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Review

Recent progress in the study of methylated tumor suppressor genes in gastric cancer

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Abstract

Gastric cancer is one of the most common malignancies and a leading cause of cancer mortality worldwide. The pathogenesis mechanisms of gastric cancer are still not fully clear. Inactivation of tumor suppressor genes and activation of oncogenes caused by genetic and epigenetic alterations are known to play significant roles in carcinogenesis. Accumulating evidence has shown that epigenetic silencing of the tumor suppressor genes, particularly caused by hypermethylation of CpG islands in promoters, is critical to carcinogenesis and metastasis. Here, we review the recent progress in the study of methylations of tumor suppressor genes involved in the pathogenesis of gastric cancer. We also briefly describe the mechanisms that induce tumor suppressor gene methylation and the status of translating these molecular mechanisms into clinical applications.

Key words Gastric cancer, methylation, tumor suppressor genes, epigenetics

Tumorigenesis is a well-known multi-step process involving inactivation of tumor suppressor genes and activation of oncogenes. Tumor suppressor genes can be inactivated by gene mutation, gene deletion, or gene silencing [1]. Evidence has shown that cancer is caused by both genetic and epigenetic alterations [2,3]. Epigenetic information is defined as heritable information other than the DNA sequence [4]. Epigenetic changes, particularly DNA methylation, have been demonstrated to be an important factor in cancer initiation and progression. Methylation of CpG islands in a promoter region inhibits gene transcription by interfering with transcription initiation and serves as an alternative mechanism of inactivating tumor suppressor genes without gene mutations [1]. Some methylated genes identified in human cancers are classic tumor suppressor genes, which are inherited with one mutant defective allele. According to Knudson's two-hit model, complete inactivation of a tumor suppressor gene requires loss of function of both gene copies [5]. Epigenetic silencing of the remaining wild-type allele can be considered the second hit in this model. Thus, in addition to gene mutations, epigenetic gene silencing is another mechanism that fosters malignant transformation by reducing tumor suppressor gene activity and promoting aberrant activation of oncogenic signaling pathways. Epigenetic alterations may occur at different stages of tumorigenesis and malignant progression [6,7], but some researchers believe that aberrant methylation takes place before genetic alterations. Identification of tumor suppressor genes silenced by CpG methylation is expected to reveal the molecular mechanism of tumorigenesis and potential tumor biomarkers.

Gastric cancer is one of the most common malignancies and a leading cause of cancer mortality worldwide [8]. In gastric cancers, tumor suppressor genes are inactivated more frequently by promoter methylation than by mutations [9]. A number of tumor suppressor genes, including *hMLM1*, *p14*, *p15*, *p16*, *GSTP1*, *RASSF1*, *COX-2*, *APC*, *CDH1*, *CDH4*, *DAP-K*, *THBS1*, *TIMP-3*, *RARβ*, *MGMT*, *CHFR*, *DCC*, *RUNX3*, *TSLC1* and *14-3-3 sigma*, are known to be silenced by hypermethylation in gastric cancer and have been reviewed before [10,11]. Accumulation of aberrant methylations is thought to promote carcinogenesis through activation of common cancer pathways. For instance, RAS, which

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regulates a signal transduction pathway linking plasma membrane receptors to many signals for growth, differentiation, and other functions, is frequently dysregulated in human neoplasms. *RAS* mutations have been found in only a small proportion of human gastric cancers, suggesting that other mechanisms may be involved in the activation of *RAS* signaling in gastric tumors. Indeed, methylation of the Ras-association domain family (*RASSF*) genes[12,13] and the RAS protein activator-like 1 (*RASAL1*) gene[14] has been frequently detected and has led to activation of the RAS signaling pathway. Identification of methylated tumor suppressor genes and critical genetic pathways may impact the prognosis and treatment strategy selection for patients with gastric cancer.

We review here the recent progress in identifying methylated tumor suppressor genes involved in gastric cancer pathogenesis and the current status of translating that knowledge to clinical applications. Many methylated tumor suppressor genes have been identified in gastric cancer (Table 1). Because of their functional involvement in various cellular pathways that prevent cancer formation, inactivation of these genes through hypermethylation likely contributes to gastric cancer tumorigenesis. Here, we describe the genes whose functions in gastric carcinogenesis have been proposed.

Methylated Tumor Suppressor Genes that Regulate Cell Cycle and Apoptosis

Dysregulated proliferation is a hallmark of tumor cells. Defects in many of the molecules that regulate the cell cycle have been implicated in cancer initiation and progression. These include p53, Rb, p107 and pRb2/p130, and CDK inhibitors (p15, p16, p18, p19, p21, p27), all of which act to prevent cell cycle progression until all repairs to damaged DNA have been completed. In this section, we will review tumor suppressor genes that regulate cell cycle and apoptosis that have been found to be methylated in gastric cancers.

Tumor suppressor genes which are most recently found to be methylated in gastric cancers regulate cell cycle progression and apoptosis. Kim *et al*.[63] reported that promoter methylation of early B-cell factor-3 (*EBF3*) was detected in 40.4% (42/104) of gastric cancer tissues but not in normal gastric tissues. The EBFs are a group of four highly conserved, DNA-binding transcription factors with an atypical zinc-finger domain and a helix-loop-helix domain. Functional analysis demonstrates that EBF3 represses gastric cancer cell growth and migration but activates cell cycle arrest and apoptosis. EBF3 up-regulates p21 and p27 in gastric cancer cells but represses CDK2, cyclin D1, cyclin D2, and Rb as previously reported in different types of cancers[64,65].

Bcl-2–like 10 (BCL2L10) protein is a member of the Bcl-2 family. Interestingly, in addition to their central roles in apoptosis regulation, the Bcl-2 family of proteins influences the cell cycle, specifically the transition between quiescence and proliferation. *BCL2L10* methylation was detected in 44.5% of gastric cancers and 21.34% of normal gastric mucosae ($P < 0.05$) by bisulphite sequencing[66]. The pro-apoptotic effect of BCL2L10 and growth promotion by siRNA targeting *BCL2L10* in gastric cancer cells suggests that BCL2L10 may be a tumor suppressor by inducing apoptosis through mitochondrial pathways.

The iroquois homeobox protein 1 (*IRX1*) gene is located on chromosome 5p15.33, which is a cancer susceptibility locus. *IRX1* transcription was suppressed by hypermethylation in gastric cancer, and restoring IRX1 expression in SGC-7901 and NCI-N87 gastric cancer cells inhibited tumor growth, invasion, and tumorigenesis *in vitro* and *in vivo*. IRX1 directly targets bradykinin receptor B2 (*BDKRB2*), an angiogenesis-related gene, as well as histone H2B type 2-E (*HIST2H2BE*) and fibroblast growth factor 70 (*FGF7*), cell proliferation and invasion related genes. Hypermethylation of *IRX1* was detected not only in primary gastric cancer tissues but also in the peripheral blood cells of gastric cancer patients, suggesting that IRX1 could potentially serve as a biomarker for gastric cancer[67].

The CKLF-like MARVEL transmembrane domain-containing family (CMTM) is a novel family of proteins linking chemokines and the transmembrane-4 superfamily. Wang *et al*.[68] reported that *CMTM3* is frequently methylated in many carcinoma cell lines and some primary tumors including gastric cancer, resulting in loss of its expression at both the mRNA and protein levels. Ectopic restoration of *CMTM3* expression in tumor cells leads to the suppression of cell growth and induction of apoptosis with caspase-3 activation, suggesting that CMTM3 may function as a tumor suppressor.

Hypermethylation signals were also observed in some cultured and primary gastric cancers with little or no expression of transcription factor SOX2, a SOX transcription factor[69]. Among the 52 patients with advanced gastric cancers who were tested, those having *SOX2* gene methylation in their cancer tissues had a significantly shorter survival than did those without the methylation ($P = 0.006$). Exogenous expression of *SOX2* inhibited cell growth through apoptosis and cell cycle arrest. *SOX2*-overexpressing cells exhibited characteristics of apoptosis, such as DNA laddering and caspase-3 activation. Cell cycle arrest was associated with decreased levels of cyclin D1 and phosphorylated Rb as well as an increased p27 level.

Cheng *et al*.[70] reported that zinc-finger transcription factor ZNF382 functions as a tumor suppressor in many types of carcinomas including gastric cancer. Ectopic expression of *ZNF382* in tumor cells in which the gene

Table 1. Recently identified methylated tumor suppressor genes in gastric cancer

Gene	Chromosomal location	Frequency of methylation (%)		Basic function	Reference
		Tumor tissue	Cell line		
RASAL1	12q23–q24	9.5% (2/21)	60% (6/10)	GAP1 family of GTPase-activating protein, acting as a suppressor of RAS function	[14]
EDNRB	13q22.3	[a]mean 50.42%	/	G protein coupled receptor superfamily	[15]
LMX1a	1q22–q23	82% (41/50)	100% (5/5)	Nucleotide, transcription, regulation	[16]
XRCC1	19q13.31	76.4% (26/34)	/	Nucleotide, repair, base excision repair	[17]
TFPI2	7q	83% (15/18)	100% (9/9)	Playing a major role in cell migration and tumor invasion	[18]
TFF2	21q22.3	83% (15/18)	/	/	[19]
WWOX	16q23.1	33% (24/73)	40% (2/5)	Putatively involved in regulation of apoptosis	[20]
TSP1	6p12.3	35.4% (34/96)	/	Cysteine-rich secretory protein (CRISP) family, motor/ Contractile, structural protein	[21]
HOPX	4q12	[b]84% (67/80)	/	Transcription factor	[22]
GATA4/5	8p23.1/20q13.33	53.8% (43/80); 61.3% (49/80)	/	GATA zinc finger transcription factor family	[23]
RECK	9p13.3	47.5% (19 /40)	/	Acting as a negative regulator for matrix metalloproteinase 9 and 2	[24]
GADD45G	9q22.1–q22.2	[c]63% (63/100)	/	Ribosomal protein L7AE/gadd45 family, signaling growth factor, DNA damage response and cell growth arrest	[25]
LRP1B	2q21.2	61% (45 /74)	100% (4/4)	Low density lipoprotein receptor gene family (LDLR), transducer of extracellular signals, and may be involved in signal transduction	[26]
HAI2/SPINT2	19q13.2	75% (30/40)	100% (4/4)	Kunitz family of serine protease inhibitor, playing an important regulatory role in pericellular activation of hepatocyte growth factor/scatter factor (HGF/SF)	[27]
UNC5C	4q22.3	25% (9/36)	/	Dependence receptor family, unc-5 family	[28]
RELN	7q22.1	100% (15 /15)	100% (9/9)	Reelin family, signal transduction	[29]
Claudin-11	3q26.2–q26.3	100% (18/18)	100% (5/5)	Claudin family, adhesion, major structural components of tight junction (TJ) strands	[30]
CDH3	16q22.1	69% (25/36)	/	Cadherin superfamily of calcium dependent cell-cell adhesion glycoproteins	[31]
CDH5	16q22.1	73% (11/15)	100% (7/7)	Cadherin superfamily of calcium dependent cell-cell adhesion glycoproteins	[32]
GRIK2	6q16.3	70% (19/27)	75% (3/4)	Glutamate-gated ion channel (TC 1.A.10) family, receptor membrane , transport channel	[33]
SLC19A3	2q36.3	51% (52/101)	57% (4/7)	Reduced folate family of micronutrient transporter genes, may contribute to resistance to apoptosis in the tumors	[34]
PTCH1a	9q22.32	32% (55/170)	/	Patched family, signaling , receptor	[35]
SOCS6	18q22.2	46.8% (22/47)	44.4% (4/9)	SOCS protein family, regulatory , signaling	[36]
BTG4	11q23.1	73.7% (28/38)	100% (5/5)	Tob/TBG1 family of growth inhibitory gene, antiproliferative activity, being able to induce G_1 arrest	[37]
Vimentin	10p13	38% (14/37)	/	Intermediate filament family, structural protein,	[38]
DLEC1	3p22.2	34% (30/89)	100% (17/17)	Tumor suppressor gene(s), putatively involved in regulation of the expression of the telomere	[39]
ZIC1	3q24	94.6% (35/37)	100% (7/7)	Transcription factor	[40]
Hsulf-1	8q13.3	81.3% (13/16)	100% (3/3)	Sulfatase family, enzyme	[41]
MAL	2q11.1	65.8% (133/202)	/	MAL proteolipid family, cellular trafficking transport	[42]
FBLN1	22q13.31	84% (86/102)	71% (5/7)	Structural protein	[43]
TCF4	18q21.2	[d]mean 3.3%	/	Basic helix-loop-helix (BHLH) family of transcription factors, Wnt signaling pathway	[44]
CACNA2D3	3p21.1	30% (24/80)	/	Alpha-2/delta subunit family, having voltage-gated ion channel activity	[45]
DCBLD2	3q12.1	[a]mean 12.2%	36.4% (4/11)	Neuropilin family, adhesion	[46]
PKD1	16p13.3	[a]mean 19.5%	72.7% (8/11)	Polycystin family, involved in cell-cell/matrix interaction and the regulation of several signalling pathways linked to cell proliferation	[47]

(*To be continued*)

Table 1. Recently identified methylated tumor suppressor genes in gastric cancer (*continued*)

Gene	Chromosomal location	Frequency of methylation (%)		Basic function	Reference
		Tumor tissue	Cell line		
TSPYL5	8q22.1	63.9% (23/3)	70% (7/10)	Nucleosome assembly protein (NAP) family, involved in nucleosome assembly	[48]
IQGAP2	5q13.3	47% (28/59)	33.3% (3/9)	Ras GTPase-activating protein family, Wnt/beta-catenin signaling pathway	[49]
TMS1	16p12–p11.2	32.1% (26/81)	/	CARD containing adaptor protein family, adaptor, signal transduction	[50]
DAPK1	9q21.33	22.2% (18/81)	/	Protein kinase superfamily, enzyme, signaling, adhesion inhibitory effect, inducing death coupling the control of apoptosis to metastasis	[51]
NMDAR2B	12p13.1	61% (17/28)	60% (6/10)	Glutamate-regulated family of ion channels, receptor membrane, transport channel, glutamate signaling pathway	[52]
CD99	Xp22.32	16.9% (15/89)	/	CD99 family, adhesion, antigen, signal transduction mediated death signaling (novel death pathway)	[53]
ADRA1B	5q33.1	70.6% (24/34)	/	G protein coupled receptor superfamily, signal transduction, mediating its action by association with G proteins that activate a phosphatidylinositol-calcium second messenger system	[54]
VEGFC	4q34.3	29% (9/31)	36.4% (4/11)	PDGF/VEGF growth factor family, signal transduction	[55]
SMAD4	18q21.2	5% (4/73)	0% (0/9)	Dwarfin (DWA/B)/Smad family, DNA associated, transcription factor, critical mediator of TGFB and BMP signaling pathways	[56]
NES1/hk10	19q13.33	90.9% (10/11)	71.4% (5/7)	Kallikrein family, peptidase S1 family, enzyme	[57]
IGFBP3	7p12.3	67% (16/24)	46% (6/13)	Sterol desaturase family, signaling cytokine growth factor	[58]
DFNA5	7p15.3	52% (46/89)	100% (12/12)	Gasdermin family, plays a role in the TP53-regulated cellular response to genotoxic stress probably by cooperating with TP53	[59]
LIMS2	2q14.3	53% (51/96)	80% (8/10)	PINCH protein family, novel LIM domain-containing gene, adhesion, regulatory	[60]
HLTF	3q24	38% (98/256)	/	SWI/SNF family of chromatin remodeling complex, actin-dependent regulator of chromatin structure CHFR-mediated downregulation of HLTF may help protect against cancer	[61]
BMP2	20p12.3	42.9% (24/56)	50% (2/4)	Transforming growth factor-beta (TGFB) superfamily, signaling growth factor, activating PI-3 kinase/Akt pathway	[62]
EBF3	10q26.3	40.4% (42/104)	60% (6/10)	Cell cycle progression and apoptosis	[63]
SST	3q28	93% (30/32)	100% (7/7)	A primary inhibitor of gastrin-stimulated gastric acid secretion	[64]
HIN1	5q35.3	57.8% (26/45)	80% (4/5)	Uteroglobin/Clara cell secretory protein family, secretory, signaling cytokine, tumor suppressor may be mediated through the AKT signaling pathway	[65]
IRX1	5p15.33	51.9% (8/15)	100% (7/7)	TALE/IRO homeobox family, transcription factor	[67]
CMTM3	16q22.1	44% (28/63)	68.8% (11/16)	Chaperone/stress, signaling	[68]
SOX2	3q26.33	16.2% (12/74)	20% (2/10)	Transcription factor, can inhibit beta-catenin-driven reporter gene expression	[69]
ZNF382	19q13.12	63.6%（7/11）	100%（15/15）	Transcription factor	[70]
UCHL1	4p13	77% (53/69)	88.2% (15/17)	Ubiquitin carboxyl-terminal hydrolase family 1, enzyme, transcription factor	[71]
hSRBC	11p15.4	41% (46/111)	73% (11/15)	STICK (substrates that interact with C-kinase) superfamily of PKC-binding protein,	[72]
OPCML	11q25	64% (7/11)	100% (17/17)	Immunoglobulin superfamily, adhesion	[73]
PLCD1	3p22.2	62% (61/98)	84% (16/19)	Enzyme, signal transduction	[77]
DLC1–i4	8p22	82% (9/11)	12.5% (2/16)	RhoA pathway	[80]
Sox17	8q11.23	/	100% (2/2)	Transcription factor,	[92]
POPDC3	6q21	64.5% (15/18)	73% (8/11)	Unknown/unspecified	[93]
BVES	6q21	69% (53/76)	73% (8/11)	Interacting with GEFT, a GEF for Rho-family GTPases, and colocalizing in adult skeletal muscle	[93]
PCDH10	4q28.3	82% (85/104)	94% (16/17)	Protocadherin subfamily, cadherin superfamily of calcium dependent cell-cell adhesion glycoproteins	[95]
FBP1	9q22.32	33% (33/101)	57% (4/7)	FBPase class 1 family, enzyme, catalyzing the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate	[97]
LRRC3B	3p24.1	/	90.9% (10/11)	IFN Signaling gene	[98]

a, pyrosequencing; b, Q-MSP; c, high-resolution melting (HRM) analysis; d, methylight; e, bisulfit sequencing.

was silenced significantly reduced clonogenicity and proliferation and induced apoptosis. Cheng *et al*. [70] further found that ZNF382 inhibited NF-κB and AP-1 signaling and down-regulated the expression of multiple oncogenes, including *MYC, MITF, HMGA2, CDK6, STAT3, STAT5B, ID1*, and *IKBKE*, most likely through heterochromatin silencing.

Methylation of the ubiquitin carboxyl-terminal hydrolase L1 (*UCHL1*) [71] was detected in primary digestive tumors, including 77% (53/69) of gastric carcinomas, but not in or occasionally in paired adjacent non-tumor tissues. Restoring *UCHL1* expression in cell lines where the gene had been silenced significantly inhibited their growth and colony formation ability by inhibiting cell proliferation, causing cell cycle arrest in G_2/M phase, and inducing apoptosis through the intrinsic caspase-dependent pathway. Moreover, UCHL1 directly interacts with p53 and stabilizes p53 through the ubiquitination.

Similarly, SDR-related gene product that binds to c-kinase (*hSRBC*), another novel methylated tumor suppressor gene in gastric cancer, increased the stability of p53 and expression of p53 target genes, such as $p21^{Waf1}$, *PUMA*, and *NOXA*. *hSRBC*-mediated cell cycle arrest and apoptosis were abolished by blockade of p53 function [72]. These results suggested that epigenetic inactivation of *hSRBC* contributes to the malignant progression of gastric tumors, in part, through attenuated p53 response to stress. Moreover, opioid binding protein/cell adhesion molecule-like gene (*OPCML*) is also a stress- and p53-responsive gene, but this response is epigenetically impaired when the *OPCML* promoter becomes methylated. Ectopic expression of *OPCML* led to significant inhibition of both anchorage-dependent and -independent growth of carcinoma cells in which the gene had been silenced[73].

As reviewed above, many methylated tumor suppressor genes in gastric cancer contribute to the dysregulation of the important aspects of tumorigenesis: cell cycle and apoptosis. The understanding of these molecular mechanisms will provide accesses to novel molecular therapeutic strategies for inhibiting oncogenic activity of signaling pathways.

Methylated Tumor Suppressor Genes that Regulate Cell Adhesion and Invasion

Morbidity in most cancer patients is not due to primary cancer but to metastatic disease. The high death rate of gastric cancer strikingly correlates with the high metastatic capacity of most gastric cancers. Thus, understanding tumor progression to the metastatic state and changes in highly aggressive cells is important for the development of novel approaches to diagnose and treat aggressive malignancies.

Detachment of cells with increased motility from a primary tumor is the first step of cancer invasion and metastasis. Gastric cancer cells with fibroblastoid morphological changes show increased motility and invasiveness due to decreased cell-cell adhesion, which is reminiscent of epithelial-mesenchymal transition (EMT) during embryonic development. The dynamic regulation of cell-cell adhesion is crucial for developmental processes including tumorigenesis. With reduced cell-cell adhesiveness, cancer cells can disobey the social order and result in destruction of the histological structure[74]. A common and early requirement for cell motility is actin polymerization, which drives the formation of cell protrusions that are used to adhere to the extracellular matrix, define the direction of migration and initiate cell crawling. Animal cells respond to signaling at the plasma membrane by remodeling their actin cytoskeleton[75]. On the other hand, the movement of cells into a tightly woven extracellular matrix also require an active proteolytic system, which can cleave a path for cell migration[76]. So the genes regulating the host cell cytoskeleton rearrangement and proteolytic activity also have important functions in cell morphogenesis and motility.

Our group reported that epigenetic inactivation of phospholipase C delta 1 (*PLCD1*) is common and tumor-specific in gastric cancer and that *PLCD1* acts as a functional tumor suppressor in gastric carcinogenesis[77]. Located at the important tumor suppressor locus 3p22, *PLCD1* encodes an enzyme that regulates energy metabolism, calcium homeostasis, and intracellular movements. Ectopic expression of *PLCD1* in gastric tumor cells with silenced *PLCD1* dramatically inhibited clonogenicity and migration, possibly through down-regulation of MMP7 expression and hampered cyto-skeletal reorganization via phosphorylation and inactivation of cofilin.

Deleted in liver cancer 1 (*DLC1*) is another tumor suppressor gene involved in the regulation of cytoskeletal organization and other functions and is frequently methylated in many cancers, including gastric cancer[78,79]. Recently, its new isoform, DLC1 isoform 4 (*DLC1-i4*), was also found to be silenced epigenetically, to have tumor inhibitory properties in multiple carcinomas, and to be regulated by p53[80].

E-cadherin is essential for cell-cell adhesion of epithelial cells. The down-regulation of E-cadherin may favor dissociation of cancer cells from one another, facilitating their invasion through the basal membrane. Although mutation and allelic loss have been confirmed as major mechanisms for E-cadherin (*CDH1*) gene inactivation in many malignancies [81], *CDH1* promoter methylation could be frequently detected in gastric carcinoma[82].

As a co-partner of E-cadherin, β-catenin not only is critical for cell adhesion in the membrane and cytoplasm of cells, but also plays a role as a transcription activating protein in nuclei[83]. Aberrant activation of the Wnt/β-catenin signaling pathway is frequently found in many cancers, including gastric cancer[84,85]. In addition to genetic deletions and point mutations, a number of negative regulators of Wnt signaling, including secreted frizzled-related protein (*SFRP*), dickkopf homolog 2 (*DKK2*), dickkopf homolog 2 (*DKK3*) and WNT inhibitory factor 1 (*WIF1*), were frequently methylated in gastric cancer[86-91]. Recently, other genes involving Wnt pathway were also reported as methylated tumor suppressor genes in gastric cancer. For example, SRY-box containing gene 17 (*SOX17*) was reported to be indispensable for embryonic development and a candidate tumor suppressor gene that antagonizes the canonical Wnt/β-catenin signaling pathway in colorectal cancer. Treatment with a demethylating agent induced *SOX17* expression in gastric cancer cells, thus indicating the down-regulation of *SOX17* by methylation. Transgenic expression of *SOX17* suppressed dysplastic tumor development in the stomach of K19-Wnt1/C2mE mice by suppressing Wnt activity. These results suggested that SOX17 protects benign tumors from malignant progression at an early stage of tumorigenesis, and down-regulation of *SOX17* contributes to malignant progression through promotion of Wnt activity[92].

The Popeye domain-containing (*POPDC*) genes *BVES*, *POPDC2*, and *POPDC3* encode proteins that regulate cell-cell adhesion and cell migration during development. *BVES* and *POPDC3* were reported to be hypermethylated in 69% and 64% of the gastric cancer tissues, respectively. Knockdown of *POPDC3* in SNU-216 cells caused an increase in cell migration and invasion. Promoter hypermethylation is a causal event for long-term repression of *BVES* and *POPDC3*, whereas EGF stimulation is an immediate repression mechanism for both genes in gastric cancer. *BVES*, *POPDC3*, and E-cadherin mRNAs were down-regulated and *Snail* mRNA expression was up-regulated in EGF-induced EMT in SNU-216 cells[93].

Interestingly, accumulating evidence suggests that a major subfamily within the cadherin superfamily, protocadherins, frequently act as tumor suppressor genes. Inactivation of these genes through promoter methylation is significantly correlated with tumor development[81]. Recent studies have shown that the main structural and functional properties of protocadherins are distinct from those of classical cadherins. They may not have the strong cell-cell adhesion activity but do have other functions, such as mediating specificity of cell-cell interactions and signal transduction[94]. Yu *et al*.[95] reported that protocadherin 10 (*PCDH10*) was silenced or down-regulated in 94% (16 of 17) of gastric cancer cell lines. Furthermore, *PCDH10* methylation was detected in 82% (85 of 104) of gastric tumors compared with 37% (38 of 104) of paired non-tumor tissues ($P < 0.001$). Re-expression of *PCDH10* reduced colony formation *in vitro* and tumor growth *in vivo*. It also inhibited cell proliferation, induced cell apoptosis, and repressed cell invasion, possibly through up-regulation of the pro-apoptosis genes *Fas*, *caspase 8*, *Jun*, and *CDKN1A*; the anti-proliferation gene *FGFR*; and the anti-invasion gene *HTATIP2*. *PCDH10* methylation at early stages of gastric carcinogenesis is an independent prognostic factor.

Methylated Tumor Suppressor Genes with Other Functions

In cancer cells, glucose is often converted into lactic acid, which is known as the "Warburg effect." The reason that cancer cells have a higher rate of aerobic glycolysis, but not oxidative phosphorylation, remains largely unclear[96]. Recently, fructose-1,6-bisphosphatase-1 (FBP1), which functions to antagonize glycolysis, was reported to be down-regulated through the NF-κB pathway in RAS-transformed NIH3T3 cells. *FBP1* was hypermethylated in 57% (4/7) of gastric cancer cell lines and 33% (33/101) of gastric carcinomas. Inhibition of NF-κB restored *FBP1* expression, partially through demethylation of *FBP1* promoter. Restoration of *FBP1* expression suppressed anchorage-independent growth, indicating the relevance of *FBP1* down-regulation in carcinogenesis[97].

Leucine-rich repeat-containing 3B (LRRC3B) is an evolutionarily highly conserved leucine-rich repeat-containing protein, but its biological significance is unknown. Recently, *LRRC3B* was identified as a putative tumor suppressor gene and is reportedly silenced in gastric cancers by epigenetic mechanisms[98]. Stable transfection of *LRRC3B* in gastric cancer cell line SNU-601 inhibited anchorage-dependent and anchorage-independent colony formation. Moreover, *LRRC3B* expression suppressed tumorigenesis in nude mice. Microarray analysis of *LRRC3B*-expressing xenograft tumors showed induction of immune response-related genes and IFN signaling genes. Hematoxylin and eosin (H&E)–stained sections of *LRRC3B*-expressing xenograft tumors showed lymphocyte infiltration in the region. These results suggested that *LRRC3B* silencing in cancer may play an important role in tumor escape from immune surveillance.

DNA Methylation and Helicobacter Pylori Infection in Gastric Cancer

As reviewed above, aberrant DNA methylation of promoter CpG islands is one of the major inactivating

mechanisms of tumor suppressor genes and is deeply involved in gastric carcinogenesis. Moreover, in gastric cancer, DNA methylation from the pre-malignant till the most aggressive stage of cancer can be defined. For instance, many genes such as *THBD, LOX*, *HRASLS*, *FLNc*, and *HAND1* were found to be infrequently methylated in non-cancerous gastric mucosae, in addition to their frequent methylation in cancers [99]. Similar findings were reported for *CDH1, DAPK, p14, THBS1*, and *TIMP-1*[100]. The presence of trace amounts of methylation in non-cancerous gastric mucosae suggested that some gastric carcinogens could have induced the methylation. The most important gastric carcinogenic factor is *Helicobacter pylori* (*H. pylori*) infection, which increases the risk of developing gastric cancers by 2.2- to 21.0-fold [101,102]. Recent studies to quantify DNA methylation changes induced by the *H. pylori* infection in light of imflammatory reactions have been performed in both animal models and in the clinical setting. The findings suggest that *H. pylori* infection induces aberrant methylation in gastric mucosae and that levels of accumulated methylation are associated with gastric cancer risk. Notably, although *H. pylori* infection is important to trigger inflammation capable of inducing aberrant DNA methylation, some inflammation processes appear to be critical in induction of aberrant DNA methylation[103-105]. Indeed, the inflammation induced by *H. pylori* infection, not *H. pylori* itself, was critically involved in methylation induction. *H. pylori* may induce methylation of promoters containing CpG islands by release of reactive oxygen species (ROS) and nitric oxide (NO) and by activation of DNA methyltransferase [106,107]. More recently, Sepulveda *et al.* [108] suggested that permanent DNA methylation after *H. pylori* eradication may occur in stem cells, thus promoting tumorigenesis. Considering the significant involvement of aberrant DNA methylation of CpG islands in human cancers, further identification of the inducing factors and mechanisms can be expected to provide novel targets for cancer prevention.

In addition to regional hypermethylation, global hypomethylation is also purported to be a hallmark of cancer cells [109] and is known to cause chromosomal instability as an early event during gastric carcinogenesis[110]. However, Yoshida *et al.* [111] reported that *H. pylori* infection potently induces Alu and Sata hypomethylation in gastric mucosae as an early event during gastric carcinogenesis, whereas global hypomethylation is present only in some individuals. Thus, the use of hypomethylation as a risk marker has not been considered realistic.

Clinical Application

Aberrant hypermethylation of promoter regions of specific genes is a key event in the formation and progression of cancer. Various tumor-specific aberrant DNA methylations have been identified. An accumulating number of studies have reported that gene silencing by DNA methylation may be established at an early stage in the multistep process of carcinogenesis. Methylations of specific genes or methylation patterns of groups of genes were found to be associated with chemotherapy response and prognosis. Thus, aberrant DNA methylation can be applied to cancer diagnostics in three ways: as a marker to detect cancer cells or cancer-derived DNA, as a marker to predict prognosis, and as a biomarker for the assessment of therapeutic response. Methylation analysis has an advantage in that it can be performed using chemically stable DNA (compared to RNA). Moreover, detecting gene methylation is easier than detecting gene mutation because the exact location of a mutation is usually unknown, making it difficult to specifically amplify DNA molecules with an embedded mutation in excess of wild-type molecules. Detection of aberrant methylation can provide confirmation of the presence of intact cancer cells or cancer-derived DNA in bodily fluids, such as blood, urine, sputum, saliva, and stools. Recently, the methylations of *BNIP3, CHFR, CYP1B1*, *MINT25*, *SFRP2*, and *RASSF2* were analyzed in 107 specimens of peritoneal fluid by quantitative methylation-specific polymerase chain reaction (MSP)[112]. The results showed that DNA methylation in periotoneal fluid is a possible marker for detecting occult neoplastic cells on the peritoneum. Methylation analysis along with a cytological examination might, therefore, improve the positive detection of cancer cells in the peritoneal fluid of gastric cancer patients.

The development of the bisulfate conversion technique that reproducibly changes unmethylated cytosines to uracil but leaves methylated cytosines unchanged rapidly increased progress in DNA methylation detection. Bisulfite sequencing, MSP, and combined bisulfite restriction analysis (COBRA) were all developed on the basis of bisulfite conversion[113-115]. Among these technologies, MSP is a subjective, gel-based assay and cannot provide quantitative information. To date, several real-time MSP methods, such as bisulfite treatment in combination with MethyLight [116], quantitative multiplex-MSP (QM-MSP) [117,118], or pyrosequencing [119], have been developed and used in DNA methylation studies. These methods have facilitated quantitative detection of minimal amounts of aberrant DNA methylation. For example, a recent study showed that one pyrosequencing analysis of global and site-specific DNA methylation in peripheral blood samples from 105 gastric cancer patients provides quantitative DNA methylation values that may serve as important prognostic indicators[120]. Methylated genes are predicted to be a new generation of cancer biomarkers. However, prior to clinical application, these findings

require validation in prospective clinical studies.

Epigenetic change is heritable but has plasticity. During the last few decades, an increasing number of drugs targeting DNA methylation have been improved to increase efficacy and to decrease toxicity. DNA demethylating agents have been shown to be effective therapeutics for hematological malignancies. 5-azacytidine, the most successful epigenetic drug to date, is currently recommended as the first-line treatment of high-risk myelodysplastic syndromes (MDS). New methods for gene-specific epigenetic modification are being developed and tested in solid tumors[121].

Gastric cancer is a biologically heterogeneous disease with various molecular tumor subsets that likely respond distinctly to therapy. Discovery of biomarkers that improve disease characterization may make optimized or personalized therapy possible. Methylation of genes involved in DNA repair and maintaining genome integrity (e.g. *MGMT*, *hMLH1*, *WRN*, and *FANCF*), as well as genes involved in cell cycle checkpoints (e.g. *CHFR*, *14-3-3σ* , *CDK10*, and *p73*) all reportedly influence sensitivity to chemotherapeutic drugs, suggesting that DNA methylation could serve as a molecular marker for predicting tumor responsiveness to chemotherapy [122]. An investigation of DNA methylation using specialized high-throughput platforms can potentially be applied to further stratify patients to individualized therapies.

Conclusions

Methylated tumor suppressor genes are being intensively investigated in gastric cancer, but the underlying functions and mechanisms need to be carefully examined. Future investigations of the connections between *H. pylori* infection, gastric cancer, and epigenetic changes will greatly expand our understanding. We believe that information obtained from studying DNA methylation will have an impact on cancer prevention, diagnostics, and treatment, and will contribute to cancer elimination. However, proper selection of methylation markers is crucial for sensitive and specific detection, and further technological advances are necessary in the future.

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