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ORIGINAL ARTICLE

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위암에서 *Helicobacter pylori* CagA에 따른 RUNX3의 메틸화 및 발현의 소실과 임상병리학적 특성과의 관계

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RUNX3 Methylation, Loss of RUNX3 Expression and Clinicopathologic Findings according to *Helicobacter pylori* CagA in Gastric Carcinoma

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Background/Aims: *Helicobacter pylori* cytotoxin-associated gene A (CagA) has been suggested to be involved in the inactivation of Runt-related transcription factor 3 (RUNX3), a known gastric carcinoma tumor suppressor gene. It remains unclear how *H. pylori* CagA initiates or maintains *RUNX3* promoter methylation and inactivates its protein expression in gastric carcinoma.
Methods: *RUNX3* promoter methylation status, RUNX3 expression, and *H. pylori* CagA were investigated in 76 sample pairs of gastric carcinoma tissue. The patients' medical records were reviewed. The association between *RUNX3* methylation or loss of RUNX3 expression and clinicopathologic variables according to *H. pylori* CagA status were investigated.
Results: In gastric carcinoma patients with *H. pylori* CagA-positive infection, *RUNX3* methylation did not show association with lymphatic invasion, venous invasion, and TNM stages. However *RUNX3* methylation was observed more frequently in poorly differentiated adenocarcinoma and signet ring cell carcinoma (77.8% *vs.* 20.0%, p=0.023) in early stage. In gastric carcinoma patients with *H. pylori* CagA-positive infection, loss of RUNX3 expression did not show association with lymphatic invasion, venous invasion, and TNM stages. However loss of RUNX3 expression was observed more frequently in early gastric carcinoma than in advanced gastric carcinoma (84.2% *vs.* 75.0%, p=0.51), but this difference was not significant.
Conclusions: In gastric carcinoma patients with *H. pylori* CagA-positive infection, *RUNX3* methylation or loss of RUNX3 expression did not show correlation with lymphovascular invasion and TNM stages. In early gastric carcinoma patients with *H. pylori* CagA-positive infection, *RUNX3* methylation was observed more in poorly differentiated adenocarcinoma and signet ring cell carcinoma. **(Korean J Gastroenterol 2015;66:75-84)**

Key Words: *Helicobacter pylori*; CagA; RUNX3; Methylation; Carcinoma

INTRODUCTION

Helicobacter pylori infection occurs in over 80% of all gastric carcinoma patients[1] with the bacterium classified as a group I human carcinogen for gastric adenocarcinoma.[2] *H. pylori* infection is more associated with early gastric carcinoma patients than with non-neoplastic lesions or advanced gastric carcinoma patients.[3] Cytotoxin-associated gene A (CagA) is a significant virulence factor of *H. pylori*.[4] The CagA pathogenicity island also encodes a type IV secretion system

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for injection of Cag A into epithelial cells of the stomach, where it causes disruption of the cell cytoskeleton, apical junction complex, and other intracellular activities.[5,6] Infection with CagA-positive strains of *H. pylori* further increases the risk for non-cardiac gastric cancer as compared to the risk associated with *H. pylori* infection alone.[7]

Since 2002, Runt-related transcription factor 3 (*RUNX3*) has been recognized as a tumor suppressor gene for gastric cancer.[8] RUNX3 is frequently inactivated in gastric cancer through the following four mechanisms: aberrant methylation of the gene's promoter, protein mislocalization, histone modification, and hemizygous deletion.[8,9] RUNX3 inactivation by methylation occurs at the CpG site by the addition of a methyl group, subsequently converting to 5-methylcytosine. In a recent meta-analysis, an association between methylation of the *RUNX3* promoter and gastric carcinoma was reported, confirming the role of *RUNX3* as a tumor suppressor gene.[10] The same study found that the *RUNX3* promoter methylation status was not correlated with either TNM staging or lymphatic and venous invasion.[10] However, RUNX3 inactivation by protein mislocalization is a marker for the loss of tumor suppression and impairment of transforming growth factor-β signaling in gastric carcinoma.[9]

The relationship between *H. pylori* CagA and *RUNX3* methylation or protein mislocalization has only recently been elucidated in early gastric cancer tissue. *H. pylori* 16S RNA may be an independent risk factor for *RUNX3* methylation in patients with early gastric carcinoma.[11] The protein mislocalization of RUNX3, because of which it moves from the nucleus to the cytoplasm, may be a major mechanism underlying the association between *H. pylori* infection and gastric carcinoma development.[9] RUNX3 inactivation occurs early during the progression to malignancy.[10,12,13]

In the current study, we investigated the association between *RUNX3* methylation and various clinicopathological variables in gastric carcinoma patients according to their *H. pylori* CagA-positive infection status. We also examined the relationship between RUNX3 protein expression and the clinicopathological variables according to CagA-positive *H. pylori* infection.

SUBJECTS AND METHODS

1. Patients

Seventy-six patients with primary gastric carcinoma were included in the current study. All patients underwent curative or palliative subtotal or total gastrectomy. None of them received chemotherapy or radiotherapy preoperatively. For the analysis of the methylation status by methylation specific PCR, endoscopic biopsy of macroscopically neoplastic and non-neoplastic mucosa were collected, snap-frozen, and stored at −70°C for isolation of genomic DNA. For immunohistochemical staining of the RUNX3 protein, formalin-fixed and paraffin-embedded operative samples were prepared from the tissues of the 76 patients. The patients' information including age, gender, tumor site, lymphatic invasion, venous invasion, and TNM tumor classification according to the American Joint Committee on Cancer/Union for International Cancer Control staging system for gastric cancer (7th edition, 2010) were documented. Histological tumor differentiation statuses were determined on the basis of the World Health Organization classification of pathology and the genetics of tumors of the digestive system.[14] Rapid urease test, H&E stain, and modified Giemsa stain were performed for evaluation of *H. pylori* infection status. This study was approved by the Institutional Human Research Board of Ewha Woman's University Mokdong Hospital, Seoul, Korea (ECT-12-22A-23). Informed consent was obtained from all patients.

2. Methylation-specific PCR for RUNX3

Methylation-specific PCR (MSP) was performed using the bisulfite-modified DNA templates obtained from the endoscopic specimen of human gastric carcinoma tissues. Endoscopic samples from neoplastic and non-neoplastic gastric mucosa were used. The macroscopic non-neoplastic gastric mucosa was defined to be at least 5 cm away from the neoplastic gastric mucosa. Genomic DNA was obtained by proteinase K digestion and extraction using the LaboPass™ Tissue Mini kit (COSMO Genetech, Seoul, Korea). The genomic DNA concentration and purity were measured using a spectrophotometer (purity=1.7−1.9 at 260/280 nm) and the DNA was treated with sodium bisulfite according to the manufacturer's instructions. Thereafter, 2 μg of the genomic DNA was denatured with 2 M NaOH and modified with 3 M sodium bi-

sulfite for 16 h. The DNA samples were then purified using a DNA purification kit (Intron®, Seongnam, Korea) with 3 M NaOH, precipitated with ethanol, and resuspended in 20 μL distilled water. RUNX3 was amplified using the GeneAmp PCR system 9600 (Perkin-Elmer, Wellesley, MA, USA); the PCR reaction mixture (10 μL) consisted of 2 μL of the DNA template, 0.1 μL of TaKaRa hotstart *Taq* polymerase, 0.2 mM of deoxynucleotide triphosphates, 0.5 μM of each primer (sense and antisense), 1 μL of the primer 10× PCR buffer, and 5.6 μL of distilled water. The PCR conditions were as follows: 95°C for 1 min and 40 cycles of denaturation at 95°C for 30 s, annealing at 66°C (methylated) or 63°C (unmethylated) for 30 s, and final 30 s extension at 72°C, followed by a final 5-min extension at 72°C. The PCR products were loaded onto a 2% agarose gel and visualized under ultraviolet (UV) transillumination. Irrespective of the methylation status in normal tissues, the presence of methylated bands and partially methylated bands was considered to indicate that the carcinoma tissues were positive for *RUNX3* methylation. The primer sequences for the methylated *RUNX3* gene were as follows: 5′-TACGAGGGGCGGTCGTACGCGGG-3′ and 5′-AAAAC GACCGACGCGAACGCCTCC-3′ (64,970-65,189 bp). The primer sequences for the unmethylated *RUNX3* gene were as follows: 5′-TTATGAGGGGTGGTTGTATGTGGG-3′ and 5′-AAAAC AACCAACACAAACACCTCC-3′ (64,970-65,189 bp). The primers for GAPDH were as follows: 5′-GCCTCAAGATCATCAGCAAT-3′ (530-549 bp) and 5′-TCCAGCTCAGGGATGACCTT-3′. Validation of the primer sets had been previously performed by bisulfate genomic sequencing of the MSP products in the carcinoma cell lines. DNA from the HT-29 (Korean Cell Line Bank [KCLB] No. 30038) and MKN-45 (KCLB No. 80103) cell lines were used as positive controls for methylated and unmethylated *RUNX3*, respectively (Fig. 1A). Distilled water was used as the negative control.

3. PCR for *H. pylori* CagA

The amplification reaction mixture (20 μL) contained 2 μL DNA template, 0.1 μL Go*Taq* polymerase, 0.2 mM dNTPs, 0.5 μM of each primer (sense and antisense), 4 μL of the primer 5× PCR buffer, and 5.6 μL distilled water. The primers for *H. pylori* CagA[15,16] were as follows: 5′-GATAACA GGCAAGCTTTTG AGG-3′ and 5′- CTGCAAAAGATTGTTTGGCAGA-3′ (1,228-1,556 bp). The PCR conditions were as follows: 94°C for 1 min followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final 5-min extension at 72°C.[15] The PCR products were loaded onto a 2% agarose gel and visualized under UV transillumination. The presence of bands in normal or carcinoma tissues indicated positivity for CagA (Fig. 1B). Endoscopic samples from neoplastic and non-neoplastic gastric mucosa were used.

4. Immunohistochemical staining for RUNX3

Immunohistochemical analysis was performed using the Bond™ Polymer DAB Detection Kit (Leica Microsystems, Weltzar, Germany) and Bond-X automated immunohistochemistry slide staining system (Leica Microsystems). Formalin-fixed and paraffin-embedded serial sections using four micron-thick samples were deparaffinized and dehydrated. The sections were heated in citrate buffer (pH 6.0, 10 mM) in a microwave for 20 min for retrieval of antigens. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 10 min followed by incubation with the primary antibodies: mouse monoclonal anti-RUNX3 antibody

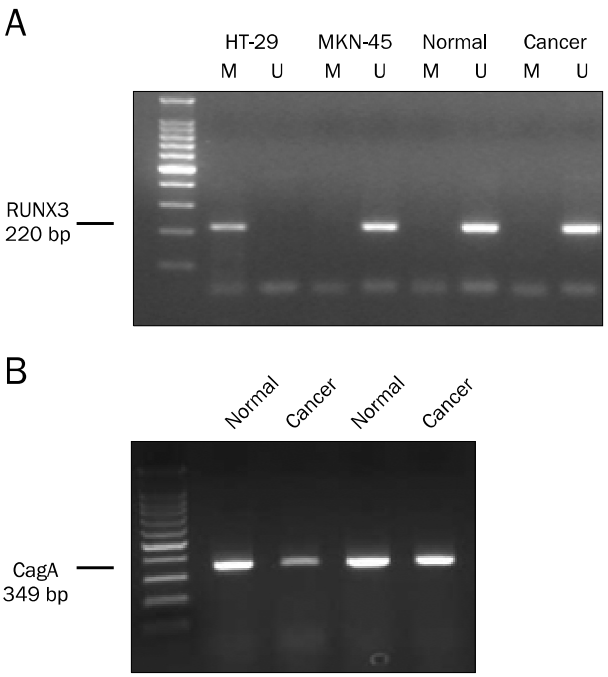

Fig. 1. Representative results of methylation-specific PCR for RUNX3 and PCR for CagA in human normal and gastric cancer tissue samples. (A) Methylation specific PCR for RUNX3. Positive control, negative control, molecular weighted marker for RUNX3 and un-methylated bands (U) were seen in cancer tissue. (B) PCR for CagA. Presence of band in normal gastric mucosa or gastric carcinoma tissue was considered positive for *Helicobacter pylori* CagA.

(eBioscience, San Diego, CA, USA). Sections were then washed in phosphate-buffered saline and incubated with horseradish-peroxidase polymer for 30 min. The reaction was visualized after incubation with 3,3-diaminobenzidine tetrahydrochloride for 5 min. The samples were then counterstained with Mayer's hematoxylin.

RUNX3 staining showed positive staining in the nuclei of mononuclear inflammatory cells and in the cytoplasmic regions of goblet cells of gastric glands with intestinal metaplasia (Fig. 2A). When no staining or only cytoplasmic staining was detected, the sample was considered negative for RUNX3 expression or was considered as showing loss of nuclear RUNX3 expression (Fig. 2B). Nuclear staining alone or both nuclear and cytoplasmic staining indicated positive RUNX3 expression. This determination was made by a pathologist.

5. Statistical analysis

The relationship between *RUNX3* methylation status and clinicopathological variables was investigated according to CagA-positive *H. pylori* infection in gastric carcinoma patients. The results of immunohistochemical staining for RUNX3 were analyzed according to *H. pylori* CagA status and the status of *RUNX3* methylation. The association between RUNX3 expression and clinicopathological variables was analyzed. Statistical analyses were performed using a two-tailed Fisher's exact test or a linear test. The results of the analyses of continuous variables are expressed as mean±standard deviation, as determined using Student's t-test. Data analyses were performed using the STATA version 12.0 (Stata

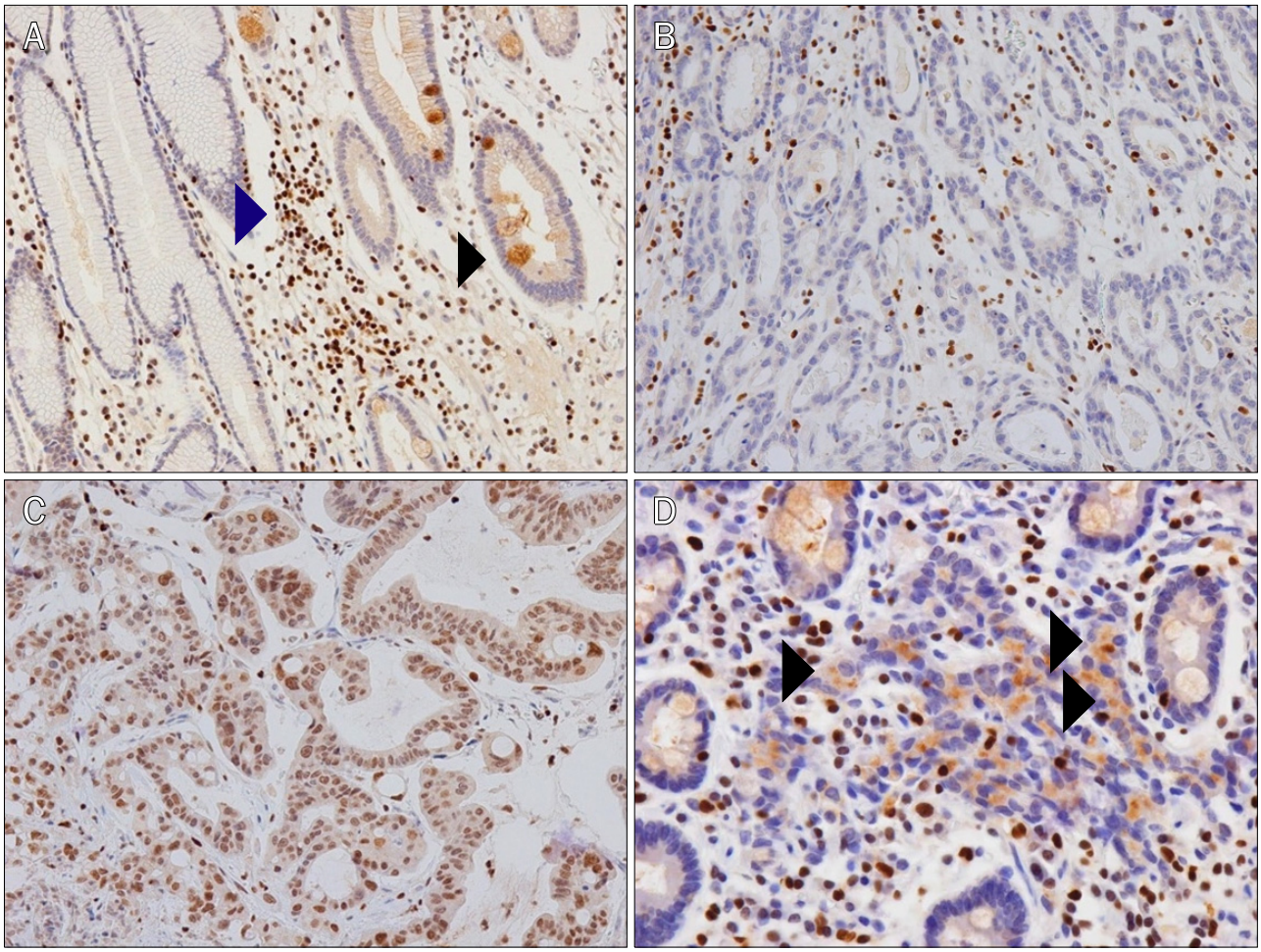

Fig. 2. Immunohistochemical stain for RUNX3 protein. (A) Adjacent mucosa surrounded by tumor showed cytoplasmic positive reaction in goblet cells (black arrowhead). The lymphocytes in lamina propria showed a nuclear positive reaction of lymphocytes (blue arrowhead) (×200). (B) The tumor cells showed a negative reaction (×200). (C) The tumor cells showed a strong brownish nuclear positive reaction (×200). (D) The tumor cells showed no nuclear positive reaction but a cytoplasmic positive reaction (cytoplasmic mislocalization) (black arrowheads) (×400).

Corp., College Station, TX, USA), and $p < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

1. Baseline characteristics in gastric carcinoma patients

The enrolled patients included 57 men and 19 women with a mean age of 61.9±1.5 years (range, 35-90 years). All 76 participants were pathologically confirmed to have adenocarcinoma or signet ring cell carcinoma, with 49 participants (64.5%) diagnosed with poorly differentiated adenocarcinoma or signet ring cell carcinoma. Fifty-nine (77.6%) patients showed *H. pylori* infection. Twenty-two (28.9%) patients showed venous invasion and 50 (65.8%) showed lymphatic invasion. Twenty-two (28.9%), 15 (19.7%), 24 (31.7%), and 15 (19.7%) patients had stage I, stage II, stage III, and stage IV disease, respectively (Table 1).

2. Correlation between *H. pylori* CagA status and RUNX3 inactivation

H. pylori CagA status did not show correlation with *RUNX3* methylation or a loss of nuclear RUNX3 expression in cancer tissue. *H. pylori* CagA status also did not show correlation with *RUNX3* methylation in normal tissues (Table 2). However, CagA-positive *H. pylori* infection was observed in 74.2% of *RUNX3* methylation occurrences (23/31 cases), and 75.4% of the cases had a loss of nuclear RUNX3 expression (43/57 cases).

3. Correlation between *RUNX3* methylation status and clinicopathological variables according to *H. pylori* CagA status in gastric carcinoma patients

The methylation status of the *RUNX3* promoter was examined in all 76 enrolled patients, including 24 early stage gastric carcinoma patients. *RUNX3* methylation was found in 31 (40.8%) cases. No correlation was found between *RUNX3* methylation status and age, gender, or *H. pylori* CagA status. Similarly, the *RUNX3* methylation status did not show correlation with histological differentiation, lymphatic invasion, venous invasion, T stage, N stage, distant metastasis, or tumor stage in any of the gastric carcinoma cases (data not shown).

Table 1. Baseline Characteristics in Gastric Carcinoma Patients

Variable	Total (n=76)
Age (yr)	61.9±1.5
Gender (male)	57 (75.0)
H. pylori positive	59 (77.6)
H. pylori CagA positive	55 (72.4)
Differentiation	
W/D, M/D	27 (35.5)
P/D, SRC	49 (64.5)
Location of tumor	
Upper	12 (15.8)
Middle	17 (22.4)
Lower	45 (59.2)
Whole	2 (2.6)
Lymphatic invasion	
Negative	26 (34.2)
Positive	50 (65.8)
Venous invasion	
Negative	54 (71.1)
Positive	22 (28.9)
Depth of invasion	
T1a (mucosa)	10 (13.2)
T1b (submucosa)	14 (18.4)
T2, 3, 4	52 (68.4)
Nodal status	
N0	26 (34.2)
N1, 2, 3	50 (65.8)
Distant metastasis	
M0	61 (80.3)
M1	15 (19.7)
Stage	
I	22 (28.9)
II	15 (19.7)
III	24 (31.7)
IV	15 (19.7)

Values are presented as mean±SD or n (%).
W/D, tubular adenocarcinoma, well differentiated; M/D, tubular adenocarcinoma moderately differentiated; P/D, tubular adenocarcinoma, poorly differentiated; SRC, signet ring cell cancer.

Table 2. Correlation between *Helicobacter pylori* CagA Status and Clinicopathologic Variables in Gastric Carcinoma

Variable	*H. pylori* CagA positive (n=55)	*H. pylori* CagA negative (n=21)	p-value
Age (yr)	61.5±1.6	63.0±3.3	0.61
Gender (male)	45 (81.8)	13 (61.9)	0.14
RUNX3 methylation in normal tissue	8 (14.5)	2 (9.5)	0.72
RUNX3 methylation in cancer tissue	23 (41.8)	8 (38.1)	0.80
Loss of RUNX3 expression in cancer tissue	43 (78.2)	14 (66.7)	0.38

Values are presented as mean±SD or n (%).

Patients were divided into two groups according to their *H. pylori* CagA status, and the association between *RUNX3* methylation and pathologic variables was investigated. In gastric carcinoma patients with *H. pylori* CagA-positive infection, no correlation was found between *RUNX3* methylation and lymphatic invasion, venous invasion, T stage, N stage, distant metastasis, or tumor stage (Table 3).

In *H. pylori* CagA-negative gastric carcinoma patients, those without *RUNX3* methylation showed lymphatic invasion (92.3% *vs.* 50.0%, p=0.047), venous invasion (46.2% *vs.* 0%, p=0.046), and lymph node metastasis (100% *vs.* 50.0%, p=0.012) more frequently than those with *RUNX3* methylation (Table 3). However, in early gastric carcinoma patients with CagA-positive *H. pylori* infection, *RUNX3* methylation occurred more often in cases of poorly differentiated adenocarcinoma or signet ring cell carcinoma than in cases of well or moderately differentiated adenocarcinoma (77.8% *vs.* 20.0%, p=0.023) (Table 4).

4. Correlation between RUNX3 expression and clinicopathological variables according to *H. pylori* CagA status in gastric carcinoma patients

Immunohistochemical staining for RUNX3 was performed on samples obtained from all 76 patients. A loss of nuclear RUNX3 expression (negative expression) was found in 57 (75.0%) patients, and nuclear RUNX3 expression (positive expression) was found in 19 (25.0%) patients (Fig. 2C).

Three cases of cytoplasmic mislocalization were observed in two patients with early gastric cancer and one patient with advanced gastric cancer (Fig. 2D). Of the 31 patients with *RUNX3* methylation, 26 (83.9%) had a loss of RUNX3 expression, and 31 (68.9%) of the 45 patients without *RUNX3* methylation had a loss of RUNX3 expression (p=0.14).

Loss of nuclear RUNX3 expression (negative expression) did not show correlation with histological differentiation, lymphatic invasion, venous invasion, T stage, N stage, distant

Table 3. Correlation between *RUNX3* Methylation and Clinicopathologic Variables according to *Helicobacter pylori* CagA Status in Gastric Carcinoma (n=76)

Variable	*H. pylori* CagA positive (n=55)			*H. pylori* CagA negative (n=21)		
	RUNX3 methylation positive (n=23)	*RUNX3* methylation negative (n=32)	p-value	*RUNX3* methylation positive (n=8)	*RUNX3* methylation negative (n=13)	p-value
Differentiation			1.00			1.00
W/D, M/D	9 (39.1)	13 (40.6)		2 (25.0)	3 (23.1)	
P/D, SRC	14 (60.9)	19 (59.4)		6 (75.0)	10 (76.9)	
Lymphatic invasion			0.78			0.047
Negative	8 (34.8)	13 (40.6)		4 (50.0)	1 (7.7)	
Positive	15 (65.2)	19 (59.4)		4 (50.0)	12 (92.3)	
Venous invasion			0.55			0.046
Negative	15 (65.2)	24 (75.0)		8 (100)	7 (53.8)	
Positive	8 (34.8)	8 (25.0)		0 (0)	6 (46.2)	
Depth of invasion			0.40			0.24
T1a	5 (21.7)	3 (9.3)		2 (25.0)	0 (0.0)	
T1b	4 (17.4)	7 (21.9)		1 (12.5)	2 (15.4)	
T2, 3, 4	14 (60.9)	22 (68.8)		5 (62.5)	11 (84.6)	
Nodal status			1.00			0.012
N0	9 (39.1)	13 (40.6)		4 (50.0)	0 (0)	
N1, 2, 3	14 (60.9)	19 (59.4)		4 (50.0)	13 (100)	
Distant metastasis			1.00			1.00
M0	19 (82.6)	27 (84.4)		6 (75.0)	9 (69.2)	
M1	4 (17.4)	5 (15.6)		2 (25.0)	4 (30.8)	
Stage			0.55			0.29
I	6 (26.1)	11 (34.4)		3 (37.5)	2 (15.4)	
II	8 (34.8)	6 (18.8)		1 (12.5)	0 (0)	
III	5 (21.7)	10 (31.2)		2 (25.0)	7 (53.8)	
IV	4 (17.4)	5 (15.6)		2 (25.0)	4 (30.8)	

Values are presented as n (%).
W/D, tubular adenocarcinoma, well differentiated; M/D, tubular adenocarcinoma, moderately differentiated; P/D, tubular adenocarcinoma, poorly differentiated; SRC, signet ring cell cancer.

Table 4. Correlation between *RUNX3* Methylation and Clinicopathologic Variables according to *Helicobacter pylori* CagA Status in Early Gastric Carcinoma (n=24)

Variable	*H. pylori* CagA positive (n=19)			*H. pylori* CagA negative (n=5)		
	RUNX3 methylation positive (n=9)	*RUNX3* methylation negative (n=10)	p-value	*RUNX3* methylation positive (n=3)	*RUNX3* methylation negative (n=2)	p-value
Differentiation			0.023			0.40
W/D, M/D	2 (22.2)	8 (80.0)		0 (0)	1 (50.0)	
P/D, SRC	7 (77.8)	2 (20.0)		3 (100)	1 (50.0)	
Lymphatic invasion			0.65			0.10
Negative	5 (55.6)	7 (70.0)		3 (100)	0 (0)	
Positive	4 (44.4)	3 (30.0)		0 (0)	2 (100)	
Venous invasion			0.58			0.40
Negative	7 (77.8)	9 (90.0)		3 (100)	1 (50.0)	
Positive	2 (22.2)	1 (10.0)		0 (0)	1 (50.0)	
Depth of invasion			0.37			0.40
1a	5 (55.6)	3 (30.0)		2 (66.7)	0 (0)	
1b	4 (44.4)	7 (70.0)		1 (33.3)	2 (100)	
Nodal status			0.65			0.10
N0	5 (55.6)	7 (70.0)		3 (100)	0 (0)	
N1, 2, 3	4 (44.4)	3 (30.0)		0 (0)	2 (100)	
Stage			0.63			1.00
I	6 (66.7)	8 (80.0)		3 (100)	2 (100)	
II	3 (33.3)	2 (20.0)		0 (0)	0 (0)	

Values are presented as n (%).
W/D, tubular adenocarcinoma, well differentiated; M/D, tubular adenocarcinoma, moderately differentiated; P/D, tubular adenocarcinoma, poorly differentiated; SRC, signet ring cell cancer.

metastasis, or tumor stage in any of the gastric carcinoma patients (data not shown). In gastric carcinoma patients with CagA-positive *H. pylori* infection too, no association was found between the loss of RUNX3 expression and histological differentiation, lymphatic invasion, venous invasion, T stage, N stage, distant metastasis, or tumor stage (Table 5). In patients with *H. pylori* CagA infection, loss of RUNX3 expression in early gastric carcinoma patients was more than that in advanced gastric carcinoma patients (84.2% [16/19] *vs.* 75.0% [27/36]) (p=0.51), but the differences were not significant.

DISCUSSION

In our study, in gastric carcinoma patients with CagA-positive *H. pylori* infection, *RUNX3* methylation status or loss of RUNX3 expression was not found to show association with lymphatic invasion, venous invasion, or TNM stages. However, *RUNX3* methylation is more significantly found in poorly differentiated adenocarcinoma and signet ring cell carcinoma in the early stages. Loss of RUNX3 expression was found more often in early gastric carcinoma than in advanced gastric carcinoma.

The RUNX family includes RUNX1, RUNX2, and RUNX3, with each member exerting different regulatory functions in intracellular processes.[17] RUNX3, with its encoding gene located on chromosome 1p36, is involved in gastric epithelial growth[8,12] and T-cell differentiation.[18] Mislocalization of RUNX3 leading to its activation has been reported to result in a loss of nuclear RUNX3 expression in 44% of gastric carcinoma cases and cytoplasmic mislocalization in 38% of cases.[9] Inactivation of RUNX3 by promoter methylation was reported in 45-65% of all gastric carcinomas.[10,19,20]

Most reported cases of gastric carcinoma in East Asia are associated with CagA-positive *H. pylori* infection. In comparison to the Western CagA-positive *H. pylori* strain, CagA in East Asia more often causes severe gastric mucosal inflammation and is associated with severe atrophic gastritis and gastric adenocarcinoma.[21,22] In a meta-analysis, the percentage of patients sero-positive for *H. pylori* CagA in East Asia varied from 61% to 96.6% of the total number of gastric cancer patients.[23] This positive result included both current and past infections. In another meta-analysis, the rate of CagA infection, determined using PCR techniques, varied from 79% to 100% in gastric cancer.[24] The rate of the East-Asian type *H. pylori* CagA was 83%.[24] In this study, the

Table 5. Correlation between RUNX3 Expression and Clinicopathologic Variables according to *Helicobacter pylori* CagA Status in Gastric Carcinoma (n=76)

Variable	*H. pylori* CagA positive (n=55)			*H. pylori* CagA negative (n=21)		
	RUNX3 expression positive (n=12)	RUNX3 expression negative (n=43)	p-value	RUNX3 expression positive (n=7)	RUNX3 expression negative (n=14)	p-value
Differentiation			0.51			0.22
W/D, M/D	6 (50.0)	16 (37.2)		2 (28.6)	3 (21.4)	
P/D, SRC	6 (50.0)	27 (62.8)		5 (71.4)	11 (78.6)	
Lymphatic invasion			0.78			0.63
Negative	4 (33.3)	17 (40.0)		1 (14.3)	4 (28.6)	
Positive	8 (66.7)	26 (60.0)		6 (85.7)	10 (71.4)	
Venous invasion			0.30			0.12
Negative	7 (58.3)	32 (74.4)		3 (42.9)	12 (85.7)	
Positive	5 (41.7)	11 (25.6)		4 (57.1)	2 (14.3)	
Depth of invasion			0.89			0.43
T1a	1 (8.3)	7 (16.3)		0 (0)	2 (14.3)	
T1b	2 (16.7)	9 (20.9)		2 (28.6)	1 (7.1)	
T2, 3, 4	9 (75.0)	27 (62.8)		5 (71.4)	11 (78.6)	
Nodal status			0.74			0.12
N0	4 (33.3)	18 (41.9)		0 (0)	4 (28.6)	
N1, 2, 3	8 (66.7)	25 (58.1)		7 (100)	10 (71.4)	
Distant metastasis			0.66			1.00
M0	11 (91.7)	35 (81.4)		5 (71.4)	10 (71.4)	
M1	1 (8.3)	8 (18.6)		2 (28.6)	4 (28.6)	
Stage			0.20			1.00
I	2 (16.7)	15 (34.9)		2 (28.6)	3 (21.4)	
II	6 (50.0)	8 (18.6)		0 (0)	1 (7.1)	
III	3 (25.0)	12 (27.9)		3 (42.8)	6 (42.9)	
IV	1 (8.3)	8 (18.6)		2 (28.6)	4 (28.6)	

Values are presented as n (%).
W/D, tubular adenocarcinoma, well differentiated; M/D, tubular adenocarcinoma, moderately differentiated; P/D, tubular adenocarcinoma, poorly differentiated; SRC, signet ring cell cancer.

rate of CagA-positive *H. pylori* infection determined using PCR techniques[16] was 86.4%. In the subset of early gastric carcinoma patients, *RUNX3* methylation was observed at a higher frequency among cases of poorly differentiated adenocarcinoma or signet ring cell carcinoma. Other studies have shown that a higher rate of *RUNX3* methylation may be related to gastric carcinoma with undifferentiated histopathology[10] and poorly differentiated colorectal carcinoma.[25,26] There are possible explanations regarding the mechanism of *H. pylori*-induced *RUNX3* methylation. Nitric oxide (NO) has been suggested to play important roles in *RUNX3* methylation. *H. pylori* CagA might increase the level of NO or other inflammatory cytokines.[27,28] NO, along with lipopolysaccharide, then induces *RUNX3* methylation in early gastric carcinoma.[29] In this study, *RUNX3* methylation did not show association with loss of RUNX3 expression. The rate of loss of RUNX3 expression in patients with *RUNX3* methylation was 83.9%, and 68.9% in patients without *RUNX3* methylation. In patients without *RUNX3* methylation, protein mislocalization or other factors such as hypoxia[30] may be related to the loss of RUNX3 expression.

Although the pathogenesis of CagA infection has not been completely elucidated, we hypothesize that CagA infection has two probable roles in cell signaling. Phosphorylation-dependent cell signaling of CagA is known to be associated with apoptosis, cell motility, elongation, proliferation, and inflammation. Phosphorylation-independent cell signaling of CagA is known to induce the disruption of cellular junctions.[31] Recent studies have shown that *H. pylori* or *H. pylori* CagA infection plays an important role in RUNX3 expression. *H. pylori* CagA can result in a loss of RUNX3 protein expression in a phosphorylation-dependent or independent manner.[32,33] In the former, CagA undergoes phosphorylation by the proto-oncogenic tyrosine-protein kinases Src and ABL1.[34] Phosphorylated *H. pylori* CagA subsequently induce a loss of nuclear RUNX3 expression via the Src/MEK/ERK or MEK/

P38/MAPK pathways in gastric epithelial cells.[32] In a phosphorylation-independent manner, *H. pylori* CagA directly induce the ubiquitination and degradation of the RUNX3 protein in the cytoplasm of gastric carcinoma cells.[35] In addition, RUNX3 protein mislocalization or loss of nuclear RUNX3 expression is a known mechanism of RUNX3 inactivation in early-stage carcinoma in both the colon and the breast.[36,37]

In this study, cytoplasmic mislocalization was observed in three cases. Cytoplasmic mislocalization may be a mechanism of RUNX3 inactivation in early and advanced gastric carcinoma. Although the correlations between the loss of RUNX3 expression and advanced tumor stage or lymph node metastasis have been reported in gastric carcinoma,[38] in the current study, loss of RUNX3 expression was not found to show association with histological differentiation, lymphatic invasion, venous invasion, T stage, N stage, distant metastasis, or tumor stage. When classified according to *H. pylori* CagA status, loss of RUNX3 expression was not associated with clinicopathological variables. However, in patients with CagA-positive *H. pylori* infection, 43 of 55 patients showed loss of nuclear RUNX3 expression (78.2%). Loss of RUNX3 expression was observed more in early stages than advanced stages (84.2% *vs.* 75.0%) of gastric carcinoma. Clinically, it has been shown that the loss of RUNX3 expression correlates with poor survival.[39,40] This analysis was not performed in the current study.

There are a few limitations of this study. First, the sample size was small. However, this cross-sectional, descriptive study provided evidence of the importance of *H. pylori* CagA and RUNX3 in gastric carcinoma in the early stages. Second, there was no long-term follow-up data.

In conclusion, in *H. pylori* CagA-positive patients, *RUNX3* methylation or loss of RUNX3 expression did not show correlation with lymphatic invasion, venous invasion, or TNM stages. In early gastric carcinoma patients with CagA-positive *H. pylori* infection, *RUNX3* methylation was observed more frequently in poorly differentiated adenocarcinoma or signet ring cell carcinoma.

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