Epigenetic alterations in gastric carcinogenesis

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ABSTRACT

Gastric cancer is believed to result in part from the accumulation of multiple genetic alterations leading to oncogene overexpression and tumor suppressor loss. Epigenetic alterations as a distinct and crucial mechanism to silence a variety of methylated tissue-specific and imprinted genes, have been extensively studied in gastric carcinoma and play important roles in gastric carcinogenesis. This review will briefly discuss the basic aspects of DNA methylation and CpG island methylation, in particular the epigenetic alterations of certain critical genes implicated in gastric carcinogenesis and its relevance of clinical implications.

Keywords: epigenetic alterations, gastric carcinogenesis, chronic gastritis, intestinal metaplasia, dysplasia/adenoma, adenocarcinoma.

INTRODUCTION

The incidence of gastric cancer has declined; however, it continues to be the second most common malignant neoplasms across the world and the second leading cause of cancer death [1]. Epigenetics has an important role in biological research and affects many different areas of study including cancer biology [2, 3], viral latency [4-7], activity of mobile elements [8], somatic gene therapy [9-14], cloning and transgenic technologies, genomic imprinting [15, 16], and developmental abnormalities [15, 16]. Epigenetic silencing of tumor-related genes due to CpG island methylation has been recently reported in gastric carcinoma [17]. CpG islands are 0.5 to 2 kb regions rich in cytosine-guanine dinucleotides and are present in the 5' promoter region of approximately 40-50% of human genes [18]. Methylation of cytosines within CpG islands is associated with loss of gene expression by repression of transcription and is observed in tumorigenesis, as well as in physiological conditions such as X chromosome inactivation and aging [19-24].

Gastric carcinoma can arise either from precursor lesions or *de novo*. It has been demonstrated that some gastric carcinoma may arise from gastric adenomas or flat dysplasias, similar to the consequence of colorectal adenoma derived from adenocarcinoma [25-28]. The cumulative prevalence of malignant transformation of gastric dysplasia/adenoma has been reported to be greater than 10% in long-term follow-up studies [29, 30]. Epigenetic alterations have been acknowledged as an important mechanism contributing to early gastric carcinogenesis. Previous studies have characterized epigenetic abnormalities in intestinal metaplasia (IM) and adenoma, which are precursors of invasive adenocarcinoma [31, 32].

DNA METHYLATION IN CANCER

DNA methylation has become the topic of intense investigation in cancer cells. As compared with normal cells, the malignant cells show major disruptions in their DNA methylation patterns [32]. The tumor cells are characterized by significant modifications of DNA methylation system including general genome demethylation, increase in DNA methyltransferase activity, and local hypermethylation [34-35]. These modifications seem to be contradictory, since it seems difficult to explain the increase in DNA methyltransferase activity with local hypermethylation of the genome and overall demethylation on the remaining genome. This contradiction is further clarified by better understanding of the DNA methylation system. The demethylation has been estimated to involve almost exclusively the "dispersed" CpG (~80% of total content, and they are methylated in normal cells). In contrast, local

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hypermethylation occurs on the CpG islands (about 20% of total content, and are not methylated in normal cells excluding certain exceptions) does not compensate the demethylation of "dispersed" CpG to give general genome demethylation. In tumor cells the reciprocal relations between the methylation levels of the CpG islands and of "dispersed" CpG, are also preserved. The increased activity of DNA methyltransferase frequently seen in tumor cells can be one of the factors responsible for the aberrant methylation of CpG islands.

Methylation of tumor suppressor genes

Inactivation of tumor suppressor genes by DNA methylation in promoter region plays an important role in carcinogenesis. The tumor suppressor genes that undergo aberrant CpG island methylation in human cancer can affect important cellular pathways including cell cycle regulation and proliferation. For example, in p16^{INK4a}/Rb/cdk4 pathway, the cell-cycle inhibitor p16^{INK4a} is methylated in various human primary tumors and cell lines [36, 37], allowing the cancer cells to escape senescence and to proliferate Rb itself can also be occasionally inactivated by aberrant methylation [38, 39]. APC/β-catenin/E-cadherin pathway has been altered in a variety of human cancers. *APC* gene is commonly mutated in sporadic colon cancer but the role of the *APC* mutation in other tumor systems is not clear. Recently, it is been shown that aberrant methylation of *APC* is a common phenomenon in other aerodigestive tract neoplasms [40] and that *E-cadherin* promoter hypermethylation is important for the cancer biology of breast and other tumor types [41, 42].

Methylation in DNA mismatch repair gene

DNA mismatch is in the crossroad of all other cellular pathways. DNA methylation is one of the major players in causing alterations of DNA mismatch repair genes. Methylation-mediated silencing of the mismatch DNA repair gene *hMLH1* in sporadic cases of colorectal, endometrial, and gastric cancers are responsible for the high level of microsatellite instability in tumors [43-47]. The promoter hypermethylation of *MGMT* [48] that prevents the removal of groups at the O^6 position of the guanine is associated with particular type of *K-ras* and *p53* mutations [49, 50]; and the somatic inactivation of *BRCA1* by aberrant methylation altering its role in the repair of DNA double-strand breaks in breast and ovarian tumors [51].

Two types of DNA methylation

Not all of the DNA methylation are tumor specific. In colon tissues, studies have shown DNA methylation in certain genes such as *ERα*, *N33* and *MYOD* are age-

related, and this type of methylation is classified as Type A methylation. In contrast, tumor-specific methylation such as *p16* and *hMLH1* is classified as Type C methylation [17]. A similar distinction between age-related and tumor-specific methylation has also been shown in gastric cancer [52, 53]. In stomach, age-related Type A methylation genes include *E-cadherin* and *APC* [32, 53]. In addition, Waki *et al* found that methylation of *DAP-kinase*, like that of *E-cadherin*, was also age-related. The methylation of DAP-kinase in non-neoplastic epithelia frequently appears at around age 45 [54].

The significance of the detection of the methylated gene can depend on the position of the CpG sites examined. Methylation of *hMLH1* gene, which was thought to be cancer-specific [17], was found to be a common age-related event in normal colonic mucosa, when the entire *hMLH1* promoter ~700 bp region was analyzed [55]. Partial methylation on *hMLH1* promoter region is frequently present in normal colonic mucosa, especially in older patients. The *hMLH1* gene will ultimately shut down when the methylation spreads to reach a threshold [55]. Therefore, these contradictory results might have been due to analysis of different CpG sites [17, 55]. In addition, it has recently been reported that *DAP-kinase* methylation was present in virtually every tumor and normal gastric and colorectal sample when the edge of CpG islands was examined, although it turned out to be a rather infrequent, cancer-specific phenomenon when the central region of the CpG islands was analyzed [56].

CpG island methylator phenotype

Tumors with concurrent methylation in multiple genes or loci have been defined as CpG island methylation phenotype-high (CIMP-H) in colorectal and gastric cancers [17, 52]. The mechanism of concurrent hypermethylation of multiple genes remains obscure. Apparently, the increase in DNA methyltransferase activity plays an important role. Transfection of a cloned human *DNMT1* gene into immortalized human fibroblasts leads to the aberrant methylation of CpG islands in promoter regions of several genes, including *E-cadherin* and *HIC1*, but CpG islands associated with other genes (for example, *p16^{INK4a}*) are not altered in methylation state, although *DNMT1* is expressed constantly [57]. Therefore, it is evident that the increase in DNMT1 activity can play a significant role in the selective aberrant methylation of CpG islands, but not in total CpG island methylation.

CIMP-H affects only a subset of tumors and a limited number of genes. The defect that leads to CIMP-H could be either aberrant *de novo* methylation (through a mutation in DNA-methyltransferase for example) or loss of protection against *de novo* methylation through the loss of a trans-

activating factor [58-60]. These CIMP-H tumors may then develop through a pathway that heavily relies on this methylation defect, whereas others rarely show tumor suppressor gene methylation. Aberrant methylation often occurs in CpG islands outside of promoter regions in which it may not affect gene transcription [61]. An important question is whether the concordant methylation described here provides a growth advantage to affect cells or whether it just accompanies tumor development. Additional studies are necessary to clarify whether the genes methylated in cancer simply reflect the genome wide methylation defect or whether stochastic methylation of each CpG island results from selective pressures.

EPIGENETIC ALTERATIONS IN EARLY GASTRIC TUMORIGENESIS

There are two major histological types of gastric adenocarcinoma (intestinal and diffuse) according to the Lauren's classification [62]. The pathogenesis and genetic alterations for these two distinct types of adenocarcinoma are also different [63-65]. The most frequent gastric malignancy is the intestinal type, which is often preceded by sequential steps of precancerous changes, including atrophic gastritis, intestinal metaplasia, and either dysplasia or

adenoma. In contrast, the diffuse type of gastric carcinoma tends to arise *de novo* and is infrequently associated with dysplasia or adenoma [66-69]. The sequential accumulation of alternations of *APC* and *K-ras* genes, characteristic of the colorectal adenoma-carcinoma sequence, however, does not occur frequently between adenoma and intestinal type adenocarcinoma of the stomach [27, 70-77]. There are two lines of evidence indicating that not all gastric dysplastic lesions are precursor lesions for gastric carcinoma. First, gastric dysplasia can undergo spontaneous regression clinically, especially low-grade dysplastic lesions, and only 11–40% of adenoma/dysplasia progress to carcinoma [29, 78-81]. Secondly, *APC* mutations have been reported to occur more frequently in gastric adenomas than in gastric adenocarcinomas [75-77, 81].

The status of methylation in multiple genes or loci has been studied extensively in early gastric tumorigenesis following the stepwise morphologic changes as summarized in Fig. 1 and Tab. 1. Methylation of tumor suppressor genes are frequently present in the non-neoplastic gastric mucosa including chronic gastritis and intestinal metaplasia (IM). Kang *et al* [32] determined the methylation frequency of 12 genes, including *APC*, *COX-2*, *DAP-kinase*, *E-cadherin*, *GSTP1*, *hMLH1*, *MGMT*, *p16*, *p14*,
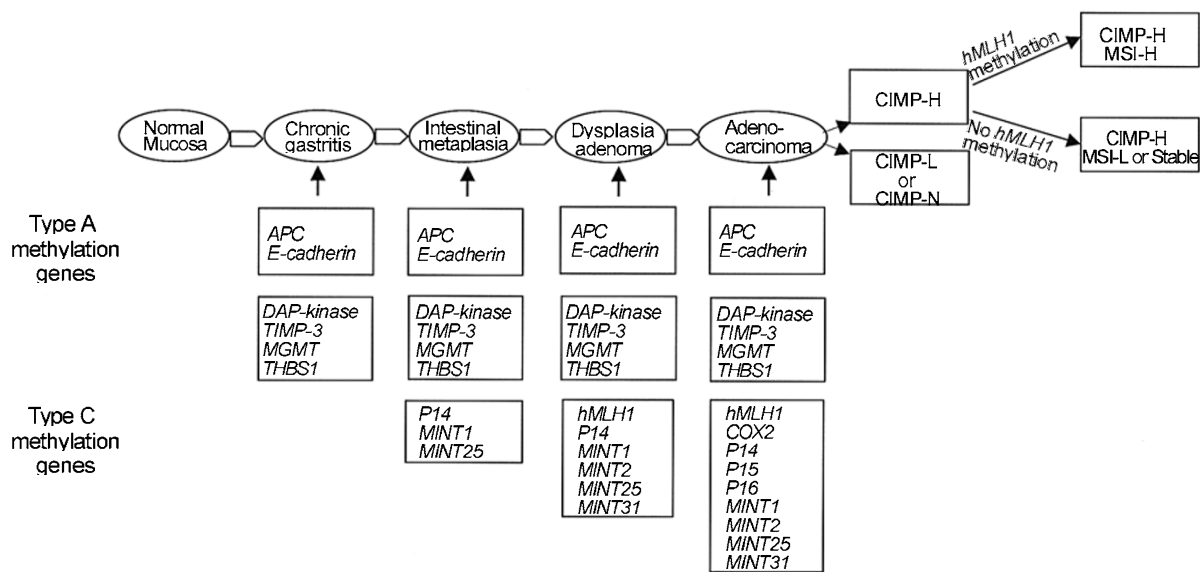

Fig. 1 Epigenetic alterations in the multistep gastric carcinogenesis pathway following the chronic gastritis-intestinal metaplasia-adenoma/dysplasia-adenocarcinoma sequence. Methylation of *APC* and *E-cadherin* genes occur frequently in normal gastric mucosa and chronic gastritis, and uniformly through the sequence is classified as Type A (age-related) methylation genes. The remaining genes are classified as Type C (tumor-specific) methylation genes. Genes such as *DAP-kinase*, *MGMT*, *TIMP3* and *THBS1* are frequently methylated in intestinal metaplasia stage, but the methylation frequency is higher in precursor lesions or adenocarcinoma, therefore also classified as Type C methylation genes. Gastric carcinoma can be further subclassified based on the extent of methylation; CIMP-high (CIMP-H) with concurrent methylation of multiple genes in >50% of assessed genes, CIMP-low (CIMP-L) with methylation in <50% of assessed genes, or CIMP-negative (CIMP-N) with no methylated genes. In tumor with CIMP-H, methylation of *hMLH1* gene is also associated with microsatellite instability-high (MSI-H) phenotype.

Tab. 1 Genes and frequency of methylation occur in the multistep gastric carcinogenesis pathway

Gene name	Normal mucosa	Chronic gastriti s	Intestinal metaplasia	Dysplasia adenoma	Adeno-carcinoma	References
APC		65	81	72	78	[32]
RASSF1A		0	0	0	8	[32]
			11		26	[82]
DAP-kinase		35	49	34	56	[32]
			39		41	[82]
		25	37	34	34	[31]
E-cadherin	15	85	72	58	68	[32]
			36		45	[82]
TIMP-3		23	44	28	81	[83]
		15	37	27	65	[32]
hMLH1		0	7	9	57	[31]
	0		22		20	[32]
					29	[82]
	0	0	6	10	35	[83]
	0	0	0	18	20	[31]
					24	[81]
					17	[85]
MGMT		15	9	10	21	[32]
GSTP1	8	0	0	0	16	[32]
			0		0	[82]
					27	[83]
COX2		2	9	4	46	[32]
p14		30	32	76	63	[32]
			8		32	[82]
p15	19		11		48	[82]
					73	[83]
p16		3	7	11	44	[32]
	19		14		45	[82]
					65	[83]
	0	0	2	12	42	[31]
	3	0	7	29	44	[81]
					20	[85]
THBS1		18	49	34	56	[32]
	17	10	35	28	48	[31]
MINT1	5	5	25	42	41	[81]
	7				37	[85]
MINT2	7	6	10	45	39	[81]
	10				34	[85]
MINT25	0	37	41	81	90	[81]
	11				55	[85]
MINT31	19	21	19	50	33	[81]
					37	[85]

RASSF1A, *THBS*1, and *TIMP3*, by methylation-specific PCR in this progression sequence. In this study five different classes of methylation behaviors were found: (1) genes only methylated in carcinoma such as *GSTP1* and *RASSF1A;* (2) genes showing low methylation frequency in chronic gastritis, IM, and gastric adenoma but significantly higher methylation frequency in carcinoma such as *COX-2*, *hMLH1*, and *p16*; (3) a gene with low and similar methylation frequency in four-step lesions such as *MGMT*, (4) genes with high and similar methylation frequency in four-step lesions such as *APC* and *E-cadherin*, and (5) genes showing an increasing tendency of the methylation frequency along the progression such as *DAP-kinase*, *p14*, *THBS1*, and *TIMP-3*. A similar result has also been shown by other studies. To *et al* showed three differential methylation patterns: methylation was more frequent in cancer than in IM (*DAP-kinase*, *p14*, *p15* and *p16*); comparable frequencies of methylation in cancer and IM (*E-cadherin* and *hMLH1*); and no methylation (*GSTP1*) [82]. Among these genes evaluated, it appears that at least methylation of *APC* and *E-cadherin* are not tumor specific but rather an age-related phenomenon (Type A methylation gene for stomach). Therefore, it is crucial to include control non-neoplastic gastric mucosa; in methylation study of gastric precursor lesions and carcinomas. It is however, difficult to classify genes such as *DAP-kinase*, *THBS1* and *TIMP-3* in which methylation is frequently detected in non-neoplastic mucosa and precursor lesions but a higher frequency of methlyation is present in carcinoma stage based on Type A or Type C methlyation gene classification (Fig. 1).

Concurrent methylation of multiple tumor-related genes has been detected in 20% of normal tissues adjacent to gastric carcinoma by Leung *et al* [83]. We have also shown concurrent methylation (CIMP-H) in 15% of IM but not in chronic gastritis or normal gastric mucosa using a panel of six genes/loci including *p16*, *hMLH1* and four CpG islands (MINT1, MINT2, MINT25 and MINT31) [81]. *MINT* loci were methylated in 6–21% of normal mucosa, and in 10–41% of IM. In particular, methylation of MINT25 was more frequent in normal/chronic gastritis mucosa contiguous with neoplasms and in IM than in noncontiguous normal/chronic gastritis mucosa. The mean methylation index and frequency of CIMP-H increased following the normal/chronic gastritis, IM, adenoma/dysplasia and early adenocarcinoma sequence indicating accumulation of methylation events may play an important role in early gastric tumorigenesis (Fig. 2) [81].

There are two morphologically distinct gastric precancerous lesions: adenoma (polypoid dysplastic mucosal lesion) and flat dysplasia. Genetic and epigenetic alterations separating these two distinct morphological precancerous lesions remain unclear. We have shown that there was no
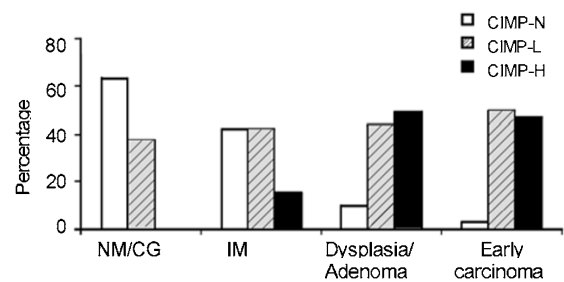

Fig. 2 The frequency of concurrent methylation in multiple genes/loci (CIMP-H) increased with the histological progression from normal (NM)/chronic gastritis (CG), to Intestinal metaplasia (IM), adenomas or dysplasias, and early adenocarcinomas. The majorities of the non-neoplastic mucosa (NM, CG and IM) are CIMP-L or CIMP-N.

difference in the frequency of individual gene/loci or in frequency of CIMP-H between flat dysplasias (50%) and polypoid adenomas (51%) [81]. Interestingly, we have found that methylation of *p16* gene was more frequent in adenocarcinoma-associated dysplasia/adenoma and adenocarcinomas as compared to adenoma/dysplasia unassociated with adenocarcinoma, indicating methylation of *p16* gene may contribute to the malignant transformation of gastric precursor lesions [81].

EPIGENETIC ALTERATIONS IN GASTRIC CARCINOMA

The roles of epigenetic alterations in the pathogenesis of gastric carcinomas have been recently elucidated. Toyota *et al* first demonstrated that about one-half of all gastric cancers had frequent methylation in multiple genes/loci including *p16*, *hMLH1* and multiple tumor specific CpG islands (MINT1, MINT2, MINT12, MINT25, and MINT31) that seemed to be methylated *de novo* during cancer progression and play an important role in gastric carcinogenesis [52]. Subsequently, numerous other genes with methylation have been found in gastric carcinomas as shown in Fig. 1 and Tab. 1.

Similar to colorectal cancer, the presence of methylation of *hMLH1* gene is strongly associated with loss of hMLH1 protein expression and MSI-H phenotype in gastric carcinomas [52, 81, 83]. In contrast to gastric carcinoma, methylation of *hMLH1* genes in gastric precursor lesions appears to be less frequently associated with MSI-H phenotype and loss of hMLH1 protein expression [81]. The reason for this discrepancy is not clear, but it is possible that *hMLH1* methylation precede the loss of protein expression.

Genetic alteration of *E-cadherin* gene has been frequently detected in gastric carcinomas, especially for diffuse type adenocarcinoma. Tamura *et al* reported that *E-cadherin*

promoter hypermethylation was seen in 27 (51%) of 53 primary gastric carcinomas, including 15 (83%) of 18 undifferentiated (diffuse) type, and *E-cadherin* promoter hypermethylation was seen at similar frequencies in both early and advanced cases [84]. However, the presence of *E-cadherin* gene methylation in non-neoplastic gastric mucosa makes difficult to determine its role in gastric carcinogenesis.

In gastric cancer, CIMP-H has been described in 41% of the tumors by Toyota *et al* [52]. We have also detected CIMP-H in 31% of the gastric carcinomas using a similar panel of genes/loci [85]. Etoh *et al* have shown that increased DNMT1 protein expression correlated significantly with DNA methylation of multiple CpG islands in poorly differentiated gastric cancers, suggested that DNMT1 may play a significant role in the development of poorly differentiated gastric cancers by inducing frequent DNA methylation of multiple CpG islands [86].

CLINICAL IMPLICATIONS

The clinical significance of presence of epigenetic alterations in gastric cancer remains unclear. It would be important to know whether the presence or absence of certain epigenetic changes affects the prognosis. This would also help in modifying initial patient treatment options and monitoring response to therapy. It is particularly important to identify any specific epigenetic alterations in gastric precursor lesions for predicting malignant transformation since not all the gastric precursor lesions carry the same malignant transforming potential. Epigenetic silencing of a tumor suppressor gene could be the rate-limiting step that initiates the series of events leading to an invasive malignant tumor. Methylation of *p16* gene appears to be a promising candidate to serve this purpose as shown in our study [81] and supported in a population-based study by Sun *et al* that aberrant methylation of *p16* promoter CpG islands might be useful to predict the malignant potential of dysplasia identified specifically in gastric biopsies [87].

The prognosis of methylation of single genes/loci is unclear in gastric cancer, but it has been reported that methylation of *MGMT* gene was associated with advance stage and poor prognosis [88]. We have recently shown that concordant methylation of multiple gene/loci (CIMP-H) is associated with better survival but is not an independent predictor of prognosis in resected gastric cancer [85]. The sensitivity and specificity of DNA methylation markers in cancer diagnosis depends on several factors, including the type of cancer and the gene to be studied, the type of body fluid to be used, and the techniques involved. The assay needs to be standardized and shown to be useful in a prospective fashion before it can become clinically useful.

A more comprehensive methylation profiling for gastric cancer will be needed to achieve this goal.

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