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Comparative analysis of DNA methylation between primary and metastatic gastric carcinoma

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Abstract. Metastasis is a multi-step process involving many biomolecular changes and DNA methylation is one such molecular change. Although differences in DNA methylation have been reported in matched primary and metastatic mammary carcinoma, no such differences have been reported in gastric carcinoma. Accordingly, to investigate whether DNA methylation profiles in metastatic gastric carcinoma differ from those of their primary counterparts, we investigated the DNA methylation of eleven genes, *ADAM23*, *CDH1*, *FHIT*, *FLNC*, *GSTP1*, *ITGA4*, *LOX*, *RUNX3*, *THBS1*, *TIMP3*, and *UCHL1* in 74 matched human primary gastric carcinomas, lymph node metastases, non-neoplastic gastric mucosal, and uninvolved lymph node tissues by utilizing methylation-specific PCR. Seven of these genes (*ADAM23*, *FLNC*, *ITGA4*, *LOX*, *RUNX3*, *TIMP3*, and *UCHL1*) showed cancer-specific methylation, and three (*CDH1*, *FHIT*, and *THBS1*) showed cancer-unrelated methylation. *GSTP1* was rarely methylated in any tissue type. Of the seven genes that showed cancer-specific methylation, *FLNC* was more frequently methylated in metastatic gastric carcinomas than in their primary counterparts (p=0.004). In addition, the average number of methylated genes in metastatic tumors was greater than that in primary tumors (p=0.004). The high-methylation group (cases with three or more genes methylated in primary tumors) was found to contain more women (p=0.031) and diffuse type tumors by Lauren classification (p=0.022). DNA methylation profiles were not found to affect prognosis. We suggest that promoter methylation of *FLNC* may be involved in the lymph node metastasis of gastric carcinoma and that the DNA methylation statuses of metastatic tumors should be considered in node-positive gastric carcinoma.

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Introduction

Metastasis is a multi-step process that involves the release of cells from a primary site, their invasion into adjacent connective tissues, transmigration across the basement membrane, intravasation, tumor emboli formation, extravasation, and establishment of new growth at target organs. To successfully perform these tasks, genes responsible for cellular attachment, apoptosis, drug metabolism and various signal transductions must undergo appropriate genetic or epigenetic changes (1-3).

It is generally accepted that the majority of cancers produce heterogeneous clones while they grow at primary sites due to their inherent genetic instabilities, and that clonal evolution eventually creates metastasis-competent clones (4). These metastasis-competent clones detach from their primary sites and disseminate systematically via lymphatic or vascular channels and attach in regional lymph nodes or distant sites. The metastatic deposits produced were initially believed to have the same molecular profiles as the primary tumors. However, this point of view has recently been challenged, because molecular alterations, such as, DNA methylation and protein expression differences have been demonstrated in primary and metastatic cancers (5-9).

DNA methylation is a type of epigenetic change, which is defined as a heritable change in gene expression not involving alterations in the nucleotide sequence. In human cancers, DNA methylation is detected as global hypomethylation or as hypermethylation of specific promoter CpG islands (10). In particular, the hypermethylation of specific promoter CpG islands often results in the silencing of important tumor suppressor genes, and thus, promotes the development and progression of human cancers. Differences in the DNA methylation profiles of primary and metastatic cancers have been reported in several studies on breast cancers (5,6) and gastric cancers (7). However, it has not been established whether the DNA methylation profiles of metastatic deposits differ from those of primary tumors.

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death worldwide (11). Although gastric cancer is now being increasingly detected in its early stage due to the introduction of screening endoscopy

in Korea and Japan, many gastric cancers are still detected when advanced. Furthermore, when detected in an advanced stage, prognosis largely depends on the presence of lymph node or distant metastases.

In gastric cancer, DNA methylation has been reported to occur mainly during the early stage of carcinogenesis (12) and to increase during multi-step carcinogenesis (13) or to affect even histologically normal mucosa (14). More recently, DNA methylation has been reported in an advanced stage disease and in associated lymph node metastases (15). Nevertheless, differences between the DNA methylation profiles of primary and metastatic gastric cancer have not been well documented.

To determine whether DNA methylation profiles are different between primary and metastatic gastric carcinomas, we investigated the promoter methylation of 11 putative tumor suppressor genes, namely, *a disintegrin and metalloproteinase domain 23 preproprotein* (*ADAM23*), *cadherin-1* (CDH1), *fragile histidine triad gene* (*FHIT*), *gamma-filamin* (*FLNC*), *glutathione S-transferase pi* (*GSTP1*), *integrin alpha 4* (*ITGA4*), *lysyl oxidase* (*LOX*), *runt-related transcription factor 3* (*RUNX3*), *thrombospondin 1* (*THBS1*), *tissue inhibitor metalloproteinase 3* (*TIMP3*) and *ubiquitin carboxylterminal esterase L1* (*UCHL1*) in 74 human gastric carcinomas and in matched lymph node metastases by methylation-specific PCR (MSP). These 11 genes were selected because it has been reported that their promoters are methylated and their respective target mRNAs are silenced in gastric carcinoma (16-21) and because some of them are known to be functionally related to metastasis, i.e., *ADAM23*, membrane adhesion and cell migration, *CDH1*, cell adhesion, *FLNC*, organization of actin polymerization and cell motility, *ITGA4*, cell-extracellular matrix, cell-cell interactions, *THBS1*, angiogenesis, and *TIMP3*, extracellular matrix degradation. Corresponding non-neoplastic mucosa and uninvolved lymph node tissues were included for control purposes, because some genes are frequently methylated in chronic gastritis and intestinal metaplasia, and because methylation has also been associated with aging (14).

Materials and methods

Patients and samples. The files of surgically resected gastric cancer cases examined at the Department of Pathology, Seoul National University College of Medicine between January 1, 1996 and December 31, 1996 were initially included. Age, gender, tumor location, gross type (according to Borrmann classification), lymphatic invasion, tumor stage (22), and a history of preoperative chemotherapy or radiotherapy were evaluated by reviewing medical charts and pathology records. Those who had undergone preoperative chemotherapy or radiotherapy were excluded. Glass slides were reviewed to determine histological types according to the World Health Organization (WHO) and Lauren classifications.

For the analysis of promoter methylation, cases fulfilling the following requirements were selected: i) presence of adenocarcinoma occupying more than total area of 50 mm^2 at both the primary and metastatic lymph node sites; ii) the presence of viable tumor cells occupying >60% of each tumor focus; and iii) the absence of tumor-obscuring inflammation. Finally, a total of 74 cases were selected and these 74 cases constituted the study population. The mean age of these 74 patients was 57.7 years and the male to female ratio was approximately 2.3:1. In terms of tumor stage, there was one case of stage I, 15 cases of stage II, 25 of stage III, and 33 of stage IV. By Lauren classification, 44 cases were of the intestinal type, 28 cases were of the diffuse type, and 2 cases were of the mixed type.

Clinical outcomes were followed from date of surgery till death or December 31, 2004, which resulted in a mean follow-up of 35 months (range 1-108 months). Cases lost to follow-up and those that died of a cause other than gastric cancer were censored during the survival rate analysis.

DNA extraction. The DNA was extracted from four tissue paraffin-blocks of: i) primary gastric cancers; ii) matched lymph node metastases; iii) non-neoplastic gastric mucosa; and iv) uninvolved lymph nodes. Targeted areas were labeled with a marking pen on H&E-stained slides. Paraffin-blocks were sectioned at 10 μm, and sections were de-paraffinized and re-hydrated by xylene and alcohol. Prepared 50 mm^2 areas, corresponding with regions labeled on H&E-stained slides, were excised by scalpel blade taking care to prevent contamination. These tissues were then suspended in 0.5% Tween-20 buffer, heated at 90˚C for 10 min, and digested with proteinase K at 55˚C for 90 min with gentle agitation. Digested samples were reacted with 2.5% Chelex 100 at 99˚C for 10 min and centrifuged at 15,000 rpm for 5 min. Supernatants were stored at 4˚C until required.

Bisulfite modification. Aliquots of 2.5 μg of DNA with 10 μg of salmon sperm DNA as a carrier, were heated at 99˚C for 5 min, and denatured in 0.3 N NaOH at 37˚C for 15 min. To these denatured DNAs were added 2.5 M sodium metabisulfite and 125 mM hydroquinone (pH 5.0), and samples were then incubated at 55˚C for 16 h in the dark. Samples were then desalted using the Wizard DNA Clean-Up System (Promega, Madison, WI, USA), and desulfonated in 0.3 N NaOH at 37˚C for 15 min. The modified DNAs were then precipitated with ammonium acetate and cold alcohol and centrifuged at 15,000 rpm for 20 min keeping them under 4˚C. Pellets so obtained were dissolved in 20 μl of distilled water and stored at 4˚C until use.

Methylation-specific polymerase chain reaction (MSP). Reaction mixtures consisted of 5 μl of Premix Ex Taq™ (Takara, Kyoto, Japan), each 5 pmol of forward and of reverse primers of both methylated and unmethylated alleles, 30~50 ng of modified DNA template and 5 μl of distilled water. PCR was hot-started at 95˚C for 5 min, and this was followed by 35 or 40 cycles of denaturation at 95˚C for 30 sec, annealing for 30 sec, and extension at 72˚C for 1 min. A final 10-min extension at 72˚C completed each PCR. Annealing temperatures and cycle numbers were individually optimized for each of the primer sets by identifying an annealing temperature that produced only one specific band and the minimal cycle number that produced PCR products (Table I). The amplifications were carried out in ABI 2720 Thermal Cycler (Perkin-Elmer, CA, USA). After amplification, each PCR product was electrophoresed in 2.5% agarose gel and

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MSP primer sets and experimental conditions.

Gene		Forward primer	Reverse primer	Ta[a]	Cy[b]	Ref[c]
ADAM23	M	GGGCGTACGTTCGTTTC	CAACGACTACGAAAACTACCG	60	35	(30)
	U	GGGGTGGGGGTGTATGTTT	ACACAACCACTTCAACAACTACA	60	35	
CDH1	M	TTAGGTTAGAGGGTTATCGCGT	TAACTAAAAATTCACCTACCGAC	60	40	(13)
	U	TAATTTTAGGTTAGAGGGTTATTGT	CACAACCAATCAACAACACA	60	35	
FHIT	M	TTGGGGCGCGGGTTTGGGTTTTTACGC	CGTAAACGACGCCGACCCCACTA	68	40	(20)
	U	TTGGGGTGTGGGTTTGGGTTTTTATG	CATAAACAACACCAACCCCACTA	66	40	
FLNC	M	GAGAGAGAGTTAGAGAGCGGTCGAGC	GACCACGAAACTCGCTACGCTACG	66	35	(27)
	U	GAGAGAGAGTTAGAGAGTGGTTGAGT	AACCACAAAACTCACTACACTACA	60	35	
GSTP1	M	TTCGGGGTGTAGCGCTCGTC	GCCCCAATACTAAATCACGACG	62	40	(18)
	U	GATGTTTGGGGTGTAGTGGTTGTT	CCACCCCAATACTAAATCACAACA	60	40	
ITGA4	M	TAGAGTTATTTCGCGTTTTGCG	CTTCGAATACTCGCGCTACTT	60	35	(17)
	U	GTTTAGAGTTATTTTGTGTTTTGTG	AAAACTTCAAATACTCACACTACT	60	35	
LOX	M	GAATAAATAGTTGAGGGGCGGTC	GCGACAATCCCGAAAAACG	64	35	(15)
	U	TGTGAATAAATAGTTGAGGGGTGGTT	CAACACAACAATCCCAAAAAACA	64	35	
RUNX3	M	TTACGAGGGGCGGTCGTACGCGGG	AAAACGACCGACGCGAACGCCTCC	68	35	(16)
	U	TTATGAGGGGTGGTTGTATGTGGG	AAAACAACCAACACAAACACCTCC	68	40	
THBS1	M	TGCGAGCGTTTTTTTAAAAGC	TAAACTCGCAAACCAACTCG	60	35	(13)
	U	GTTTGGTTGTTGTTTATTGGTTG	CCTAAACTCACAAACCAACTCA	60	35	
TIMP3	M	CGTTTCGTTATTTTTTGTTTTCGGTTTC	CCGAAAACCCCGCCTCG	59	35	(19)
	U	TTTTGTTTTGTTATTTTTTGTTTTTGGTTTT	CCCCCAAAAACCCCACCTCA	59	35	
UCHL1	M	GGTTCGGTCGTATTATTTCGC	ACTACATCTTCGCGAAACGCCCG	62	35	(21)
	U	GGTTTGGTTGTATTATTTTGT	ACTACATCTTCACAAAACACCCA	58	40	

[a]Annealing temperatures (°C); [b]numbers of cycles; [c]reference numbers.

visualized by ethidium bromide staining using ultraviolet light. The integrity and completeness of bisulfite conversions of modified DNAs were estimated using unmethylated *GSTP1* gene because *GSTP1* was rarely methylated in the present series. A sample was considered to have been methylated when an unequivocal specific band of a predicted size was detected in the reaction performed with methylated primer sets. Samples showing an equivocal band were re-subjected to MSP and those showing a specific band consistently were considered to have been methylated. SssI methylase-treated DNA or DNA from gastric cancer cell lines with known methylation status for each gene were used as positive controls. The distilled water without template was used as a negative control.

Statistical analyses. The differences in DNA methylation frequencies between primary and metastatic gastric carcinoma were estimated using NcMemar's test. The differences between numbers of methylated genes in primary and metastatic carcinomas were estimated using Wilcoxon's signed rank test. The χ^2 test or Fisher's exact test (two-sided) were used to determine the significance of associations between the DNA methylation frequencies and clinicopathologic factors. Multivariate logistic regression analysis was used to confirm the presence of an independent significant association between the DNA methylations of multiple genes and clinicopathologic factors. Survival curves were estimated using the Kaplan-Meier product-limit method and the significance of differences between the survival curves were determined using the log-rank test. Multivariate analysis was performed using Cox's proportional hazards model. Results were considered to be statistically significant when the p-value was <0.05. All statistical analyses were conducted using SPSS ver. 12.0 (SPSS, Chicago, IL).

Results

Frequency of DNA methylation. Representative MSP results for 11 genes are shown in Fig. 1, and all results are shown in Fig. 2 and Table II. The frequencies of DNA methylation of the 11 genes in primary gastric carcinomas ranged from 1 case (1.4%, *GSTP1*) to 54 cases (73.0%, *ITGA4*), and the

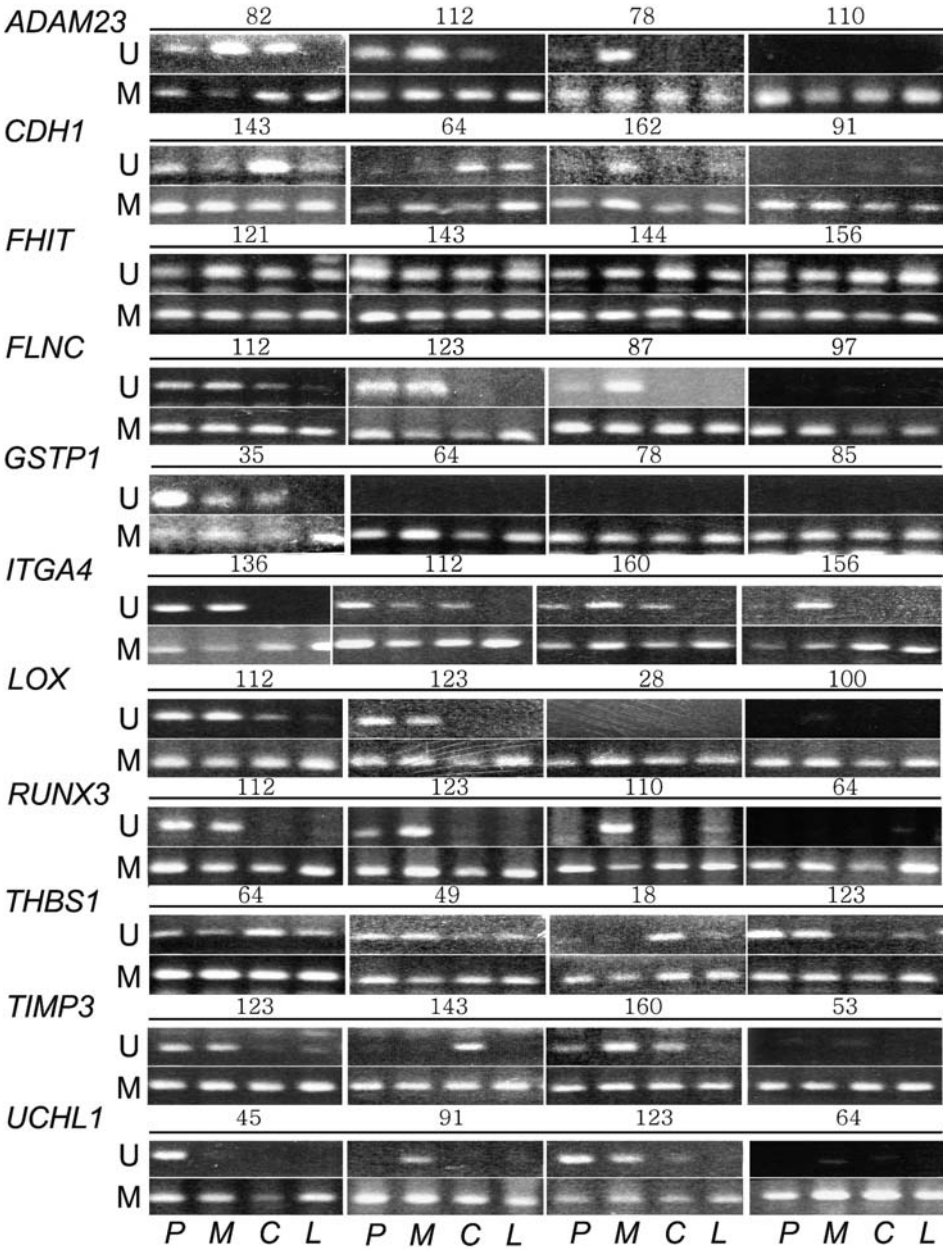

Figure 1. Representative MSP results of 11 genes in primary (*P*) and metastatic (*M*) gastric carcinomas and in corresponding non-neoplastic mucosal (*C*), and uninvolved lymph node tissues (*L*). Four methylated (M) bands and four unmethylated (U) bands are demonstrated for each case. The four bands represent results of *P*, *M*, *C* and *L* samples. Case numbers are shown at the top.

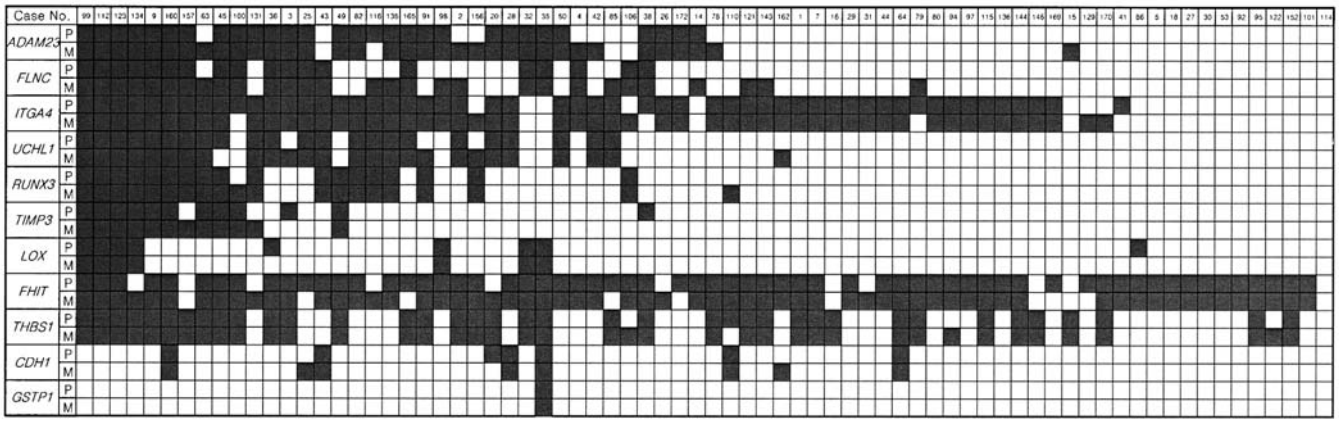

Figure 2. MSP results of individual samples. Empty squares (❑) represent the absence of a methylated allele, and filled squares (■) the presence of a methylated allele. No, number; P, primary gastric carcinoma; M, metastatic gastric carcinoma.

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Frequency of DNA methylation in primary and metastatic gastric carcinomas, and in corresponding non-neoplastic nd uninvolved lymph node tissues.

Gene	Primary gastric carcinoma (n=74) (%)	Metastatic gastric carcinoma (n=74) (%)	Non-neoplastic mucosa (n=63) (%)	Uninvolved lymph nodes (n=46) (%)	p-value[a]
ADAM23	29 (39.2)	35 (47.3)	6 (9.5)	2 (4.3)	<0.001
CDH1	7 (9.5)	8 (10.8)	8 (12.7)	11 (23.9)	0.5
FHIT	63 (85.1)	63 (85.1)	53 (84.1)	43 (93.5)	1.0
FLNC	19 (25.7)	28 (37.8)	0 (0)	1 (2.2)	<0.001
GSTP1	1 (1.4)	1 (1.4)	0 (0)	0 (0)	1.0
ITGA4	54 (73.0)	55 (74.3)	9 (14.3)	2 (4.3)	<0.001
LOX	9 (12.2)	7 (9.5)	1 (1.6)	0 (0)	0.008
RUNX3	18 (24.3)	20 (27.0)	2 (3.2)	0 (0)	<0.001
THBS1	38 (51.4)	40 (54.1)	32 (50.8)	27 (58.7)	1.0
TIMP3	12 (16.2)	12 (16.2)	3 (4.8)	0 (0)	0.039
UCHL1	23 (31.1)	26 (35.1)	5 (7.9)	1 (2.2)	<0.001
Average number of methylated genes	2.2[b]	2.5[b]	0.3[c]	0.1[c]	0.004[b] 0.011[c]

[a]Significances of differences in promoter methylation between primary gastric carcinoma and non-neoplastic mucosa by McNemar's test. [b,c]p-value by Wilcoxon's signed rank test.

Table III. Changes in the methylation status from primary to metastatic gastric carcinomas.

	Changes in methylation status				
	Primary gastric → Metastatic gastric carcinoma				p-value[c]
Gene	U[a] → U	U → M[b]	M → U	M → M	
ADAM23	38	7	1	28	0.07
FLNC	46	9	0	19	0.004
ITGA4	16	4	3	51	1.0
LOX	65	0	2	7	0.5
RUNX3	54	2	0	18	0.5
TIMP3	60	2	2	10	1.0
UCHL1	47	4	1	22	0.375

[a]Unmethylated; [b]methylated; [c]p-value by McNemar's test.

frequencies in metastatic gastric carcinomas were somewhat different. Genes were divided into two groups based on patterns of DNA methylation frequency in corresponding non-neoplastic mucosal and uninvolved lymph node tissues. One group showed a 'cancer-specific pattern' in which the DNA methylation was observed exclusively in tumor tissues and rarely in non-neoplastic mucosa or uninvolved lymph node tissues. The other group showed a 'cancer-unrelated pattern' in which methylation frequencies in non-neoplastic mucosa or uninvolved lymph node tissues were comparable to those in tumor tissues. The genes that showed a 'cancer-specific pattern' were *ADAM23*, *FLNC*, *ITGA4*, *LOX*, *RUNX3*, *UCHL1*, and *TIMP3*, whereas *CDH1*, *FHIT*, and *THBS1* showed a 'cancer-unrelated pattern' (Table II). *GSTP1* methylation appeared to be 'cancer-specific' but its frequency was extremely low. Consequently, only the seven genes showing a 'cancer-specific' pattern of DNA methylation were selected for further analysis.

Comparison of DNA methylation frequencies in primary and metastatic gastric carcinomas. Changes in the methylation status of the seven genes selected above (*ADAM23*, *FLNC*, *ITGA4*, *LOX*, *RUNX3*, *UCHL1*, and *TIMP3*) are listed in Table III. Of these, *ADAM23* and *FLNC* tended to be more frequently methylated in metastatic gastric carcinomas, and this difference in *FLNC* methylation was significant (p=0.004). The remaining five genes also tended to show a higher frequency of methylation in metastatic gastric carcinomas. The majority (64 of 74 cases, 86.5%) of gastric carcinomas showed methylation of at least one of these 7 genes in either a primary or metastatic site. The average number of genes methylated in the 74 cases increased from 2.2 for primary gastric carcinomas to 2.5 for metastatic gastric carcinomas and this difference was statistically significant (p=0.004).

Association between the frequency of DNA methylation and clinicopathologic factors. Promoter methylations of *FLNC* and *TIMP3* were found to be associated with a female gender (p=0.007 and p=0.03, respectively). *RUNX3* methylation was found to be associated with an advanced T class (p=0.028)

Table IV. Associations between the high-methylation group and clinicopathological factors in gastric carcinoma.

Clinicopathological factors	Methylation group		p-value[a]	Logistic regression	p-value[b]
	Low (%)	High (%)		Odds ratio (95% CI)	
Median survival (months)	17	8	0.299[c]		
Age			0.917	-	-
<65	34 (61.8)	21 (38.2)			
≥65	12 (63.2)	7 (76.8)			
Gender			0.031		0.030
Male	37 (69.8)	16 (30.2)		1.00	
Female	9 (42.9)	12 (57.1)		3.655 (1.133-11.791)	
Lauren classification			0.022		0.014
Intestinal	33 (75.0)	11 (25.0)		1.00	
Diffuse	12 (42.9)	16 (57.1)		3.957 (1.323-11.840)	
Mixed	0 (0)	2 (100)			
Lymphatic invasion			0.723	-	-
Absent	20 (64.5)	11 (35.5)			
Present	26 (60.5)	17 (39.5)			
Vascular invasion			0.913	-	-
Absent	39 (61.9)	24 (38.1)			
Present	7 (63.6)	4 (36.4)			
T class[d]			0.231	-	-
T1 + T2	23 (70.0)	10 (30.0)			
T3 + T4	23 (56.1)	18 (43.9)			
N class[d]			0.030		0.172
N1 + N2	30 (73.2)	11 (26.8)		1.00	
N3	16 (48.5)	17 (51.5)		2.095 (0.725-6.056)	
Distant metastasis			0.583	-	-
Absent	39 (60.9)	25 (39.1)			
Present	7 (70.0)	3 (30.0)			
pTNM stage[d]			0.030		0.172
I + II + III	30 (73.2)	11 (26.8)		1.00	
IV	16 (48.5)	17 (51.5)		2.095 (0.725-6.056)	

[a]p-value by the χ^2 test. [b]p-value corrected by multivariate logistic regression. [c]p-value by the log-rank test. [d]Staging according to the AJCC Cancer Staging Manual (6th edition).

and *TIMP3* methylation with an advanced pTNM stage (p=0.02). However, no relation was found between the methylation status of any of 7 genes and prognosis.

Since methylation of multiple genes has been shown to be associated with a poor prognosis in esophageal adenocarcinoma (23) and bladder cancer (24), we investigated the association between the number of genes methylated in primary tumors and clinicopathological factors including prognosis. To divide cases into two groups according to the number of methylated genes in primary tumors, we arbitrarily divided cases into high-methylation (cases in which three or more genes were methylated in their primary sites) and low-methylation (cases in which two or fewer genes were methylated in their primary sites) groups because the average number of methylated genes in primary tumor was 2.2 (15). The high-methylation group was found to contain a greater number of female patients (p=0.03) and diffuse type tumors (p=0.02), and to be associated with an advanced N class and pTNM stage (p=0.03). However, multivariate logistic regression analysis showed that the N class and pTNM stage were not associated with high-methylation group. Median survival of high-methylation group tended to be shorter than that of low-methylation group (8 months vs. 17 months), but this difference was not significant (Table IV).

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The present study demonstrated that DNA methylation profiles in metastatic gastric carcinoma were different from those in primary gastric carcinoma. Specifically, *FLNC* promoter was more frequently methylated in metastatic gastric carcinomas than in primary gastric carcinomas and the average number of methylated genes in metastatic gastric carcinomas was greater than that in primary gastric carcinomas. These findings indicate that the aberrant DNA methylation of *FLNC* may play a role in the metastasis of gastric carcinoma.

Differences between the methylation statuses of primary and metastatic tumors have been initially described in breast cancer. In one study (5), seven genes (*MINT1*, *MINT2*, *MINT31*, *INK4A*, *CDH1*, *RAR-ß2* and *THBS2*) were analyzed in 6 breast cancers, and the methylation status of these genes in the metastatic sentinel lymph nodes was different from those in their primary counterparts. In another study (6), six genes (*RASSF1A*, *APC*, *TWIST*, *CDH1*, *GSTP1* and *RAR-ß2*) were analyzed in 29 breast cancers, and promoter methylation of *CDH1* was increased during sentinel lymph node metastasis. Consistent with these previous reports, we found that the DNA methylation profiles of metastatic gastric carcinomas differed from those of their primary counterparts. In addition, we found that *FLNC* promoter methylation was significantly higher in metastatic than in primary tumors.

FLNC, a member of the filamin family, has been known to organize actin polymerization in response to various signals (25). The aberrant methylation of *FLNC* promoter in gastric carcinoma was initially found using a genome scanning technique, namely, methylation-sensitive representational difference analysis (26). In the present study, the frequency of *FLNC* methylation in this report was 25.7% (19 of 74 cases) which is lower than that reported previously (41.3%: 31 of 75 cases) (15). Functionally, *FLNC* is known to play a crucial role in muscle development and in the maintenance of muscle structural integrity (27). However, the biological role of *FLNC* in other organs is poorly understood, and thus, speculation concerning the role of *FLNC* methylation in the metastasis of gastric cancer is premature.

ADAM23 is a member of the ADAM family of disintegrins and metalloproteinase which are involved in control of growth factors, cytokines, membrane adhesion, and cell migration (28). The overexpressions of most ADAM family members usually accelerate tumorigenesis, whereas *ADAM11* and *ADAM23* inhibits tumorigenesis (29). In gastric carcinoma, the aberrant methylation of *ADAM23* promoter has been reported to occur in 43.8% (7 of 16 cases) (30). In the present study, the frequency of *ADAM23* methylation was comparable to that of the previous report and tended to increase during metastasis. However, this increase during metastasis was not statistically significant.

UCHL1, also called *PGP9.5* (protein gene product 9.5), is a member of the ubiquitin carboxyl-terminal hydrolase family and is involved in the processing of ubiquitin precursors and of ubiquitinated proteins (31). In gastric carcinoma, promoter methylation of *UCHL1* has been reported to occur in 48.4% (15 of 31 cases) and to be more frequent in diffuse type gastric carcinomas than in the intestinal type (32). Consistent with this previous report, we also observed that *UCHL1* methylation tended to be more frequent in diffuse type gastric carcinoma than in the intestinal type (p=0.057, data not shown).

RUNX3, a runt-related transcription factor, is known to have a tumor-suppressor activity and to be silenced in gastric carcinoma either by hemizygous deletion or by promoter hypermethylation (16). In one study, *RUNX3* promoter methylation was detected in 75% of primary gastric carcinomas (n=22) and in 100% of cells from malignant ascites (7). However, in contrast to this previous report, in the present study, *RUNX3* methylation was identified in 24.3% of primary gastric carcinomas and was not found to be increased in metastases. These differences may have been caused by selection bias in the previous report, in which all patients had peritoneal dissemination. In addition, it is also possible that *RUNX3* methylation plays a selective role in this process.

Frequencies of the methylations of the 11 genes in corresponding non-neoplastic mucosa of the present series were similar to those reported previously (7,13,15,32), although the overall frequencies of *CDH1* and *TIMP3* methylation were lower than those reported previously. These findings and our findings concerning methylation frequencies in uninvolved lymph nodes, which have not been reported yet, show that *CDH1*, *FHIT* and *THBS1* are methylated in a cancer-unrelated fashion. Furthermore, this finding is supported by previous reports which concluded that *CDH1*, *TIMP3* and *THBS1* show aging-related methylation (14,33). However, it should be added that in the present study the methylation pattern of *TIMP3* was found to be cancer-specific, which disagrees with the findings of a previous report (14). In addition, in the present study, the extensive methylation of the *FHIT* gene in non-neoplastic gastric mucosa and uninvolved lymph nodes is reported for the first time.

The reason why genes showing cancer-specific methylation (*ADAM23*, *FLNC*, *ITGA4*, *LOX*, *RUNX3*, *TIMP3* and *UCHL1*) were occasionally methylated in non-neoplastic mucosa and uninvolved lymph nodes is unclear. Since most of non-neoplastic mucosal tissues in our cases showed either *Helicobacter pylori* infection or intestinal metaplasia (data not shown), the occasional methylation of these genes in non-neoplastic mucosa cannot be explained by *Helicobacter pylori* infection or intestinal metaplasia. Instead, we would be more inclined to believe that this pattern of methylation in non-neoplastic mucosa reflects ongoing carcinogenesis at the molecular level in histologically non-neoplastic mucosa. DNA methylation in uninvolved lymph nodes is also intriguing, although it may have possibly resulted from contaminating tumor cells which could be present in 10 μm sections used for DNA extraction without being detected in H&E-stained slides.

The association of high-methylation group with advanced N class and pTNM stage found in the present study is supported by a previous report in which DNA methylation was associated with tumor progression in gastric cancer (15). Furthermore, in the present study, the average number of genes methylated was greater in metastatic tumors than in primary tumors. These findings suggest that the methylations of multiple genes might be involved in metastasis. However,

multivariate logistic regression analysis showed that only the female gender and diffuse type tumor by Lauren classification were independently associated with high-methylation group. It was of interest to find that an independent association of high-methylation group with diffuse type tumor, not with N class or pTNM stage, which suggests that the more frequent methylation in diffuse type tumors is attributable to an intrinsic property of these tumors rather than to their association with an advanced stage. An analysis of the methylation status of a large number of genes in an extended series of gastric cancer would probably shed additional light on this issue.

Because the methylation of the 11 genes examined in this study has been reported to be correlated with loss of expression of their target mRNAs, the losses of the specific biological functions of these genes may have contributed to metastasis. However, this may not be the case because *FLNC* methylation, which was found to be increased in metastatic tumors, was not found to be associated with tumor progression, and conversely, the methylations of *RUNX3* and *TIMP3*, which were associated with an advanced T class or pTNM stage, were not increased in metastatic tumors. Thus, it seems more reasonable that accumulation of DNA methylation may be caused by long-standing proliferation during tumor progression, given that increased methylation of multiple genes has been reported to be associated with hepatic cirrhosis (34) and with ulcerative colitis (35) where long-standing inflammation and proliferation also occur.

Although the prognostic significance of the methylation of individual genes or multiple genes were not established in the present study, the high-methylation group tended to show a poorer prognosis. We suggest that an analysis of a larger number of samples is necessary to determine the effects of promoter methylation of multiple genes on prognosis.

One limitation of our study is that the 11 genes investigated are unlikely to be representative of global methylation profiles. Ideally, high-throughput global DNA methylation profiling techniques (36) should be used to reveal the real 'methylation signatures' of primary gastric carcinomas and their lymph node metastases. However, these high-throughput techniques require well-preserved fresh tissues which were not available to us. Nevertheless, despite this limitation, we did find that the *FLNC* promoter is more frequently methylated in metastatic gastric carcinoma than in its primary counterpart.

Based on the results mentioned above, it is evident that some portion of metastatic gastric carcinomas consist of various tumor cell clones that differ epigenetically from their primary counterparts. These differences may result from the generation of heterogeneous clones with different methylation profiles during primary tumor progression and their subsequent clonal selection during metastasis. The precise biological meanings of the DNA methylation differences found between primary and metastatic gastric carcinomas require further investigation.

In summary, the present study demonstrates that *FLNC* promoter is more frequently methylated in metastatic gastric carcinomas than in their primary counterparts, and that the number of methylated genes is greater in metastatic gastric carcinomas than in their primary counterparts. Although we failed to identify a clinical factor related to *FLNC* methylation, we did find that multiple gene methylation was independently associated with the female gender and a diffuse type. These differences between the methylation profiles of primary and metastatic gastric carcinoma indicate that *FLNC* promoter methylation or those of multiple genes may be involved in the metastasis of gastric carcinoma. Consequently, we suggest that an investigation of metastatic tumors should be undertaken to characterize the methylation profile of node-positive gastric carcinoma, and that the heterogeneous methylation in primary and metastatic lesions should be considered when applying a new therapeutic strategy of modifying the methylated DNA.

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