Short Communication

Promoter Methylation Status of *E-Cadherin*, *hMLH1*, and *p16* Genes in Nonneoplastic Gastric Epithelia

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Silencing of tumor suppressor and tumor-related genes by hypermethylation at promoter CpG islands is one of the major events in human tumorigenesis. Promoter methylation is also present in nonneoplastic cells as an age-related tissue-specific phenomenon that precedes the development of neoplasia. To clarify the significance of promoter methylation in nonneoplastic gastric epithelia as a precancerous signal, we investigated promoter methylation status of *E-cadherin*, *hMLH1*, and *p16* genes in nonneoplastic cells of various organs obtained at autopsy, and compared the results with those of nonneoplastic epithelia of a cancerous stomach. Methylation of these genes was not seen in nonneoplastic cells of organs from people who were 22 years and younger (0%, 0 of 6). In contrast, *E-cadherin* and *p16* were methylated in nonneoplastic gastric epithelia of persons who were 45 years or older. The numbers were 86% (12 of 14) and 29% (4 of 14), respectively. *E-cadherin* methylation occurred preferentially in the intestines, whereas *p16* methylation was almost restricted to the stomach. For samples obtained from patients with stomach cancer, methylation was frequently observed in both neoplastic and corresponding nonneoplastic gastric epithelia: 47% (44 of 94) and 67% (63 of 94) for *E-cadherin*, 32% (30 of 94) and 24% (23 of 94) for *hMLH1*, and 22% (21 of 94) and 44% (41 of 94) for *p16*, respectively. *hMLH1* methylation was not seen in nonneoplastic gastric epithelia from autopsy samples but occurred significantly in samples from nonneoplastic tissues of individuals with stomach cancer. Therefore, detection of *hMLH1* methylation in nonneoplastic gastric epithelia may be useful for screening patients who may be at risk of developing gastric cancer. (Am J Pathol 2002, 161:399–403)

DNA methylation is a regulatory mechanism associated with loss of function of the target gene.^{1,2} In various types of human tumors and cells that are aging, CpG islands on promoters of genes that are important in tumor generation are observed to be methylated.^{3,4} Estrogen receptor methylation in human colonic mucosa is known to be hypermethylated with age,⁵ as are the promoters of *IGF2*, *MYOD1*, *N33*, *PAX6*, and *Versican* in the normal colon.^{6,7} Although the mechanism of age-related methylation is unknown, it is clear that age-related methylation affects only a subset of genes with intensities that vary between tissues.^{7,8} Several factors may contribute to age-related methylation, such as exogenous carcinogens, endogenously generated reactive oxygen species, and host genetic differences.⁸ In gastric cancers, the loss of function of *E-cadherin*, *hMLH1*, and *p16* genes is linked to hypermethylation of CpG islands within their promoters.^{9–12} Methylation of these genes in nonneoplastic gastric epithelia was recently reported.^{9,13–15} We reasoned that this phenomenon during aging may be an early warning signal for the beginning of cancer in the gastric epithelia. To test this hypothesis, we have compared the promoter methylation status of *E-cadherin*, *hMLH1*, and *p16* genes in nonneoplastic cells of organs obtained at autopsies from young and old cadavers, with neoplastic and nonneoplastic gastric epithelia of patients with stomach cancer.

Materials and Methods

Samples

Nonneoplastic mucosal tissue samples were obtained from the upper, middle, and lower portions of the stomach, as well as the esophagus, duodenum, jejunum, ileum, colon, rectum, liver, pancreas, lung, and kidney at autopsies. The autopsies consisted of 11 males and 9

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females, ranging in age from 0.7 to 87 years (53 years in average excluding a stillborn infant). Ninety-four gastric cancer samples and their matching nonneoplastic gastric tissues were obtained at surgery from 94 patients (56 males and 38 females). The patients ranged in age from 32 to 89 years (64 years in average). All samples were snap-frozen and stored at -80°C until processed. The tumors consisted of 62 early and 32 advanced, made up of 42 differentiated and 52 undifferentiated by histological examination. Genomic DNA was extracted using standard procedures.

Bisulfite Modification and Methylation-Specific Polymerase Chain Reaction (MSP)

Treatment of DNA samples with bisulfite converts all unmethylated cytosines to uracils, while leaving methylated cytosines unaffected. Briefly, 2 μg of genomic DNA was denatured by treatment with NaOH and modified by sodium bisulfite. The samples were then purified using Wizard DNA purification resin (Promega, Madison, WI), treated with NaOH, recovered in ethanol, and resuspended in 30 μl of distilled water. Amplification was achieved in a 20- μl reaction volume containing 2 μl of GeneAmp PCR Gold Buffer (PE Applied Biosystems, Foster City, CA), 1.0 mmol/L of MgCl_2 , 1 μl of each primer, 0.2 mmol/L of dNTPs, and 1 U of Taq polymerase (AmpliTaQ Gold DNA Polymerase, PE Applied Biosystems). Hot start polymerase chain reaction (PCR) was performed in a thermal cycler (GeneAmp 2400, PE Applied Biosystems) for 35 cycles, each of which consisted of denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 30 seconds, followed by a final 7-minute extension at 72°C . A positive control and negative control (distilled water without DNA) were included for each amplification. The PCR products were separated on a 6% nondenaturing polyacrylamide gel. Primer sequences for MSP were: sense, 5'-GGT GAA TTT TTA GTT AAT TAG CGG TAC-3'; and antisense, 5'-CAT AAC TAA CCG AAA ACG CCG-3' (204 bp) for methylated DNA of *E-cadherin*, sense, 5'-GGT AGG TGA ATT TTT AGT TAA TTA GTG GTA-3'; and antisense, 5'-ACC CAT AAC TAA CCA AAA ACA CCA-3' (211 bp) for unmethylated DNA of *E-cadherin* (accession no. L34545),¹⁰ and sense, 5'-ACG TAG ACG TTT TAT TAG GGT CGC-3'; and antisense, 5'-CCT CAT CGT AAC TAC CCG CG-3' (115 bp) for methylated DNA of *hMLH1* (accession no. AB017806),¹¹ and sense, 5'-GGG TCG GAG GGG GTT TTT TC-3' (nucleotides 1075 to 1094); and antisense, 5'-CAA CCG CCG AAC GCA CTC GA-3' (nucleotides 1152 to 1171) (97 bp) for methylated DNA of *p16* (accession no. X94154). All DNA samples were checked for bisulfite modification using the primer set for unmethylated *E-cadherin*.

Preparation of Positive Control

Sss-I methylase (New England BioLabs, Inc., Beverly, MA) was used to methylate 100 μg of peripheral blood

DNA, and was modified by sodium bisulfite as described above.

Statistical Analysis

Statistical comparisons were performed using Fisher's exact test. A $P < 0.05$ was considered significant.

Results

Methylation in Autopsy Samples

Methylation of *E-cadherin*, *hMLH1*, and *p16* was not detected in nonneoplastic cells of any organs obtained from individuals who were 22 years and younger (0 of 6). In contrast, methylation of *E-cadherin* and *p16* in nonneoplastic gastric epithelia was present in 86% (12 of 14) and 29% (4 of 14) of individuals who were 45 years or older, respectively. *E-cadherin* methylation occurred preferentially in the intestines, whereas *p16* methylation was almost restricted to the stomach of the elderly (Table 1 and Figure 1). Methylation of *hMLH1* was not found in any organ in all age groups except for a nonneoplastic lung tissue obtained from a 78-year-old male who died of lung cancer (Table 1).

Methylation in Neoplastic and Nonneoplastic Gastric Epithelia of Gastric Cancer Patients

Methylation of *E-cadherin*, *hMLH1*, and *p16* was present in both neoplastic and nonneoplastic gastric epithelia, distributed as follows: 47% (44 of 94) and 67% (63 of 94) for *E-cadherin*, 32% (30 of 94) and 24% (23 of 94) for *hMLH1*, and 22% (21 of 94) and 44% (41 of 94) for *p16*, respectively (Figure 2). *E-cadherin* methylation was significantly more frequent in females than in males ($P < 0.05$), and in histologically undifferentiated tumors than in differentiated ones ($P < 0.05$). *hMLH1* methylation was also significantly more frequent in females than in males ($P < 0.05$), and in patients older than 50 years of age than in those younger than 50 years of age ($P < 0.05$). Methylation of *p16* did not correlate with any clinicopathological characteristic. The results are summarized in Table 2.

Discussion

Our findings suggest that *E-cadherin* and *p16* methylation in nonneoplastic cells is age-related, whereas *hMLH1* methylation lacks any association with age in nonneoplastic tissues. *E-cadherin* and *p16* methylation occurs in nonneoplastic gastric epithelia at approximately 40 years of age. The incidence of gastric cancer rises up in this age group and points to an association between age-related methylation and gastric cancer development. In addition, a marked increase in methylated genes from nonmetaplastic mucosa to intestinal metaplasia, which is very common in the Japanese elderly population, has been reported.¹⁵ In a recent publication, age-related

Table 1. Methylation Status of *E-Cadherin*, *hMLH1*, and *p16* in Nonneoplastic Tissues of Autopsies

Gene	Age (y)	Sex*	Cause of death†	Organs**												
				S(U)	S(M)	S(L)	Es	Du	Je	Il	Co	Re	Li	Pa	Lu	Ki
<i>E-cadherin</i>		f	Still born infant		—											
	0.7	f	COARC		—		—	—	—	—	—		—	—	—	—
	8	m	AML	—	—	—	—		—		—	—		—		—
	15	f	RDS		—											
	16	m	Brain tumor		—											
	22	m	Brain tumor	—	—	—							—	—	—	—
	45	m	Liver cancer	M	M	M	—	—	M	M	M	—	—	—	—	—
	47	m	Brain tumor	M	M	M	—	—	—	—	—	—	—	—	—	—
	49	m	Lung cancer		—		—									
	55	m	Rectal cancer	M	M	M	—			M		M	—	—	—	M
	60	f	Lung cancer	M	M	M	—	—	M	M	M	—	—	—	—	—
	67	m	Pancreatic cancer	M	M	M	—	M	M	—	—	—	—	—	—	—
	68	f	Ovarian cancer	M	M	M	—					—	—	—	M	—
	69	f	Ovarian cancer	M	M	M	—	M	M		M		—	—	—	—
	73	f	Malignant lymphoma	—	—	—	—	M	M	M	—	—	M	—	M	—
	77	m	Lung cancer	M	M	M	—	M	M	—	M		—	—	—	—
	78	m	Lung cancer	—	M	M	—	—	—	M	M	M	—	—	M	—
	78	m	Aneurysm	M	M	—	—	—	M		M	M	—	—	—	M
	86	f	Colon cancer		M		M			—	M		—	—	—	—
	87	f	Lung cancer	M	M	—	M	M	—	—	—	—	—	—	—	—
<i>hMLH1</i>		f	Stillborn infant		—											
	0.7	f	COARC		—		—	—	—	—	—		—	—	—	—
	8	m	AML	—	—	—	—		—		—	—		—		—
	15	f	RDS		—											
	16	m	Brain tumor		—											
	22	m	Brain tumor	—	—	—							—	—	—	—
	45	m	Liver cancer	—	—	—	—	—	—	—	—	—	—	—	—	—
	47	m	Brain tumor	—	—	—	—	—	—	—	—	—	—	—	—	—
	49	m	Lung cancer		—		—									
	55	m	Rectal cancer	—	—	—	—			—		—	—	—	—	—
	60	f	Lung cancer	—	—	—	—	—	—	—	—	—	—	—	—	—
	67	m	Pancreatic cancer	—	—	—	—	—	—	—	—	—	—	—	—	—
	68	f	Ovarian cancer	—	—	—	—					—	—	—	—	—
	69	f	Ovarian cancer	—	—	—	—	—	—		—		—	—	—	—
	73	f	Malignant lymphoma	—	—	—	—	—	—	—	—	—	—	—	—	—
	77	m	Lung cancer	—	—	—	—	—	—	—	—	—	—	—	—	—
	78	m	Lung cancer	—	—	—	—	—	—	—	—	—	—	—	M	—
	78	m	Aneurysm	—	—	—	—	—	—	—	—	—	—	—	—	—
	86	f	Colon cancer		—		—			—	—		—	—	—	—
	87	f	Lung cancer	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>p16</i>		f	Stillborn infant		—											
	0.7	f	COARC		—		—	—	—	—	—		—	—	—	—
	8	m	AML	—	—	—	—		—		—	—		—		—
	15	f	RDS		—											
	16	m	Brain tumor		—											
	22	m	Brain tumor	—	—	—							—	—	—	—
	45	m	Liver cancer	M	—	M	—	—	—	—	—	—	—	—	—	—
	47	m	Brain tumor	—	—	—	—	—	—	—	—	—	—	—	—	—
	49	m	Lung cancer		—		—								—	—
	55	m	Rectal cancer	—	—	—	—			—		—	—	—	—	—
	60	f	Lung cancer	—	—	—	—	—	—	—	—	—	—	—	—	—
	67	m	Pancreatic cancer	M	M	M	—	M	—	—	—	—	—	—	—	—
	68	f	Ovarian cancer	—	—	—	—					—	—	—	—	—
	69	f	Ovarian cancer	M	M	M	—	—	—		—		—	—	—	—
	73	f	Malignant lymphoma	—	—	—	—	—	—	—	—	—	—	—	—	—
	77	m	Lung cancer	—	—	—	—	—	—	—	—	—	—	—	—	—
	78	m	Lung cancer	—	—	—	—	—	—	—	—	—	—	—	—	—
	78	m	Aneurysm	M	M	—	—	—	—	—	—	—	—	—	—	—
	86	f	Colon cancer		—		—			—	—		—	—	—	—
	87	f	Lung cancer	—	—	—	—	—	—	—	—	—	—	—	—	—

*f, female; m, male.

†COARC, coarctation of aorta; AML, acute myelogenous leukemia; RDS, respiratory distress syndrome.

**S(U), upper third portion of the stomach; S(M), middle third portion of stomach; S(L), lower third portion of stomach; Es, esophagus; Du, duodenum; Je, jejunum; Il, ileum; Co, colon; Re, rectum; Li, liver; Pa, pancreas; Lu, lung; Ki, kidney; blank, not done; —, unmethylated; M, methylated.

Figure 1. MSP of autopsy samples (67-year-old male, see Table 1). **a:** *E-cadherin*. **b:** *hMLH1*. **c:** *p16*. **d:** Unmethylated sequence-specific PCR for *E-cadherin*. Methylated PCR products are present in lanes 1–3, 5, and 6 for *E-cadherin* (**a**), in none for *hMLH1* (**b**), in lanes 1–3 and 5 for *p16* (**c**). Unmethylated *E-cadherin* PCR products are present in all the samples (**d**). **Lanes:** 1, stomach (upper); 2, stomach (middle); 3, stomach (lower); 4, esophagus; 5, duodenum; 6, jejunum; 7, ileum; 8, colon; 9, rectum; 10, liver; 11, pancreas; 12, lung; 13, kidney; P, positive control; DW, distilled water; and SM, size marker.

methylation of *E-cadherin* was detected in normal control samples of the urinary bladder obtained at autopsies in patients older than 70 years of age, as well as in urinary bladder neoplasms that developed in very old individuals.¹⁶ *E-cadherin* methylation was frequently detected in nonneoplastic tissues of autopsies in the present study, however, it remained uncertain which cell type was affected by methylation. Laser microdissection may help to resolve this issue. Methylation frequencies of *E-cadherin* and *p16* in gastric cancer tissue were 47% and 22%, respectively, falling within the ranges reported previously.^{9,10,14} The methylation of *E-cadherin* and *p16* leads

Figure 2. MSP of samples from patients with stomach cancer. **a:** *E-cadherin*. **b:** *hMLH1*. **c:** *p16*. **d:** Unmethylated sequence-specific PCR for *E-cadherin*. Methylated PCR products are present in T and N of M225, M251, Y78, and Y83, in N of M240, and in T of Y27 for *E-cadherin* (**a**), in T and N of M240, in N of M251, and in T of Y78 for *hMLH1* (**b**), and in T and N of M240 and Y83, in N of Y78, and in T of Y27 for *p16* (**c**). Unmethylated *E-cadherin* PCR products are present in all the samples (**d**). **Lanes:** T, tumor DNA; N, normal DNA; P, positive control; DW, distilled water; and SM, size marker.

to the loss of protein expression and cancer-related pathology.^{9,12,17} *hMLH1* was methylated in 32% of neoplastic and 24% of nonneoplastic tissues of patients with stomach cancer. The methylation at this promoter region of *hMLH1* also correlated well with gene silencing.¹¹ The proportion of *hMLH1* methylation was increased in older patients, similarly to a previous report.¹⁸ Interestingly, *hMLH1* methylation was absent in nonneoplastic gastric epithelia obtained at autopsies. These results suggest that methylation of *hMLH1* in nonneoplastic gastric epithelia is pathological and may serve as a useful marker for gastric cancer development. In a separate study, we

Table 2. Correlation of Methylation Status and Clinicopathological Characteristics in Gastric Cancer

Clinicopathological characteristics (n)	Promoter methylation status					
	<i>E-cadherin</i>		<i>hMLH1</i>		<i>p16</i>	
	M	U	M	U	M	U
Sex						
Male (56)	20	36	11	45	10	46
Female (38)	24	14	19	19	11	27
	$P < 0.05$		$P < 0.05$		NS	
Age						
≤50 (17)	11	6	2	15	6	11
50 < (77)	33	44	28	49	15	62
	NS		$P < 0.05$		NS	
Location						
Upper third (17)	10	7	4	13	5	12
Middle third (34)	16	18	10	24	10	24
Lower third (41)	16	25	16	25	6	35
Unknown (2)	2	0	0	2	0	2
	NS		NS		NS	
Histological type						
Differentiated (42)	13	29	12	30	9	33
Undifferentiated (52)	31	21	18	34	12	40
	$P < 0.05$		NS		NS	
Stage						
Early (62)	30	32	22	40	16	46
Advanced (32)	13	19	8	24	5	27
	NS		NS		NS	
Lymph node metastasis						
Present (27)	10	17	9	18	5	22
Absent (64)	32	32	21	43	16	48
Unknown (3)	2	1	0	3	0	3
	NS		NS		NS	

M, methylated; U, unmethylated; NS, not significant.

detected a frequent *hMLH1* methylation in nonneoplastic gastric epithelia not only adjacent to, but also far from each tumor exhibiting a high frequency of microsatellite instability.¹⁹

We conclude that detection of *hMLH1* methylation in nonneoplastic gastric epithelia may be a useful screening method for identifying individuals who may be at risk of developing gastric cancer, although additional studies of the prevalence of *hMLH1* methylation is needed in larger populations with other nonneoplastic gastric disease. The high frequencies of *E-cadherin* and *p16* methylation in nonneoplastic gastric epithelia from autopsies and gastric cancer patients rule out the use of the two genes as useful markers.

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