Promoter methylation status of tumor suppressor and tumor-related genes in neoplastic and non-neoplastic gastric epithelia

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Summary. A number of tumor suppressor and tumor-related genes exhibit promoter hypermethylation with resulting gene silencing in human cancers. In addition, several gene promoters have also been shown to become hypermethylated in non-neoplastic cells during aging. To assess the physiological consequence and clinical significance of gene promoter methylation in gastric epithelia, our laboratory has studied the methylation status of tumor suppressor and tumor-related genes, including APC, DAP-kinase, DCC, E-cadherin, GSTP1, hMLH1, p16, PTEN, RASSF1A, RUNX3 and TSLC1, in neoplastic and non-neoplastic gastric epithelia. The tumor suppressor and tumor-related genes, except APC, were generally unmethylated in non-neoplastic gastric epithelia obtained from younger individuals. The frequencies of methylation increased with age to varying degrees in various genes, although GSTP1 and PTEN methylation was completely absent in both neoplastic and non-neoplastic gastric epithelia. The methylation frequencies in each gene were found to be comparable in neoplastic and non-neoplastic gastric epithelia, except the methylation of RUNX3 and TSLC1, which was mostly cancer-specific (P<0.01). When methylation frequencies were compared between non-neoplastic gastric epithelia from cancer-bearing and non-cancer-bearing stomachs, hMLH1 and p16 methylation was more frequent in those from cancer-bearing stomachs (P<0.01). Promoter methylation in tumor suppressor and tumor-related genes initially occurs in non-neoplastic gastric epithelia, increases with age, and ultimately silences gene function to constitute a field-defect that may predispose tissues to gastric cancer evolution. In clinical applications RUNX3 and TSLC1 methylation may be utilized as molecular diagnostic markers, and hMLH1 and p16 methylation as predictors of malignancy in the stomach.

Key words: Gastric cancer, Tumor suppressor gene, Promoter hypermethylation, Age-related methylation

Introduction

Carcinogenesis is a multistep process in which genetic and epigenetic alterations accumulate. As for gastric cancer, mutations of p53 and E-cadherin genes, as well as loss of heterozygosity (LOH) on certain chromosomal regions, have been identified as frequent structural (genetic) alterations (Tamura, 2002). In addition, gastric cancers often display hypermethylation in CpG islands; CpG-rich sequences in the promoters of housekeeping genes that are generally protected from methylation (Tamura, 2002). The promoter methylation of tumor suppressor and tumor-related genes in gastric cancer has been examined so far in genes including APC, COX2, DAP-kinase, DCC, E-cadherin, GSTP1, hMLH1, MGMT, p14, p15, p16, PTEN, RASSF1A, RUNX3, 14-3-3 sigma, THBS1, TIMP-3 and TSLC1 (Fleisher et al., 1999, 2001; Suzuki et al., 1999; Toyota et al., 1999a, 1999b; Endoh et al., 2000; Tamura et al., 2001, 2002; Tsuchiya et al., 2000; Byun et al., 2001; Leung et al., 2001; Kang et al., 2001, 2002; Sato et al., 2001, 2002; Honda et al., 2002; Lee et al., 2002; Li et al., 2002; Sakata et al., 2002; Satoh et al., 2002; To et al., 2002; Waki et al., 2002, 2003a). These genes function in signal transduction (APC, PTEN and RASSF1A), apoptosis (DAP-kinase and RUNX3), cell-to-cell or cell-to-matrix adhesion (DCC, E-cadherin, TIMP-3 and TSLC1), DNA repair (hMLH1, MGMT and GSTP1), cell-cycle regulation (COX2, p14, p15, p16 and 14-3-3 sigma) and angiogenesis (THBS1). Therefore, any loss in function of these genes is likely to contribute to carcinogenesis, a scenario typified by the loss of function of hMLH1 leading to mutator pathway carcinogenesis of tumors exhibiting high-frequency microsatellite instability (MSI-H) and targeted gene mutations (Fleisher et al., 1999; Suzuki et al., 1999).

Promoter methylation is not cancer-specific. For
example, estrogen-receptor (ER) methylation was shown to increase during aging in normal human colonic mucosa (Issa, 2000). It was hypothesized that the cells that originated colonic tumors were the ones in which the ER gene became hypermethylated (Issa, 2000). Promoter methylation in other genes, such as *IGF2*, *MYOD1*, *N33*, *PAX6* and *Versican*, also increases with age in the normal colon (Issa, 2000). While its mechanism is not known, it is clear that age-related methylation affects only a subset of genes, suggesting a gene-specific susceptibility to this process (Issa, 2000). Furthermore, there are considerable tissue-specific differences in the extent of age-related methylation (Issa, 2000). Several factors may modulate age-related methylation, such as exogenous carcinogens, endogenously-generated reactive oxygen species, and genetic differences in individual susceptibility (Issa, 2000; Paz et al., 2002).

In our laboratory, the promoter methylation status of tumor suppressor and tumor-related genes, including *APC*, *DAP-kinase*, *DCC*, *E-cadherin*, *GSTP1*, *hMLH1*, *p16*, *PTEN*, *RASSF1A*, *RUNX3* and *TSLC1*, has been studied in neoplastic and non-neoplastic gastric epithelia obtained at autopsy or surgery (Endoh et al., 2000; Tamura et al., 2000, 2001; Tsuchiya et al., 2000; Sato et al., 2001, 2002; Honda et al., 2002; Sakata et al., 2002; Waki et al., 2001, 2002; Honda et al., 2003a, b). Here I review physiological consequences of promoter methylation in gastric epithelia, and propose a possible clinical application for detecting methylated genes as a molecular diagnostic marker.

### Age-related methylation of tumor-suppressor and tumor-related genes in gastric epithelia and epithelial cells of other tissue types

To clarify the physiological consequence of age-related methylation of tumor suppressor and tumor-related genes, methylation status of *APC*, *DAP-kinase*, *E-cadherin*, *GSTP1*, *hMLH1*, *p16*, *RASSF1A* and *RUNX3* genes were studied in non-neoplastic gastric epithelia and other non-neoplastic cells of different tissue types obtained at autopsy, and the results were compared between patients younger than 32 years old (n=11) and those older than 42 years old (n=27) (see Waki et al., 2003b, for detail). In the study, we have demonstrated the significant differences of susceptibility to age-related methylation among genes in different organs (Fig. 1) (Waki et al., 2003b). As for non-neoplastic gastric epithelia, methylation was absent in younger individuals, except in *APC* (promoter 1A) (Fig. 1). Methylation of one of the promoters (promoter 1A) is not oncogenic because the other (promoter 1B) is protected from methylation and thus *APC* is not inactivated (Tsuchiya et al., 2000). Hence, *APC* methylation (promoter 1A), though present in younger individuals, does not contribute to gastric carcinogenesis. Methylation of other tumor suppressor and tumor-related genes was present at variable frequencies in non-neoplastic gastric epithelia from elderly individuals (Fig. 1). Methylation of *APC*, *E-cadherin* and *DAP-kinase* was present in the majority of samples, while that of *p16* and *RUNX3* was found at intermediate frequencies, and that of *RASSF1A*, *hMLH1* and *GSTP1* was rare or absent (Fig. 1). Thus, the susceptibility to age-related methylation differed significantly among genes in gastric epithelia, although methylation generally increased with age. There were also differences in methylation frequencies depending on the site in the stomach from which the sample was taken. *RUNX3* and *hMLH1* methylation was frequent in the lower portion of the stomach (Waki et al., 2003a). The exact reasons for these phenomena are unclear. However, the antral location of gastric cancer is known to be susceptible to methylation of several tumor suppressor and tumor-related genes (Honda et al., 2002). Intestinal metaplasia, especially that of the incomplete type, commonly arises in the antrum and then expands toward the body of the stomach, and may be predisposed to promoter methylation of these genes.

### Promoter methylation status of tumor suppressor and tumor-related genes in neoplastic and non-neoplastic gastric epithelia

Methylation frequencies of the tumor suppressor and tumor-related genes in neoplastic and corresponding non-neoplastic gastric epithelia, obtained at surgery, were detected by MSP, except for those of *TSLC1* which were studied by bisulfite-SSCP (single-strand conformation polymorphism analysis) (Honda et al., 2002). These frequencies are illustrated in Figure 2, together with those measured in non-neoplastic gastric epithelia from non-cancer-bearing patients older than 42 years old obtained at autopsy, where available. Interestingly, the methylation frequencies in neoplastic and corresponding non-neoplastic gastric epithelia were mostly comparable for each gene. For instance, *APC* methylation was frequent in both neoplastic and non-neoplastic gastric epithelia, whereas *RASSF1A* methylation was rare in both (Fig. 2). However, there were several exceptions. *RUNX3* and *TSLC1* methylation was more frequently observed in neoplastic than in corresponding non-neoplastic gastric epithelia (P<0.01) (Fig. 2), i.e., cancer-specific methylation. Methylation frequencies for *p16* and *hMLH1* were significantly higher in non-neoplastic gastric epithelia from cancer-bearing stomachs (obtained at surgery) than that from non-cancer-bearing (obtained at autopsy) stomachs (P<0.01) (Fig. 2). For *APC*, *DAP-kinase* and *E-cadherin*, methylation frequencies were higher in non-neoplastic gastric epithelia than in neoplastic. This phenomenon might be due to the sensitivity of MSP, which detects even one methylated allele sparsely distributed in non-neoplastic tissues (Nakagawa et al., 2001), in 100,000 unmethylated alleles (Herman et al., 1996). It is also possible that methylation of these genes, at least at the CpG sites examined by MSP, does not
Fig. 1. Percentages of promoter methylation of tumor suppressor and tumor-related genes in each organ. 

A. Incidences in subjects who are less than 32 years old (n=11).

B. Incidences in subjects who are older than 44 years old (n=27).

S(U): stomach (upper); S(M), stomach (middle); S(L): stomach (lower);
Es: esophagus;
Du: duodenum;
Je: jejunum;
Il: ileum;
Co: colon;
Re: rectum;
Li: liver;
Pa: pancreas;
Ki: kidney.

The numbers in parentheses are the number of samples examined for each organ. See Waki et al., (2003b) for detail.
immediately make them oncogenic. APC methylation has been proven not to be as described above.

Differences in methylation patterns among genes may correspond to type A (aging-specific) and type C (cancer-specific) methylation previously described for gastric and colorectal cancers (Toyota et al., 1999a,b). Type A methylation arises as a function of age in normal colorectal cells, potentially affecting genes that regulate the growth and/or differentiation of these cells. Such methylation could account, in part, for the hyperproliferative state that is thought to precede tumor formation in the colon. In contrast, type C methylation affects only a subset of tumors, which then evolve along a global hypermethylation pathway (Toyota et al., 1999b). However, hMLH1 methylation, once thought to be cancer-specific (Toyota et al., 1999b), was found to be a common age-related event in normal colonic mucosa when the entire hMLH1 promoter ~700-bp region was analyzed (Nakagawa et al., 2001). hMLH1 methylation is partial in normal colonic mucosa and increases with age, spreading to reach a threshold, and ultimately shutting down protein expression (Nakagawa et al., 2001). Therefore, these contradictory results might be due to the analysis of different CpG sites in these studies (Nakagawa et al., 2001). Moreover, affected cells were distributed throughout multiple sites of the colon (Nakagawa et al., 2001). If critical CpG sites for each indication of gene silencing are more precisely analyzed, age-related methylation may be found to be cancer-specific. This hypothesis is supported by the observation that DAP-kinase methylation was present in virtually every tumor and normal gastric and colorectal sample when the edge of a CpG island was examined, yet upon analysis of the central region of the CpG island, the methylation was determined to be a more infrequent, cancer-specific phenomenon (Satoh et al., 2002). Alternatively, age-related methylation may not be immediately oncogenic, but gradually spread to inactivate gene function. Thus, the differences of methylation patterns may not provide any evidence for the existence of any CpG island methylator phenotype, and some (or cancer cells) are simply more hypermethylated than others (or non-neoplastic cells) (Paz et al., 2003). Therefore, the significance of methylated gene detection depends on the position of the examined CpG sites. It is possible that age-related methylation causes additional genetic and epigenetic alterations. However, specific circumstances may be required for these additional alterations to occur, because age-related methylation of several genes was also observed in the small intestine where tumor evolution is extremely rare (Waki et al., 2003b). Specific comments for each gene are listed below:

**APC**

The APC gene is responsible not only for the hereditary cancer syndrome familial adenomatous

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**Fig. 2.** Frequencies of gene-promoter methylation in neoplastic and non-neoplastic gastric epithelia. RUNX3 and TSLC1 methylation was more frequent in neoplastic than corresponding non-neoplastic gastric epithelia (P<0.01). Methylation frequencies for p16 and hMLH1 were higher in non-neoplastic gastric epithelia from cancer-bearing stomachs than that from non-cancer-bearing stomachs (P<0.01). Methylation frequency in non-neoplastic gastric epithelia from non-cancer-bearing stomachs was not available for DCC, TSLC1 or PTEN.
polyposis, but also for sporadic colorectal cancer development due to mutations within its coding sequence (Nishisho et al., 1991; Miyoshi et al., 1992). Loss of APC function results in nuclear accumulation of β-catenin, a transcriptional activator that binds to the Tcf-Lef (T cell factor/lymphoid enhancer factor) family of transcription factors, ultimately leading to loss of cellular growth control (Morin et al., 1997; Sparks et al., 1998). APC mutations occur frequently in colorectal tumors (Miyoshi et al., 1992) and gastric adenoma (Tamura et al., 1994; Jin et al., 2002; Lee et al., 2002), but rarely in gastric and other extracolonic cancers (Ogasawara et al., 1994). On the other hand, promoter hypermethylation of APC has been found frequently in many kinds of human cancers, including gastric cancer (Tsuchiya et al., 2000; Esteller et al., 2001; Jin et al., 2001). We detected methylated alleles of promoter 1A, which is not oncogenic, in both neoplastic (83%; 33/40) and corresponding non-neoplastic (98%; 39/40) gastric epithelia in the great majority of cases (Tsuchiya et al., 2000). Among the tumor suppressor and tumor-related genes examined in our laboratory, age-related methylation occurred the earliest in APC, and was detected even in a 2-year-old male patient who died of myocarditis (Waki et al., 2003b).

**DAP-kinase**

Death-associated protein kinase (DAP-kinase, 160kDa in size) is a Ca^{2+} calmodulin-regulated serine/threonine kinase that participates in several apoptotic systems initiated by interferon-γ, TNF-α, activated Fas, and detachment from the extracellular matrix (Cohen et al., 2001). Loss of DAP-kinase expression associated with its promoter methylation has been reported in various carcinomas including gastric cancer (Esteller et al., 2001; Kang et al., 2001; To et al., 2002). DAP-kinase methylation has been detected frequently in both neoplastic and non-neoplastic gastric epithelia (Kang et al., 2001; To et al., 2002), the results are similar to our laboratory data: 43% (40/93) of neoplastic and 73% (68/93) of corresponding non-neoplastic gastric epithelia (Waki et al., 2003a). No significant association between DAP-kinase methylation and clinicopathological characteristics including disease-free survival of gastric cancer patients was found, except that this methylation was more frequent in the undifferentiated histological type than in the differentiated type (Waki et al., 2003a).

**DCC**

DCC encodes a membrane-bound protein of the immunoglobulin-cellular adhesion molecule (CAM) family which is found in axons of the central and peripheral nervous system and in differentiated cell types of the intestine (Hedrick et al., 1994). This gene frequently exhibited loss of expression and LOH, although mutations were absent in gastric cancer (Sato et al., 2001). Because the promoter sequence of DCC has not yet been identified, we designed primers flanking the start codon for use in the MSP analysis of the methylation status (Sato et al., 2001). DCC methylation frequently occurred in both neoplastic (75%; 45/60) and corresponding non-neoplastic (72%; 43/60) gastric epithelia (Sato et al., 2001). The methylation status of DCC significantly correlated with the loss of DCC expression in primary tumors (p<0.01) (Sato et al., 2001). However, further analyses on the DCC promoter CpG islands are necessary to confirm this issue once the promoter sequences are identified.

**E-cadherin**

E-cadherin complexes and connects actin filaments with α-, β-, and γ-catenins, which are themselves involved in tumorigenesis, making the E-cadherin-mediated cell adhesion system a likely target of inactivation in human tumors (Hirohashi, 1998). Somatic mutations of the E-cadherin gene are most frequent in gastric carcinoma of the undifferentiated-scattered (diffuse) type (Becker et al., 1994; Tamura et al., 1996) and in lobular carcinoma of the breast (Kanai et al., 1994), both of which show histological features consistent with loss of cell-to-cell adhesiveness. In addition, mutations of E-cadherin are frequently accompanied by a loss of its wild-type allele, leading to, presumably, complete inactivation (Tamura et al., 1996). In addition to the classic two-hit inactivation of the E-cadherin gene described above, E-cadherin is silenced by CpG methylation in a variety of carcinomas including gastric carcinoma (Tamura et al., 2000, 2001; Esteller et al., 2001), especially the undifferentiated type, at the early stage (Tamura et al., 2000, 2001). E-cadherin methylation was frequently observed in both neoplastic (47%; 44/94) and corresponding non-neoplastic (67%; 63/94) gastric epithelia (Waki et al., 2002). Age-related E-cadherin methylation is frequently present in gastric epithelia from around 45 years of age (Waki et al., 2002); however, this methylation may be partial or mono-allelic as described above.

**GSTP1**

The glutathione S-transferase gene GSTP1 catalyzes intracellular detoxification reactions, including the inactivation of electrophilic carcinogens, by conjugating chemically reactive electrophiles to glutathione (Lee et al., 1994). The GSTP1 promoter is hypermethylated in carcinomas of the prostate and other organs (Esteller et al., 2001), although not in gastric cancer (To et al., 2002). We confirmed the absence of GSTP1 methylation in neoplastic (0/10) and corresponding non-neoplastic (0/10) gastric epithelia (unpublished data).

**hMLH1**

MSI-H is associated with hypermethylation of the human mut-L homologue 1 (hMLH1) mismatch repair gene promoter and with diminished hMLH1 expression
in early and advanced gastric cancers (Fleisher et al., 1999; 2001; Endoh et al., 2000), especially in differentiated carcinomas of the foveolar subtype (Endoh et al., 2000). This methylation was detected in 32% (30/94) of neoplastic and 24% (23/94) of corresponding non-neoplastic gastric epithelia (Waki et al., 2002), and in 6% (4/70) of non-neoplastic gastric epithelia from non-cancer-bearing stomachs. Methylation was significantly more frequent in non-neoplastic gastric epithelia from cancer-bearing stomachs than in those from non-cancer-bearing stomachs (P<0.01).

p16

p16 binds to CDK4 and inhibits the catalytic activity of the CDK4/cyclin D enzymes, and acts in a regulatory feedback circuit with CDK4, D-type cyclins and retinoblastoma protein (Serrano et al., 1993). p16 is frequently inactivated by homozygous deletion or promoter hypermethylation in many types of human cancers (Herman et al., 1995). p16 methylation was observed in neoplastic (22%; 21/94) and corresponding non-neoplastic (44%; 41/94) gastric epithelia (Waki et al., 2002). In addition, age-related methylation of p16 preferentially occurred in the stomach, but was uncommon in other organs (Waki et al., 2002, 2003b). Similar to the situation involving hMLH1 methylation, p16 methylation was more frequent in non-neoplastic gastric epithelia from cancer-bearing stomachs as compared to those from non-cancer-bearing stomachs (P<0.01).

PTEN

PTEN was identified as a tumor suppressor gene, encoding a dual-specificity phosphatase (Li et al., 1997) that dephosphorylates both tyrosine phosphate and serine/threonine phosphate residues. The in vivo role of PTEN appears to be the dephosphorylation of phosphotidylinositol 3,4,5-triphosphate. Mutations of PTEN were very rare (2%; 1/58), and promoter methylation of this gene was completely absent in both neoplastic and non-neoplastic gastric epithelia (0/58) (Sato et al., 2002). In addition, PTEN expression was preserved in all 10 gastric carcinoma cell lines studied (Sato et al., 2002). Therefore, we concluded that PTEN does not participate in gastric carcinogenesis as a tumor suppressor gene (Sato et al., 2002). Although there was another report describing frequent PTEN methylation associated with tumor progression, metastasis, and survival of gastric cancer patients (Kang et al., 2002), this study probably analyzed the PTEN pseudogene (Zysman et al., 2002). The PTEN pseudogene, but not PTEN, was found to be predominantly methylated in cell lines and primary tumors (Zysman et al., 2002).

RASSF1A

The RASSF1 locus encodes several major transcripts by alternate mRNA splicing (Dammann et al., 2000). RASSF1A, one of the several transcripts, encodes a predicted peptide with a Ras association domain and a predicted NH2-terminal diacylglycerol- binding domain, and may play a role as an effector molecule in the Ras-activated growth inhibition signaling pathway (Dammann et al., 2000). Mutations of RASSF1A are uncommon, whereas silencing by promoter methylation is frequent in carcinomas including gastric carcinoma (Byun et al., 2001; Dammann et al., 2003). This methylation also occurred in a small proportion of non-neoplastic gastric epithelia (To et al., 2002). In our laboratory, RASSF1A methylation was detected in 13% (6/47) of neoplastic and 8% (4/52) of non-neoplastic gastric epithelia (unpublished data), similar to frequencies reported previously (To et al., 2002).

RUNX3

The RUNX3, one of three mammalian runt-related genes, is a recently identified tumor suppressor gene that frequently shows a loss of expression due to hemizygous deletion and hypermethylation in gastric cancer (Li et al., 2002). RUNX3 methylation was detected in 45% (42/93) of neoplastic and 8% (7/93) of corresponding non-neoplastic gastric epithelia (Waki et al., 2003a), being significantly more frequent in the former (P<0.01).

TSLC1

TSLC1 protein is predicted to comprise an extracellular domain containing three immunoglobin-like C2 type fragments, a transmembrane domain and a short cytoplasmic domain similar to that of glycophorin C, leading to its designation as an immunoglobin superfamily member. TSLC1 protein has structural homology to the extracellular domains of the cell adhesion proteins NCAM1 and NCAM2, and thus may participate in cell to cell and/or cell to matrix adhesion (Kuromachi et al., 2001). TSLC1 is frequently silenced by concordant promoter hypermethylation and LOH in non-small cell lung cancer (NSCLC), hepatocellular carcinoma, and pancreatic cancer (Kuromachi et al., 2001). TSLC1 was methylated in 16% (15/97) of primary gastric cancers, but not in any of their corresponding non-neoplastic gastric epithelia, as analyzed by the bisulfite-SSCP method (Honda et al., 2002). TSLC1 methylation was not accompanied by LOH and might be bi-allelic in gastric cancer. Methylation preferentially occurred in tumors at the lower portion of the stomach (Honda et al., 2002).

Conclusions

Methylation of tumor suppressor and tumor-related genes initially occurs in non-neoplastic gastric epithelia. Though not immediately oncogenic, methylation increases with age and ultimately inactivates gene function to constitute a field-defect where gastric cancer
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may be prone to develop. As for clinical applications, the detection of cancer-specific RUNX3 and TSLC1 methylation may be used as a diagnostic tool in biopsy samples, gastric juice and serum. Furthermore, detection of methylated hMLH1 and p16 in non-neoplastic gastric epithelia can be utilized as a precancerous signal.

References


