table 2).

Table 1
Genes commonly methylated in gastric cancer.

Functions	Gene	Assay	Methylation prevalence (%)			References
			Normal	Para-cancer	Cancer	
DNA repair	*hMLH1*	MSP	20.0	N/A	8.8–72.9	[61,66,67,229,230]
	MGMT	MSP	5.7–8.0	N/A	26.7–36.8	[67–69,229,230]
Cell cycle	*CDKN1C*	Q-MSP	66.7	96.0	36.0	[182]
	IGFBP3	Q-MSP	N/A	N/A	58.3	[231]
	P16	MSP	3.8–35.0	N/A	21.3–45.0	[31,67,75,79–82,229,230]
	TCF4	Pyrosequencing	40.0	N/A	67.0	[232]
	PRDM5	MSP, BS	0.0	N/A	50.0–88.0	[233]
Cell adherent/invasion/migration	*CDH1*	MSP	16.0–36.1	N/A	50.6–84.0	[31,75,90,182,229]
	FLNc	MSP	8.0	N/A	37.0–41.3	[67,229]
	GRIK2	Q-MSP	7.41–30.0	N/A	50.0–66.6	[97,98]
	HOXA10	Q-MSP	7.4	0.0	24.0	[182]
	LOX	MSP	12.0	N/A	27.0–41.3	[229,234]
	TIMP3	MSP	3.8	N/A	13.2	[230]
	TSP1	MSP	3.1	N/A	35.4	[235]
Cell growth/differentiation	*HAI-2/SPINT2*	MSP	0.0	N/A	75.0	[100]
	HOXA1	Q-MSP	18.5	72.0	48.0	[182]
	HoxD10	MSP	0.0	N/A	85.7	[104]
	NDRG2	MSP	20.0	N/A	54.0	[236]
	RARRES1	Q-MSP	3.0–51.9	84.0	10.0–36.0	[171,182]
	SHP1	MSP	20.8		25.0	[230]
Apoptosis	*BNIP3*	Q-MSP	15.0	N/A	39.0–65.0	[97,112]
	CACNA1G	Q-MSP	11.1	4.0	48.0	[182]
	CMTM3	MSP	14.0	N/A	44.0	[237]
	DAPK	MSP	24.5–42.2	N/A	30.9–83.2	[75,112,116,230]
	GPX3	Pyrosequencing	39.0	N/A	30.1–60.0	[127,128]
	GSTP1	MSP	1.9	N/A	20.6	[230]
	PCDH10	MSP, BS	37.0	N/A	82.0	[238]
	PCDH17	MSP	N/A	N/A	95.0	[239]
	RBP1	Q-MSP	44.4	80.0	64.0	[182]
	SFRP2	Q-MSP	10.0–20.0	N/A	55.0–73.3	[97,240]
Transcriptional regulation	*ZNF545*	MSP	0.0	27.0	51.9	[241]
	CHD5	Q-MSP	20.0	N/A	40.0	[97,134]
	HLTF	MSP	8.3–12.0	N/A	45.8–53.3	[229,242]
	ZIC1	MSP	N/A	N/A	94.6	[144]
	RUNX3	Q-MSP	7.4	8.0	56.0–75. 2	[141,182,243]
Ras pathway	*hDAB2IP*	MSP	6.0	N/A	46.0	[244]
	HRASLS	MSP	N/A	N/A	40.0–46.0	[67,229,234]
	RASSF1A	MSP	5.7	N/A	45.6–61.8	[116,149,230]
	RASSF2	Q-MSP	35.0	N/A	14.0–70.0	[67,75,97,245]
	RKIP	MSP	4.1	N/A	62.1	[246]
STAT pathway	*SOCS-1*	MSP	12.0	N/A	44.0	[156–158]
Wnt pathway	*APC*	MSP	37.7	N/A	52.9	[230,247]
	Dkk-3	MSP	34.6	N/A	67.6	[163,166]
	SFRP5	Q-MSP	66.7	76.0	56.0	[182]
Retinoic acid pathway	*RARß*	MSP	16.0–20.0	N/A	36.0–50.7	[75,116,229]
	CRBP1	MSP	0.0	N/A	33.0	[171]
Others	*KL*	MSP	0.0		47.5	[181,182]
	ITGA4	Q-MSP	29.6	24.0	96.0	[182]
	CDKN2A	MSP, Q-MSP	29.6	20.0	30.4–36	[182]
	TP73	Q-MSP	3.7	0.0	24.0	[182]
	BTG4	MSP	0.0	N/A	73.7	[248]
	DACT1	MSP, BS	0.0	N/A	29.3	[249]
	NPR1	MSP	N/A	N/A	42.5	[231]
	ECRG4	MSP	6.7	53.3	69.4	[250]
	EDNRB	Pyrosequencing	6.5	N/A	50.4	[250]
	CHFR	Q-MSP	5.0	N/A	48–65	[97,182]
	HACE1	Q-MSP	N/A	N/A	26.0	[31]
	LRP1B	Q-MSP	23.0	N/A	61.0	[251]
	NR3C1	Q-MSP	15.0	N/A	24.0–30.0	[97,182]
	TFPI2	Q-MSP	0.0	N/A	18.0–80.9.0	[127,175,176]

4.1. DNA repair

During replication, the primary function of the eukaryotic DNA mismatch repair (MMR) system is to recognize and correct mismatched base pairs within the DNA helix [60,61]. Microsatellite instability (MSI), mainly caused by mismatch repair defect, is a common phenomenon in gastric cancer [62–64]. *hMLH1*, which encodes a mismatch repair enzyme, is activated in response to DNA damage, further inducing apoptosis of tumor cells. Epigenetic changes involving promoter methylation of *hMLH1* have been implicated in the development of various types of gastric cancer [65–67]. In addition, our previous study suggests that *hMLH1* methylation is closely associated with poor prognosis of gastric cancer patients [67].

O6-methylguanine-DNA methyl-transferase (*MGMT*) is a DNA-repair enzyme that protects cells from the carcinogenic effects of alkylating agents by removing adducts from the O6 position of guanine. *MGMT* is inactivated by promoter methylation in human cancers, including gastric cancer [67–69]. Significantly, promoter methylation-mediated *MGMT*

Table 2
Correlation of gene methylation with clinical outcomes in gastric cancer.

Functions	Gene	Correlation with clinical outcomes	References
DNA repair	*hMLH1*	Association with poor prognosis	[67]
	MGMT	Association with lymph node metastasis, TNM stage and poor survival	[67,68,73,75]
Cell cycle	*p16*	Correlation with poor tumor differentiation, lymph node metastasis, and poor survival	[38,67,91–93]
	TCF4	Correlation with tumor size, Lauren classification, depth of invasion, and lymph node metastasis	[232]
Cell adherent/invasion/migration	*CDH1*	Association with worse prognosis, tumor size, lymph vascular invasion, infiltration depth, lymph node and distant metastasis	[93,182]
	FLNc	Association with a poor prognosis	[67]
	LOX	Association with depth of tumor invasion, lymph node metastasis, TNM stage and poor survival	[234]
	TIMP3	Associated with tumor localization	[116]
	TSP1	Correlation with TNM stage	[235]
Cell growth/differentiation	*HoxD10*	Association with poor prognosis	[104]
	HAI-2/SPINT2	Association with poor differentiation and lymph node metastasis	[107]
	NDRG2	Association with lymph node metastasis, tumor invasion, Borrmann classification and TNM stage	[236]
Apoptosis	*BNIP3*	Association with poor survival	[112,122]
	CACNA2D3	Correlation with lymph node metastasis	[38]
	DAPK	Correlation with poorly differentiated tumors and lymph node metastasis	[75,112,114,116]
	GPX3	Correlation with lymph node metastasis	[127,128]
	PCDH10	Association with poor survival	[238]
	PCDH17	Correlation with low tumor stage and lymph node metastasis	[239]
Transcriptional regulation	*HLTF*	Association with TNM stage	[242]
	PAX6	Association with tumor stage, lymph node metastasis and poor prognosis	[116]
	ZNF545	Association with poor prognosis	[241]
	RUNX3	Correlation with depth of tumor invasion, lymph node and distant metastasis	[141]
Ras pathway	*RASSF1A*	Association with TNM stage and poor prognosis	[75,116,252]
	RASSF2	Association with poor prognosis, histological differentiation, depth of tumor invasion, regional lymph node and distant metastasis, and TNM stage	[67,245]
	RKIP	Association with TNM stage, histological differentiation, depth of invasion, lymph node and distant metastasis.	[246]
STAT pathway	*SOCS-1*	Association with poor prognosis and metastasis	[157]
Wnt pathway	*Dkk-3*	Association with cancer-related death	[163]
Retinoic acid pathway	*RAR-ß*	Correlation with lymph node metastasis	[116]
Others	*KL*	Association with the poor prognosis	[181]
	DACT1	Association with tumor size, lymph node and distant metastasis	[249]
	BTG4	Correlation with cell differentiation, lymph node metastasis	[248]
	ECRG4	Correlation with tumor stage	[250]
	EDNRB	Correlation with lymph node and distant metastasis	[250]
	LRP1B	Correlation with tumorigenicity in nude mice	[251]
	TFPI2	Correlation with poor prognosis	[127]
	CALCA	Correlation with lymph node metastasis	[116]
	QKI	Correlation with poor differentiation status, depth of invasion, lymph node and distant metastasis, advanced TNM stage, and poor survival	[253]

inactivation has been shown to be associated with increased frequency of G:C → A:T transition mutations in the *p53* tumor suppressor gene in brain, colorectal and lung cancer [70–72], and in the *KRAS* gene in gastric and colorectal cancer [73,74]. These observations suggest that *MGMT* inactivation may cause the ensuring mutation in genes. Moreover, *MGMT* methylation is also associated with poor clinical outcomes of gastric cancer patients [67,73,75].

4.2. Cell cycle

In normal cells, cell cycle is controlled by a complex series of signaling pathways by which a cell grows, replicates its DNA and divides. Dysregulation of cell cycle components may lead to tumor formation [76]. Tumor suppressor gene *p16* is an inhibitor of cyclin-dependent kinase 4 (CDK4) and 6 (CDK6), which bind cyclin D1 and phosphorylate the retinoblastoma protein (*Rb*) tumor suppressor genes [77,78]. Thus, *p16* contributes to the maintenance of Rb in unphosphorylated state, which inhibits cell cycle progression. Aberrant methylation of CGI is the main mechanism for *p16* inactivation, not deletions or mutations, in primary gastric cancer [79–81]. Furthermore, *p16* methylation is an early event in carcinogenesis and has been shown to significantly increase the risk of malignant transformation of epithelial dysplasia in the stomach organs in a follow up cohort study [82]. Thus, *p16* methylation may served as a prognosis predictor for precancerous lesions. It is noteworthy that *p16* methylation is closely associated with poor clinical outcomes of gastric cancer patients [67,73,75]. Particularly, *p16* methylation affected the overall prognosis in gastric cancer regardless if the patients have early-stage or late-stage tumors, suggesting that this gene plays an important role in the multistep process of gastric carcinogenesis [67].

PR (PRDI-BF1 and RIZ) domain proteins (PRDM) are a subfamily of the kruppel-like zinc finger gene products and play key roles during cell differentiation and malignant transformation [83]. *PRDM5* methylation is frequently found in colorectal and gastric cancer, and closely associated with its transcriptional silencing. Introducing *PRDM5* into gastric cancer cells using an adenoviral vector increases the fractions of G2-M and sub-G1 cells, suggesting that *PRDM5* acts as a tumor suppressor in gastric cancer [84].

4.3. Cell adherent/invasion/migration

The progression of a tumor in situ to an invasive tumor is a major prerequisite to cancer metastasis which requires the movement and invasion of cancer cells from the primary tumor into the surrounding

tissue. With cancer progression, cancer cells lose intercellular contact, becoming motile, and invading surrounding tissues. Cell–cell and cell–matrix interactions are crucially involved in neoplastic transformation and metastasis. Defective cell adhesion contributes to loss of contact inhibition of growth, an important early step in the neoplastic process [85].

CDH1 gene (also known as *E-cadherin* gene) is located on chromosome 16q22.1. The mature CDH1 protein is a transmembrane glycoprotein that is localized mainly to the adherens junctions of epithelial cells [86]. *CDH1* inactivation is thought to contribute to tumor progression through increased proliferation, invasion, and metastasis [86–88]. It is well known that *CDH1* is one of the most important tumor suppressor genes in human cancers, particularly in gastric cancer [87,88]. Several possible genetic and epigenetic mechanisms have been proposed to inactivate *CDH1* gene in gastric cancer, including gene mutations, chromosomal deletions, as well as epigenetic alterations, such as promoter methylation, histone deacetylation, and chromatin condensation [87–89]. Increasing evidences show that *CDH1* is frequently methylated in primary gastric cancer, particularly in the poorly differentiated gastric cancer and diffuse histotype [90]. It is thus considered as a common inactivating second hit for *CDH1* gene [91,92]. Moreover, *CDH1* methylation is associated with poor prognosis of gastric cancer patients [93], emphasizing its potential clinical significance.

Glutamate receptor, ionotropic, kainite 2 (GRIK2) is the second ionotropic glutamate receptor family member, which is responsible for mediating most excitatory neurotransmissions in the mammalian central nervous system (CNS) [94,95]. It has been reported that GRIK2 plays a tumor-suppressor function in gastric cancer [96]. Moreover, *GRIK2* is highly frequently methylated in gastric cancer cell lines and primary tumors, but not in adjacent normal tissues [97,98], and, at least in part, leads to gene silencing. Importantly, restoring *GRIK2* expression in gastric cancer cells decreased tumor cell migration, further demonstrating its oncosuppressor role in gastric cancer.

4.4. Cell growth/differentiation

Cell proliferation is achieved through the transition of cells from G_0/G_1 arrest into the active cell cycle. The growth signal transduction is disrupted in almost all tumor types [76]. Promoter methylation is closely associated with the transcriptional silencing of tumor-related genes and affects cell growth and differentiation in human cancers, including gastric cancer [99,100].

The homeobox (Hox) superfamily genes encode transcription factors that control cell differentiation and morphogenesis during development [101]. Emerging evidence suggests that the expression of *Hox* genes is controlled by epigenetic mechanisms, such as *HoxD10* [102]. The dysregulation of *Hox* genes may affect various pathways, which play critical roles in tumorigenesis and cancer metastasis [103]. A recent study shows that *HoxD10* is frequently methylated in primary gastric cancer tissues, but not in normal gastric tissues [104]. In addition, *HoxD10* methylation is significantly associated with poor survival of gastric cancer patients. Ectopic expression of *HoxD10* dramatically inhibits gastric cancer cell proliferation, migration and invasion, and induces cell apoptosis [104].

HAI-2/SPINT2, a novel member of the Kunitz family of serine protease inhibitors, is an endogenous inhibitor of hepatocyte growth factor (HGF) activator (HGFA) [105]. HGFA is an enzyme that transforms the inactivate, single-chain preform of HGF to its active heterodimeric form, initiating MET signaling via binding to MET receptor [106]. HAI-2/SPINT2 can inhibit HGF/MET pathway by suppressing HGFA to play its tumor suppressor function in cancer cell growth, invasion, metastasis and angiogenesis [107]. Promoter methylation and transcriptional silencing of *HAI-2/SPINT2* have been reported in several human cancers, including gastric cancer [99,107–110]. Moreover, *HAI-2/SPINT2* methylation is significantly associated with poor differentiation and metastasis in gastric cancer [107].

4.5. Apoptosis

Every cell in a multicellular organism has the potential to die by apoptosis. However, cancer is one of the scenarios where too little apoptosis occurs, resulting in malignant cells that will not die. The mechanism of apoptosis is complex and involves many pathways. Defects can occur at any point along these pathways, leading to malignant transformation, tumor metastasis and resistance to anticancer drugs [111]. Aberrant methylation of many apoptosis-related genes, such as death-associated protein kinase (*DAPK*) and Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (*BNIP3*), has been reported in various cancers [112].

DAPK is known to encode a structurally unique calcium/calmodulin-dependent serine/threonine kinase, which exerts as a positive regulator of cell apoptosis [113]. It is frequently methylated in human cancers as a tumor suppressor gene, including gastric cancer [75,114–116]. Additionally, *DAPK* methylation is significantly correlated with poorly differentiated tumor and lymph node metastasis, and poor survival in gastric cancer [112,114].

BNIP3 is a proapoptotic member of the Bcl-2 family and can be induced by hypoxia, which is an important cellular stress involved in various human diseases, including malignancies [117]. It usually occurs during cardiac ischemia and in the hypoxic regions of tumors, and it acts against prosurvival proteins, including Bcl-2 and Bcl-xl [118–120]. Promoter methylation-mediated *BNIP3* inactivation has been reported in gastric cancer [112,121,122]. Similar to *DAPK* methylation, *BNIP3* methylation is also associated with poor prognosis in gastric cancer [112].

The glutathione peroxidase family (GPX) is a major antioxidative enzyme family that catalyzes the reduction of hydrogen peroxide, organic hydroperoxide, and lipid peroxides by reduced glutathione [123,124]. Excessive ROS production in the stomach promotes DNA damage in gastric epithelial cells. Normal cells have intact anti-oxidative properties that protect cells from ROS-induced DNA damage and cell injury. Glutathione peroxidase 3 (GPX3), also named plasma glutathione peroxidase, is the only known selenocysteine containing an extracellular antioxidant isoform. *GPX3* is selectively expressed in normal human tissues, including the gastrointestinal tract [125,126]. *GPX3* methylation has been found in gastric cancer, and is significantly correlated with lymph node metastasis [127,128]. Moreover, GPX3 plays a key role in cell migration and metastasis in gastric tumorigenesis [128].

4.6. Transcriptional regulation

Chromodomain helicase DNA binding protein 5 (CHD5) belongs to a superfamily of SWI2/SNF2-related ATPases, which is one major group of chromatin remodeling proteins [129]. By regulating chromatin structure, CHD5 promotes the expression of $p19^{ARF}$ that functions to stabilize p53, which is inactivated in more than half of human cancers [130]. Suppression of *CHD5* expression by promoter methylation has been found in many cancers, including gastric cancer [131–134]. The ectopic expression of CHD5 in gastric cancer cells leads to a significant growth inhibition [134], further validating and extending the idea that chromatin remodeling proteins function in carcinogenesis.

Runt-related transcription factor 3 (RUNX3), belongs to the RUNX family of transcription factor, and acts as a tumor suppressor by regulating a series of cancer-related genes, such as *p53*, *p21*, *ATBF1*, *Notch 1*, *p27*, and *Caspace3* [135–139]. It has been reported that loss of *RUNX3* contributes to hyperplasia and intestinal metaplasia of gastric mucosa epithelial cells in an animal model [140], whereas the restoration of *RUNX3* expression activates apoptotic pathway in gastric cancer [137]. It has been observed that *RUNX3* activity is reduced by promoter methylation in gastric cancer [141]. Moreover, *RUNX3* methylation is correlated with the depth of tumor invasion, lymph node and distant metastasis, as well as lymphatic vessel invasion in gastric cancer [141].

ZIC1, a vital transcription factor with zinc finger domains, has been implicated in a variety of developmental processes, including neurogenesis and myogenesis [142,143]. Recently, ZIC1 has been documented to participate in the progression of human cancers, including gastric cancer [144,145]. *ZIC1* expression is significantly decreased in gastric cancer tissues compared with normal gastric tissues, and, accordingly, *ZIC1* is frequently methylated in gastric cancer, but not in normal gastric tissues, suggesting that it may play a tumor suppressor function in gastric cancer [144]. Indeed, ectopic expression of ZIC1 leads to the growth inhibition of gastric cancer cells by regulation of sonic hedgehog, PI3K/Akt and MAPK signaling pathways in gastric cancer [145].

4.7. Ras pathway

The Ras superfamily of GTP-binding proteins regulates a diverse spectrum of intracellular processes, including cellular proliferation and differentiation, intracellular vesicular trafficking, cytoskeletal control, and cell death [146,147]. RASSF1A, a number of the Ras association domain family, is identified as a tumor suppressor gene, which plays a critical role in cell cycle regulation, apoptosis and microtubule stability by regulating Ras signaling pathway [148]. *RASSF1A* expression is silenced by promoter methylation in a wide variety of human tumors, including gastric cancer [75,116,149], suggesting that it may play a pivotal role in human carcinogenesis. Moreover, *RASSF1A* methylation is closely associated with TNM stage and poor prognosis of gastric cancer patients [116]. Thus, RASSF1A represents a potential diagnostic and therapeutic target in gastric cancer.

4.8. STAT pathway

Cytokines are secreted proteins that regulate cellular proliferation and differentiation. The stimuli of these mediators mainly lead to the transcriptional activation of Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway [150], which is involved in initiation and progression of several cancers [151–153]. The suppressor of cytokine signaling (SOCS)-1 is identified as a protein involved in a negative feedback loop for cytokine signaling, particularly the JAK/STAT pathway [154]. Interaction of SOCS-1 with Jak1, Jak2 or Jak3 markedly reduces their tyrosine-kinase activity and suppresses the tyrosine-phosphorylation and activation of STATs [155]. It has been reported that *SOCS-1* is downregulated by promoter methylation in gastric cancer [156–158]. Moreover, *SOCS-1* methylation is significantly associated with lymph node metastasis and advanced tumor stage in gastric cancer [157]. Taken together, these observations suggest that *SOCS-1* methylation may be a useful marker for detection and evaluation of progression and metastatic potential of gastric cancer.

4.9. Wnt pathway

The Wnt/β-catenin signaling pathway has a well-established role in the regulation of cell growth and proliferation, as well as in stem cell differentiation, and its constitutive activation is commonly found in human cancers, including gastric cancer [159,160]. Interestingly, several antagonists of Wnt signaling have been identified with two functional classes: the secreted frizzled-related protein (sFRP) class and the dickkopf (Dkk) class [161].

The Wnt antagonist *Dkk-3* is downregulated by promoter methylation in various types of human cancers, including gastric cancer, which makes it a candidate tumor-suppressor gene [162–167]. The function loss of *Dkk-3* may contribute to overactivation of Wnt signaling pathway and it has a tumor-promoting effect through the dysregulation of cell proliferation and differentiation. Ectopic expression of Dkk-3 in gastric cancer cells dramatically inhibits cell growth, further implicating its tumor suppressor function [163]. Notably, *Dkk-3* methylation is significantly associated with poor prognosis of gastric cancer patients [163].

4.10. Retinoic acid pathway

Retinoids regulate the growth, differentiation, and apoptosis of normal cells during embryonic development and of premalignant and malignant cells during carcinogenesis. Most of these effects are mediated by nuclear retinoic receptors, including retinoid acid receptors (RARs) α, β, and γ [168,169]. *RARβ* encodes retinoic acid receptor beta, a member of thyroid–steroid hormone receptor superfamily of nuclear transcriptional regulator, which functions as a tumor suppressor gene in various contexts where its absence is associated with tumorigenicity and its presence causes cell cycle arrest [170]. Methylation-associated inactivation of this gene is frequently found in human cancers, including gastric cancer [75,116,167]. In addition, *RARβ* methylation is closely associated with poor prognosis of gastric cancer patients [116].

Cellular retinol-binding proteins (CRBPs), which belong to the family of fatty acid-binding proteins, are present in the circulation, and most tissues rely on the uptake and cytosolic metabolism of retinoic acid to activate RARs and RXRs. CRBPs possess high-affinity binding for retinoic acid and possibly function as chaperone-like proteins to regulate the prenuclear phase of retinoic acid signaling [171,172]. *CRBP1* methylation is found in gastric cancer, and is closely associated with low levels of mRNA expression [173], suggests that loss-of-function of *CRBP1* may contribute to gastric carcinogenesis.

4.11. Others

Many other genes are also found to be aberrantly methylated in gastric cancer. For example, TFPI2 (also known as PP5 or MSPI) is a member of the Kunitz-type serine protease inhibitors, which negatively regulate the enzymatic activities of trypsin, plasmin and VIIa–tissue factor complex [174]. Recently, *TFPI2* methylation and under-expression are commonly found in human cancer, including gastric cancer [127,175,176], thus, it has been proposed that TFPI2 may be a tumor suppressor in carcinogenesis. Notably, *TFPI2* methylation may be a significant and independent prognostic factor in gastric cancer [127].

As a hormone similar in molecular structure to insulin, IGF-1 functions in a paracrine/autocrine fashion. Binding of IGF-1 to IGF-1R, a receptor tyrosine kinase, can initiate multiple intracellular signaling critical for cell growth and survival, such as PI3K/Akt signaling pathways [177]. KL (klotho) is found as an inhibitor of IGF-1 pathways, indicating that KL may be relevant to cancer development by remodeling the interaction of tumor-initiating cells with microenvironment [178–180]. *KL* methylation is frequently found in gastric cancer and is significantly associated with poor clinical outcomes of gastric cancer patients [181,182]. Restoration of *KL* expression in gastric cancer cells indeed can inhibit cell growth and Erk phosphorylation, induce cell apoptosis and increase the expression of p21 [181], further supporting its oncosuppressor role in gastric cancer.

5. Clinical utility of methylation marker in gastric cancer

The prognosis of gastric cancer is dependent on clinical stage at diagnosis and treatment [183]. Diagnostic tools such as gastrointestinal (GI) endoscopy followed by pathological analysis or fluoroscopy have proven useful. However, its diagnostic power depends on the technical skill of the endoscopist. Moreover, GI endoscopy is neither comfortable nor risk free for patients, and it is associated with frequent morbidity. Thus, there is an urgent need to develop less-invasive and more efficient diagnostic strategies for early detection of gastric cancer.

DNA methylation is a major mechanism of inactivation of tumor-related genes, particularly tumor suppressor genes, in neoplastic

cells [19]. The advantages of gene methylation as a marker for the detection and diagnosis cancer in biopsy specimens and non-invasive body fluids, such as serum or gastric washes, have led to many studies of application in gastric cancer (Table 3). For example, high prevalence of gene methylation, such as *DAPK*, *CDH1*, *GSTP1*, *p15*, and *p16*, is found in the serum of gastric cancer patients [184]. These methylation markers detected in serum, possibly caused by circulating nucleic acid released by gastric cancer cells, is significantly correlated with gene methylation in gastric cancer tissues [185]. The quantification of serum *RUNX3* methylation has great potential value for detecting and diagnosing gastric cancer and even in the postoperative evaluation of gastric cancer patients [141]. Serum *RASSF1A* methylation in gastric cancer patients (34.0%) is significantly higher than those in benign gastric disease patients (3.3%). Importantly, although the sensitivity of serum *RASSF1A* methylation in detecting gastric and colorectal cancer is relatively low, its specificity is very high (approximate 98.3%) [186]. Promoter methylation of *p16* is frequently detected in tumor samples, but not in matched normal tissues. Moreover, *p16* methylation is an early molecular event in gastric carcinogenesis. Thus, detection of *p16* methylation in serum may be a useful biomarker for early detection of gastric cancer [187]. Similar to tissue samples, multiple genes are also concurrently methylated in the serum of gastric cancer patients [188]. Thus, serum gene methylation is common in gastric cancer and aberrant methylation in the promoter region of these genes may be a promising biomarker.

The use of stomach juice as a molecular diagnostic or prediction tool has been previously shown to be unfeasible because DNA is easily denatured by gastric acidity. Many mucosal cells can be found in stomach juice, the detection of molecular markers in stomach juice is thus a possible noninvasive approach to detect gastric cancer. It has been reported that gene methylation is successfully detected in gastric washes, including *MINT25*, *RORA*, *GDNF*, *ADAM23*, *PRDM5*, and *MLF1* [189]. In addition, these genes show frequent differential methylation between gastric cancer and normal mucosa in the training, test and validation sets. Among them, *MINT25* methylation has the best sensitivity (90%) and specificity (96%). These findings suggest that DNA from gastric washes can be an appropriate alternative to DNA from biopsied tissues for determining methylation status in gastric cancer and screening this deadly disease.

6. Current methods for DNA methylation analysis

In the past decade, there has been an explosion of interest in DNA methylation, and with it, many new and powerful techniques have been developed to facilitate DNA methylation analysis, including blotting, genomic sequencing, methylated DNA immunoprecipitation, microarray analysis, bisulfite sequencing (BS), methylation-specific PCR (MSP), quantitative methylation-specific PCR (Q-MSP), bisulfite pyrosequencing, quantum dot-based nanoassay, single-molecule real-time detection, fluorimetric assay, and electrochemical detection [190–193]. Among them, BS, MSP, Q-MSP and bisulfite pyrosequencing are the most common and important methods.

BS is the most straightforward means of detecting the methylation status of every cytosine residue within the target sequence [194]. In general, after the denaturation and bisulfite modification, the fragment of interest is amplified by PCR. The PCR products may be sequenced directly to provide an average across all molecules in the sample [195,196]. This procedure is simple and less prone to artifacts but cannot provide information about the methylation patterns of individual alleles. MSP is a simple, sensitive, and specific method for determining the methylation status of small samples of DNA, including those from paraffin-embedded or microdissected tissues. The differences between methylated and unmethylated alleles that arise from bisulfite treatment are the basic principle of MSP. MSP is a technique that has facilitated the detection of DNA methylation at CpG islands in cell lines and clinical samples including fresh/frozen tissues [197–199]. Although MSP is a simple technique that can easily be incorporated in most molecular biology laboratories, the ability to accurately determine the promoter methylation status of genes largely depends on the careful design of MSP primers as well as other steps [200]. Q-MSP is a highly sensitive assay, capable for detecting methylated alleles in the presence of a 10,000-fold excess of unmethylated alleles [201,202]. The most advantage of this technique, as compared to existing techniques, is its potential to allow the rapid screening of hundreds to thousands of samples. Unlike other techniques, Q-MSP assay is completed at the PCR step, without the need for further gel-electrophoretic separation or hybridization. However, this technique requires expensive hybridization probes, that serial dilution of fully methylated and fully unmethylated control samples must be included in each experiment to generate standard curves, and that heterogeneous DNA methylation may not be reliably detected [203]. Bisulfite pyrosequencing is a quantitative methodology for the investigation of DNA methylation of sequences up to 100-bp in length [204,205]. Biotin-labeled, single-stranded PCR products generated from bisulfite-treated DNA are used as a template with an internal primer to perform the pyrosequencing reaction. Although bisulfite pyrosequencing allows the identification of heterogeneous DNA methylation patterns, it cannot provide the information of a single allele resolution [204,206].

DNA methylation promises to be an interesting field over the next time. There has been a marked proliferation in the number of techniques available for studying DNA methylation. These methods may become important techniques to discover some aberrant methylation markers for early diagnosis and prognostic evaluation of human cancers, including gastric cancer.

Table 3
Key studies in methylation-based detection of gastric cancer.

Genes	Specimen	Assay	Coverage (%)	Sensitivity (%)	Specificity (%)	References
TFPI2	Serum	Q-MSP	10.0	N/A	N/A	[254]
RUNX3	Serum	Q-MSP	70.0	94.1	100	[141]
p16	Serum	MSP	19.0–51.9	N/A	N/A	[187,255,256]
RARβ	Serum	MSP	25.0	N/A	N/A	[255]
CDH1	Serum	MSP	25.0–57.4	N/A	N/A	[255,256]
RASSF1A	Serum	MSP	34.0	N/A	98.3	[186]
DAPK	Serum	MSP	48.1	N/A	N/A	[256]
GSTP1	Serum	MSP	14.8	N/A	N/A	[256]
p15	Serum	MSP	55.6	N/A	N/A	[256]
MINT25	Gastric washes	Pyrosequencing	90.0	90.0	95.8	[189]
RORA	Gastric washes	Pyrosequencing	57.9	60.0	85.4	[189]
GDNF	Gastric washes	Pyrosequencing	65.0	65.0	89.6	[189]
ADAM23	Gastric washes	Pyrosequencing	68.4	70.0	83.3	[189]
PRDM5	Gastric washes	Pyrosequencing	65.0	65.0	93.7	[189]
MLF1	Gastric washes	Pyrosequencing	60.0	60.0	85.4	[189]

7. Demethylation and re-expression of epigenetically silenced tumor-related genes

As mentioned above, promoter methylation of multiple tumor-related genes, particularly tumor suppressor genes, is a common molecular event, which may play a significant role in the development of gastric cancer and correlation with clinical outcomes. Despite the developments in diagnosis and treatment technologies, the prognosis of gastric cancer patients is still poor, even for those who undergo complete resection of their carcinomas. Given that DNA methylation is a potentially reversible epigenetic alteration, demethylation inhibitors are thus proposed to be potential new anticancer agents [207,208]. Currently, tumor suppressor genes are promising targets for epigenetic drug therapies because many cell cycle inhibitors and tumor suppressor genes are methylated or silenced in cancer cells. Demethylation of these genes causes re-expression of tumor suppressor genes, leading to cell cycle inhibition and apoptosis.

It is known that aberrant expression of DNMTs plays a key role in carcinogenesis, including gastric cancer [209]. Therefore, the emerging interest in the use of DNMT inhibitors as a potential strategy for cancer treatment is constantly increasing. Most of the DNMT inhibitors have been described and are divided into two families: the nucleoside analogs that have been known and studied for many years, and the non-nucleoside inhibitors which structure varies according to their inhibitory mechanism [210]. The first molecules that have been characterized as DNMT inhibitors are initially used as antimetabolites and cytotoxic agents in leukemia chemotherapies, such as 5-azacytidine (azacitidine) and 5-aza-2-deoxycytidine (decitabine). At higher doses, these compounds are cytotoxic, they are thus used at low doses in order to achieve only the demethylation effect with little cytotoxicity. Among all the nucleoside inhibitors described, azacitidine and decitabine have been approved by the FDA in 2004 and 2006, respectively, for the treatment of MDS and AML [211]. The success of azacitidine and decitabine as DNMT inhibitors in human chemotherapy prompted researchers to identify new compounds with a better pharmacokinetic profile [212,213].

A particular interest has recently emerged from non-nucleoside molecules, whose mechanism does not rely on DNA incorporation. Flavonoids (or bio flavonoids) are organic compounds mainly extracted from plants. For example, EGCG is the main polyphenol of the green tea and its preventive anti-cancerous properties have been regularly reported in the literature for many years. Another well-known molecule of this family is genistein. Recently, both EGCG and genistein have been characterized as enzymatic and cellular DNMT inhibitors, leading to demethylation and re-expression of tumor suppressor genes, such as *RARβ*, *p16INK4a* and *MGMT* [214,215]. As none of the described non-nucleoside inhibitors have entered clinical development yet, there is still a long way to go before the identification of novel, selective, non-nucleoside DNMT inhibitors [216].

In addition to drug-induced demethylation, increasing evidences indicate an interesting possibility of a demethylating enzyme functioning in the regulation of methylation. The recent discovery of the ten-eleven translocation (TET) family of 5-mC hydroxylases, including TET1, 2, and 3, which can specifically oxidize 5-hmC to 5-hydroxymethylcytosine (5-hmC), has added another dimension of complexity to our understanding of DNA methylation [217]. 5-hmC has thus been proposed as a potential intermediate for active DNA demethylation [218–220], which plays an important role in carcinogenesis. Additionally, TET proteins are not only involved in the active DNA demethylation process, they have also been shown to prevent DNA methylation by physically binding to DNA [221]. Recently, interesting clues on the role of TET proteins in tumorigenesis are quickly emerging. A very recent study reveals that TET1 suppresses breast cancer invasion through activating the tissue inhibitors of metalloproteinases [222], implicating its tumor suppressor role. Notably, the loss of TET and 5-hmC in a broad spectrum of solid tumors [223–227] is closely associated with poor prognosis of patients with melanoma [223], and gastric cancer (unpublished data). Thus, key genes affecting the generation of 5-hmC, such as *TET* genes, can be therapeutically targeted to restore 5-hmC in human cancers, including gastric cancer, thus revealing new strategies for cancer treatment.

8. Conclusions and future perspectives

Gastric cancer is a disease driven by progressive genetic and epigenetic aberrations. The role of epigenetics in the pathogenesis of cancer has come to the forefront over the last decade. It is now well established that epigenetic events, such as DNA methylation, can be driver events in the pathogenesis of gastric cancer, and that these epigenetic events cooperate with gene mutations in the progression of normal gastric mucosa to cancer, with more genes in the gastric cancer genome affected by altered DNA methylation than by gene mutations. These alterations in DNA methylation contribute to the molecular heterogeneity of gastric cancers, as illustrated by the identification of molecular subtype of gastric cancers that can be identified by their unique methylated gene signatures. Given the role of altered DNA methylation in directing the pathogenesis of gastric cancer, studying DNA methylation signatures and developing them as biomarkers for diagnosis, prognosis and direction of therapy is likely to yield clinically useful assays that will be used to direct patient care.

There are recently developed epigenetic biomarkers for the early detection of gastric cancer and efforts are in progress to develop epigenetic markers for prognostic and predictive markers relevant for therapy. However, in many important diagnostic scenarios, DNA from the cancer represents only a small fraction of the total DNA in the clinical sample, including the use of DNA from plasma, serum, urine, feces, or sputum for early diagnosis or therapeutic monitoring and the use of DNA from surgical margins or lymph nodes to monitor the extent of disease. An exciting evolution of the development of epigenetic biomarkers is the improvement of the technology, which now allows us to profile epigenetic alterations at a much higher sensitivity and genomic scale previously not possible. Digital approaches involve the counting of methylated and unmethylated fragments, one-by-one, thereby dramatically increasing the signal-to-noise ratio of the assay. Methyl-BEAMing technology, which extends the digital BEAMing (beads, emulsion, amplification and magnetics) technology to analysis of DNA methylation, addresses this need [228].

It is well known that the field of active DNA demethylation has undergone a significant acceleration in the past few years. However, it has been reported that DNA remethylation and gene re-silencing usually occur after removal of demethylation treatment, and this may significantly hamper the therapeutic value of DNA methylation inhibitors. Continued efforts to investigate these molecular mechanisms will allow for a better understanding of the role of epigenetic alterations in gastric cancer and will lead to the translation of these insights into the clinical arena.

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