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BRIEF ARTICLE

Evaluation of the relationship between dietary factors, *CagA*-positive *Helicobacter pylori* infection, and *RUNX3* promoter hypermethylation in gastric cancer tissue

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Abstract

AIM: To evaluate the relationship among *Helicobacter pylori* (*H. pylori*) infection, *CagA* status, and dietary factors with RUNX3 promoter hypermethylation.

METHODS: Gastric cancer tissue samples were collected from 184 South Korean patients. All patients were interviewed following a semi-quantitative food frequency questionnaire. The average frequencies of intake and portion sizes of 89 common food items were documented, and total intakes of calories, nutrients, vitamins, and minerals were calculated for each subject. DNA was extracted from gastric cancer tissue samples, and amplification of the *HSP60* gene was performed to detect *H. pylori* infection. Nested polymerase chain reaction (PCR) was used to detect the presence of the *CagA* gene. *RUNX3* gene expression was measured by reverse transcription-PCR, and *RUNX3* methylation status was evaluated by methylation-specific PCR. The odds ratios (ORs) and 95%CI associated with *RUNX3* promoter hypermethylation status were estimated for each of the food groups, lifestyle factors, and the interaction between dietary and lifestyle factors with *CagA* status of *H. pylori* infection.

RESULTS: Overall, 164 patients (89.1%) were positive for *H. pylori* DNA, with the *CagA* gene detected in 59 (36%) of these *H. pylori*-positive samples. In all, 106 (57.6%) patients with gastric cancer demonstrated CpG island hypermethylation at the RUNX3 promoter. *RUNX3* expression was undetectable in 52 (43.7%) of the 119 gastric cancer tissues sampled. A high consumption of eggs may increase the risk of *RUNX3* methylation in gastric cancer patients, having a mean OR of 2.15 (range, 1.14-4.08). A significantly increased OR of 4.28 (range, 1.19-15.49) was observed with a high consumption of nuts in patients with *CagA*-positive *H. pylori* infection. High intakes of carbohydrate, vitamin B1, and vitamin E may decrease the risk of *RUNX3* methylation in gastric cancer tissue, particularly in *CagA*- or *H. pylori*-negative infection, with OR of 0.41 (0.19-0.90), 0.42 (0.20-0.89), and 0.29 (0.13-0.62),

respectively. A high consumption of fruits may protect against *RUNX3* methylation.

CONCLUSION: These results suggest that the *CagA* status of *H. pylori* infection may be a modifier of dietary effects on RUNX3 methylation in gastric cancer tissue.

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Key words: Gastric cancer; *RUNX3*; *Helicobacter pylori*; *CagA*; Dietary factors

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INTRODUCTION

The incidence of gastric cancer has declined dramatically in most countries over the past 70 years, yet it remains the second most common cause of cancer-related deaths worldwide. The estimated incidence in 2008 was 989 600, and the majority of new cases occurred in developing countries[1]. Asian countries, including Japan, South Korea and China, have a particularly high incidence of gastric cancer; it is the second most frequently diagnosed cancer in the Korean population.

The development of gastric cancer appears to be the result of a complex interaction between environmental and genetic factors. Extensive epidemiology studies have shown that *Helicobacter pylori* (*H. pylori*) infection is a major risk factor for gastric cancer and its precursor lesions[2]. The risk of developing gastric cancer is estimated to increase 2 to 6 times in patients with *H. pylori* infection, as determined by retrospective case-control and prospective epidemiology studies[3]. As such, the World Health Organization and the International Agency for Research on Cancer consensus group have classified *H. pylori* as a Class I human carcinogen. More than half of the world's population is infected with *H. pylori*; its prevalence ranges from 25% in developed countries to more than 90% in developing regions[4]. Among individuals infected with *H. pylori*, a small percentage develop gastric cancer by a process influenced by bacterial virulence. The most widely studied *H. pylori* virulence factor is the *CagA* antigen, a 96-to 138-kDa protein[5]. The *CagA* gene, found on a genomic region called the *cag* pathogenicity island (PAI), is considered as a marker for enhanced virulence. Moreover, individuals infected with *CagA*-positive *H. pylori* strains have a higher risk of developing peptic ulcers and gastric cancer compared to those harboring *CagA*-negative *H. pylori* strains[6].

The human runt-related transcription factors (*RUNXs*) are important targets of the transforming growth factor (TGF)-β superfamily signaling pathway[7]. Three different *RUNX* genes have been identified as human homologues of the *Drosophila* genes *runt* and *lozenge*[8]. *RUNX1* (*AML1/CBFA2/PEBP-2aB*) is believed to be essential in hematopoiesis and is located in chromosome 21q22.3[8]. Moreover, it is targeted for translocation or mutation in acute and chronic leukemias and in myelodysplastic syndromes[9]. *RUNX2* (*AML3/CBFA1/PEBP2aA*), located on chromosome 6q21, is regarded as indispensable for the development of the musculoskeletal system and has been associated with the human bone disease cleidocranial dysplasia[10]. In humans, *RUNX3* (*AML2/CBFA3/PEBP2aC*) is found at chromosomal locus 1p36 in a region that is frequently deleted in many types of cancers. This region is therefore postulated to contain an important tumor suppressor gene[11]. The *RUNX3* protein combines with Smads and acts synergistically to regulate various target genes[7]. Little or no expression of *RUNX3* has been observed in gastric cancer[12] or in carcinomas of the liver, lung, breast, prostate, endometrium, and colon[13]. Several mechanisms are thought to be responsible for downregulating *RUNX3*, including promoter hypermethylation, loss of heterozygosity, hemizygous deletion, and mutation, and these mechanisms have been linked to carcinogenesis in a wide range of human solid tumors. Recent studies have demonstrated that loss of *RUNX3* expression is causally related to the genesis and progression of gastric cancer. Approximately 45% to 60% of surgically resected gastric cancer specimens and cell lines derived from these cancers do not express *RUNX3* due to either hemizygous deletion of the gene or hypermethylation of its promoter region[14]. Therefore, *RUNX3* is considered a tumor suppressor gene, and hypermethylation of its promoter is thought to play an important role in gastric carcinogenesis. Diets low in methyl-contributing folate, vitamins B6 and B12, and methionine and a high consumption of alcohol have been hypothesized to affect DNA methylation at CpG islands and confer an increased risk of cancer[15]. CpG island DNA methylation is also thought to occur as a result of inflammation[16]. Infection with *CagA*-positive *H. pylori* has been associated with higher grades of gastric mucosal inflammation as well as severe atrophic gastritis and is believed to play a role in the development of gastric carcinoma[17].

The purpose of this study was to evaluate the associations between dietary factors and *RUNX3* promoter hypermethylation status and to assess the combined contribution of dietary factors and *H. pylori CagA* status to *RUNX3* promoter hypermethylation in patients with gastric cancer.

MATERIALS AND METHODS

Study subjects

Between September 2003 and March 2006, 184 South Korean patients with gastric cancer were enrolled in the

Table 1 Characteristics of the study subjects

Variables	*n* (%)
All subjects	184
Age (mean ± SD), yr	59.8 ± 11.3 (range: 23-83)
Gender	
Male	124 (67.4)
Female	60 (32.6)
Helicobacter pylori infection	
Negative	20 (10.9)
Positive	164 (89.1)
CagA status	
Negative	125 (67.9)
Positive	59 (32.1)
RUNX3 methylation	
Negative	78 (42.4)
Positive	106 (57.6)
RUNX3 expression	
Positive	67 (56.3)
Negative	52 (43.7)

study. All patients were diagnosed at Chungbuk National University Hospital and Eulji University Hospital, both of which are located in the middle of the South Korean peninsula. Gastric cancer tissue samples were collected from all patients with prior consent. Patient characteristics are shown in Table 1. Tissue samples were obtained during resection surgery, immediately frozen in liquid nitrogen, and stored at -80 ℃ until needed for DNA and RNA extraction.

Patient demographics and other potential risk factors for gastric cancer were collected during direct interviews with subjects. Trained personnel interviewed the subjects using a structured questionnaire within one month of the diagnosis of gastric cancer and other benign diseases. Control subjects were questioned while undergoing routine medical examinations during hospital visits. Dietary data were collected using a semiquantitative food frequency table that had been previously evaluated for its validity and reliability[18]. The average frequency of intake and portion size of 89 common food items was documented. These items were classified into 21 food groups based on their ingredients: cereals; potatoes; nuts; noodles (pasta); breads and cakes; vegetables; mushrooms; fruits; red meats; eggs; fish and shellfish; stews; chicken; kimchi; soybean foods; soybean pastes; milk and dairy products; jams, honey, sweets and chocolates; coffee and tea; seaweeds; and alliums. Each food item was divided into high and low groups according to the median value of its distribution.

The amount of calories, nutrients, vitamins, and minerals consumed for each food item was estimated by multiplying the amount of the food item consumed by its nutrient value. Total intakes of calories, nutrients, vitamins, and minerals were calculated for each subject by summing the respective calories, nutrients, vitamins, and minerals for each food item[19]. The intake amounts of these factors were adjusted for caloric intake using the method of Willett *et al*[20].

Polymerase chain reaction amplification of heat-shock protein 60 and CagA gene

DNA was extracted from gastric cancer tissue samples using a commercially available kit (Wako, Osaka, Japan). To detect *H. pylori* infection in these samples, a nested PCR protocol was developed against heat-shock protein (*HSP60*). *HSP60* was chosen due to its high degree of sequence conservation in all biota[21].

DNA amplification of the *HSP60* gene was performed according to the nested polymerase chain reaction (PCR) method described by Varsha *et al*[22]. Forward and reverse oligonucleotides were derived from the conserved region located between bases 8085 and 8675 of the *HSP60* gene of *H. pylori* (ATCC 26695). Internal primers sets were derived from the region between bases 8162 and 8663 (Gene Bank Accession number NC-OO91; gene ID 899089). The first amplification was performed using HSP1, the sense primer (5'-AAGGCATG-CAATTTGATAGAGGCT-3'), and HSP2, the antisense primer (5'-CTTTTTTCTCTTTCATTTCCACTT-3'), in a 25-μL reaction mixture containing 2.5 μL of 10 × PCR buffer, 5 pmol of each primer, 10 ng of DNA, 200 μmol/L aliquots of each dNTP, and 1 unit of *Taq* polymerase. This PCR resulted in a 590-base pair DNA fragment.

For the second amplification, 2 μL of the primary amplification product was used in a 15-μL reaction mixture with HSPN1, the sense primer (5'-TTGATAGAG-GCTACCTCTCC-3'), and HSPN2, the antisense primer (5'-TGTCATAATCGCTTGTCGTGC-3'). Both PCR amplifications consisted of 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 58 ℃ for 40 s, and elongation at 72 ℃ for 30 s. The final amplicon (expected size, 501 base pairs) was analyzed by electrophoresis on a 2% agarose gel.

Nested PCR was also chosen as a sensitive and specific method to detect the presence of the *CagA* gene. Forward and reverse oligonucleotides were derived from the conserved region located between bases 544680 and 545161 of the *CagA* gene of *H. pylori* (ATCC 26695). Internal primers sets were derived from the region between bases 544833 and 545131 (Gene Bank Accession number NC-00921; gene ID 899089). The first amplification was performed with *CagA* N1, the sense primer (5'-TG-GCAGTGGGTTAGTCATAGCAG-3'), and *CagA* N2, the antisense primer (5'-AGGACTCTTGCAGGCGTT-GGTG-3'), in a 15-μL reaction mixture containing 1.5 μL of 10 × PCR buffer, 5 pmol of each primer, 10 ng of DNA, 200 μmol/L aliquots of each dNTP, and 1 unit of *Taq* polymerase, resulting in a 481-base pair DNA fragment. For the second amplification, 2 μL of the primary amplification product was used in a 15 μL reaction mixture with *CagA* D1, the sense primer (5'-ATAATGCTA-AATTAGACAACTTGAGCGA-3'), and *CagA* R1, the antisense primer (5'-TTAGAATAATCAACAAACAT-CACGCCAT-3'). Both PCR amplifications consisted of 35 cycles of denaturation at 94 ℃ for 30 s, annealing at

55 ℃ for 30 s, and elongation at 72 ℃ for 30 s. The final amplicon (expected size, 298 base pairs) was analyzed by electrophoresis on a 2% agarose gel.

RUNX3 hypermethylation analysis

The methylation status of the CpG island in the promoter region of *RUNX3* was determined by bisulfite treatment of DNA (Imprint DNA Modification Kit, Sigma-Aldrich, St. Louis, MO, United States) in order to convert all unmethylated cytosines to uracils while leaving methylated cytosines unchanged, followed by methylation-specific PCR. Amplifications were carried out in a 96-well plate. DNA extracted from the leukocytes of healthy individuals and treated with *Sss*I methyltransferase (New England Biolabs, Beverly, MA, United States) prior to bisulfite modification was used as a positive control for the methylated alleles. Bisulfite-modified DNA extracted from leukocytes of a healthy individual served as a positive control for the unmethylated alleles, and water was used as a negative control.

Amplification was carried out in a 20 μL reaction volume containing 10 ng of bisulfite-modified DNA and 2 μL of 10 × PCR buffer with 20 mmol/L $MgCl_2$, 4 μL of a GC-rich solution, 5 pmol of each primer for *RUNX3*, 200 μmol/L aliquots of each dNTP, and 1 unit of Faststart *Taq* DNA polymerase (Roche Applied Science, Mannheim, Germany). PCR was performed in a TaKaRa PCR Thermal Cycler Dice Gradient (Otsu, Japan) for methylated *RUNX3* with *RUNX3*-5M, the sense primer (5'-TTACGAGGGGCGGTCGTACGCGGG-3'), and *RUNX3*-3M, the antisense primer (5'-AAAACGACCGACGCGAACGCCTCC-3'), using an initial denaturation at 95 ℃ for 5 min, followed by 35 cycles of 95 ℃ for 1 min, 69.1 ℃ for 1 min, 74 ℃ for 1 min and a final extension at 74 ℃ for 7 min. For unmethylated *RUNX3,* PCR was performed using *RUNX3*-5U, the sense primer (5'-TTATGAGGGGTGGTTGTATGTGGG-3') and *RUNX3*-3U, the antisense primer (5'-AAAACAACCAACACAAACACCTCC-3') following an initial denaturation at 95 ℃ for 5 min, followed by 35 cycles of 95 ℃ for 1 min, 61.8 ℃ for 1 min, 72 ℃ for 1 min and a final extension at 72 ℃ for 7 min. The PCR product (expected size, 218 base pairs) was separated on a 3% agarose gel. To verify the PCR product, the methylation-specific PCR amplicons from 5 samples were purified with HiYield PCR DNA Extraction Kit (Real Biotech, Banqiao, Taiwan) and subjected to direct DNA sequencing (ABI 3100, Applied Biosystems, Foster City, CA, United States).

RNA isolation and reverse transcription-PCR

Total RNA was extracted from 137 gastric cancer tissue samples using a commercially available kit (Easy-Blue, Intron, Seongnam, South Korea). All samples were assayed for *RUNX3* expression. The assay failed for 18 samples due to RNA degradation. The RT reaction was performed using 1 μg of total RNA with SuperScript Ⅲ First-strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, United States). The following primer sets were used: 5'-TCTGCTCCGTGCTGCCCTCGCACTG-3' and 5'-AGGCATTGCGCAGCTCAGCGGAGTA-3' for *RUNX3* (151 bp) and 5'-ACCCACTCCTCCACCTTTG-3' and 5'-CTCTTGTGCTCTTGCTGGG-3' for *GAPDH* (178 bp). Amplification was carried out in a 15-μL reaction volume containing 0.1 μg of cDNA and 1.5 μL of 10 × PCR buffer with 20 mmol/L $MgCl_2$, 3 μL of a GC-rich solution, 5 pmol of each primer, 200 μmol/L aliquots of each dNTP, and 1 unit of Faststart *Taq* DNA polymerase. PCR was performed in the TaKaRa PCR Thermal Cycler Dice Gradient with an initial denaturation at 95 ℃ for 5 min, followed by 30 cycles (for *RUNX3)* or 25 cycles (for *GAPDH)* of 95 ℃ for 1 min, 62.3 ℃ for 1 min, 72 ℃ for 1 min and a final extension at 72 ℃ for 7 min. The PCR product was separated on a 3% agarose gel.

Statistical analysis

The amount of calories, nutrients, vitamins and minerals for each particular food type was calculated by multiplying their respective values by the level of intake. The total intake of calories, nutrients, vitamins, and minerals was calculated as the sum of each value across all food types consumed by each individual[19]. Each value was adjusted for caloric intake using the method of Willet *et al*[20]. The median value calculated for each variable was used as the grouping criterion in the unmethylated *RUNX3* group.

The odds ratios (ORs) and 95%CIs associated with the *RUNX3* methylation status were estimated for each of the food groups and lifestyle factors and for the interaction between dietary and lifestyle factors with *CagA* status of *H. pylori* infection using an unconditional logistic model that controlled for age, sex and total energy intake. The homogeneities of the ORs according to the *CagA* status were evaluated using the Breslow-Day test. *P* values less than 0.05 were considered significant.

RESULTS

Overall, 164 patients (89.1%) were found to be positive for *H. pylori* DNA, with the *CagA* gene detected in 59 (36%) of the *H. pylori*-positive samples. Overall, *CagA*-positive expression was detected in 32.1% of all patients included in our study. In all, 106 (57.6%) patients with gastric cancer demonstrated CpG island hypermethylation at the RUNX3 promoter. *RUNX3* expression was undetectable in 52 (43.7%) of the 119 gastric cancer tissues sampled.

We found a significant difference in *RUNX3* expression status between patients with and without hypermethylated *RUNX3*. *RUNX3* gene expression was detected in 71.7% of patients with unmethylated *RUNX3*. In contrast, *RUNX3* gene expression was detected in only 46.6% of patients with methylated *RUNX3* ($P = 0.0070$) (Table 2).

A high consumption of eggs was associated with a higher risk of *RUNX3* methylation, with an OR of 2.15

Table 2 Distribution of age, gender, *Helicobacter pylori* infection, *CagA* status, and *RUNX3* expression according to *RUNX3* methylation status *n* (%)

Variables	RUNX3 methylation (+)	RUNX3 methylation (-)	*P* value
Age (mean ± SD), yr	60.5 ± 11.2	58.7 ± 11.5	0.290
Gender			0.648
Male	70 (56.5)	36 (67.4)	
Female	54 (43.5)	24 (40.0)	
Helicobacter pylori infection			0.234
Negative	14 (13.2)	6 (7.7)	
Positive	92 (86.8)	72 (92.3)	
CagA status			0.997
Negative	72 (67.9)	53 (68.0)	
Positive	34 (32.1)	25 (32.0)	
RUNX3 expression			0.007
Positive	34 (46.6)	33 (71.7)	
Negative	39 (53.4)	13 (28.3)	

(95%CI, 1.14-4.08). In contrast, high fruit consumption was associated with a lower risk of RUNX3 methylation, with an OR of 0.50 (95%CI, 0.27-0.93). A large consumption of eggs or chicken increased the risk of *RUNX3* methylation in *CagA*- or *H. pylori*-negative cancer patients. A high intake of unfermented seaweeds or alliums conferred larger protective effects against *RUNX3* methylation in *CagA*-positive cancer patients compared to CagA-negative cancer patients. In homogeneity tests, however, the OR of high consumption of these food groups was similar between those with *CagA*-positive and *CagA*-negative status. In contrast, a high consumption of nuts was a significant risk factor for *RUNX3* methylation in *CagA*-positive patients. The OR of high nut intake in *CagA*-positive patients was 4.28 (95%CI, 1.19-15.49), and this value significantly differed from that in *CagA*-negative or *H. pylori*-negative cases (Table 3).

Large intakes of carbohydrates and vitamin E decreased the risk of *RUNX3* methylation in gastric cancer, with odd ratios of 0.48 (95%CI, 0.25-0.91) and 0.47 (95%CI, 0.26-0.88), respectively. When stratified according to the *CagA* status, high intakes of carbohydrates, vitamin B1, or vitamin E conferred a protective effect in *CagA*-negative or *H. pylori*-negative patients but not in *CagA*-positive patients, with statistically significant differences demonstrated for vitamins B1 and E (Table 4).

DISCUSSION

RUNX3, a member of the *RUNX* family, is a candidate tumor suppressor gene that is thought to play a role in the progression of gastric cancer. *RUNX3* is unique in that it is inactivated primarily by epigenetic silencing, unlike many other tumor suppressors, such as *p53*, that are inactivated mainly by deletion and mutation[23]. Molecular epidemiology studies have investigated the relationship between DNA hypermethylation-induced inactivation of *RUNX3* and individual risk of cancer development in various organs, including the lungs, pancreas, urinary bladder, and gastric cancers[12,24-26].

In the present study, we found that *RUNX3* methylation was observed in 58% (106/184) of our gastric cancer patients. Consistent with our results, *RUNX3* methylation was previously observed in 52.6% (30/57) of the gastric cancer specimens in a Japanese study[27]. Other investigators have reported the prevalence of *RUNX3* methylation in primary gastric cancers to be 71% (57/80) and 64% (48/75)[13,28]. These differences in methylation frequency can be explained by the fact that different regions were examined within the *RUNX3* CpG island. Homma *et al*[29] suggested that the methylation status of the regions spanning the transcription start site (No 6-8) is critical for the loss of *RUNX3* expression. In our study, we examined CpG islands within regions No 6-7 of *RUNX3* and observed levels of methylation similar to those reported by Homma *et al*[29]. Previous studies have shown that, among primary gastric cancer samples and gastric cancer cell lines, 45%-60% showed loss of *RUNX3* expression due to hypermethylation of the CpG island located in the P2 promoter region[12,30]. We found that *RUNX3* expression was undetectable in 43.7% of primary gastric cancer tissues. The significant association between *RUNX3* methylation status and its expression is consistent with the fact that promoter hypermethylation downregulates the expression of this gene.

H. pylori infection and *CagA* status did not reveal any significant association with *RUNX3* promoter hypermethylation or with *RUNX3* expression levels (Table 2). This finding suggests that *H. pylori* infection, regardless of *CagA* status, does not induce *RUNX3* methylation and that the ability of *RUNX3* to function as a tumor suppressor is not specific to *H. pylori*-related gastric cancer. A Japanese study reported a marginally significant association between *H. pylori* infection and *RUNX3* promoter hypermethylation; however, no significance was found between *CagA* positive *H. pylori* infection and *RUNX3* promoter hypermethylation[27]. Our results are further supported by a German study that demonstrated that the level of *RUNX3* mRNA expression in the gastric epithelium is not influenced by *H. pylori* infection[31]. Interestingly, it has previously been shown that *H. pylori* infection induces the ubiquitination and degradation of *RUNX3* and suppresses *RUNX3* expression in cultured gastric epithelial cells and mouse gastric epithelial cells. This discordance may be due to differences between *in vivo* and *in vitro* systems, and differences between mouse and human diseases. Furthermore, we tested the association of *H. pylori* infection with *RUNX3* promoter hypermethylation, not with *RUNX3* expression. It is possible that *H. pylori* infection suppresses the expression of *RUNX3* in gastric epithelial cells *via* pathways other than promoter hypermethylation. For example, Liu *et al*[32] reported that *CagA* can inhibit the expression of *RUNX3* *via* Src/MEK/ERK and p38 MAPK pathways.

Several dietary factors, including folate, methionine, and vitamins B12 and B6 have been reported to be involved either directly or indirectly in DNA methylation[15]. Re-

Table 3 Associations between dietary factors and *RUNX3* methylation risk, according to *CagA* status

	Total subjects		*CagA* (-) or *H. pylori* (-)		*CagA* (+)	
	Methylated/ unmethylated	OR (95%CI)[1]	Methylated/ unmethylated	OR (95%CI)[1]	Methylated/ unmethylated	OR (95%CI)[1]
Nuts						
Low	36/40	1.0	26/23	1.0	10/17	1.0
High	70/38	1.65 (0.86-3.18)	46/30	1.10 (0.49-2.49)	24/8	4.28 (1.19-15.49)[2,3]
Vegetables						
Low	65/39	1.0	47/26	1.0	18/13	1.0
High	41/39	0.58 (0.31-1.07)	25/27	0.49 (0.23-1.03)	16/12	0.78 (0.27-2.36)
Fruits						
Low	69/39	1.0	51/31	1.0	18/8	1.0
High	37/39	0.50 (0.27-0.93)[3]	21/22	0.55 (0.26-1.19)	16/17	0.32 (0.10-1.06)
Meat						
Low	39/40	1.0	26/28	1.0	13/12	1.0
High	67/38	1.68 (0.91-3.12)	46/25	1.83 (0.87-3.87)	21/13	1.30 (0.41-4.10)
Egg						
Low	32/40	1.0	21/29	1.0	11/11	1.0
High	74/38	2.15 (1.14-4.08)[3]	51/24	2.64 (1.21-5.75)[3]	23/14	1.57 (0.48-5.23)
Chicken						
Low	34/39	1.0	20/26	1.0	14/13	1.0
High	72/39	1.78 (0.93-3.41)	52/27	2.24 (1.02-4.95)[3]	20/12	0.95 (0.27-3.39)
Seaweeds						
Low	64/39	1.0	43/30	1.0	21/9	1.0
High	42/39	0.63 (0.35-1.16)	29/23	0.89 (0.43-1.87)	13/16	0.23 (0.07-0.79)[3]
Alliums						
Low	66/40	1.0	43/28	1.0	23/11	1.0
High	40/38	0.55 (0.30-1.01)	29/25	1.71 (0.34-1.48)	11/14	0.28 (0.09-0.92)[3]
Fish						
Low	61/39	1.0	40/29	1.0	21/10	1.0
High	45/39	0.69 (0.38-1.27)	32/24	0.89 (0.43-1.85)	13/15	0.44 (0.14-1.33)

[1]Adjusted for age, gender, total energy intake; [2]Odds ratio (OR) was significantly different according to CagA status in a homogeneity test; [3]*P* value < 0.05 statistically significant difference.

verse associations between these dietary factors and cancer risk, observed in epidemiological studies, have led to the hypothesis that folate, methionine, and other dietary factors confer an altered risk of gastric cancer due to their role in DNA methylation[33]. However, few data exit to support this hypothesis. Many studies have investigated potential associations between dietary factors and DNA promoter methylation in colon cancer[34,35]; however, these studies have failed to establish a consistent association. Methionine is considered to be an essential dietary component because it is the penultimate methyl donor for mammalian methylation reactions and it cannot be manufactured by the body. Consuming excessive quantities of methionine may affect DNA promoter CpG island methylation and thereby cause dysregulation of gene expression. In the present study, a high consumption of methionine-rich foods such as eggs and chicken increased the risk of *RUNX3* promoter methylation, and a high consumption of nuts increased the risk when combined with *CagA*-positive *H. pylori* infection.

Two major forms of *CagA* have been identified: East Asian *CagA* and Western *CagA*. In countries such as Japan, South Korea and China, most *H. pylori* infections contain East Asian *CagA*. The degree of inflammation, activity of gastritis, and atrophy are significantly higher in patients infected with East Asian *CagA*-positive strains compared to patients infected with *CagA*-negative or Western *CagA*-positive strains[36]. *CagA*-positive *H. pylori* infection induces progressive inflammatory changes in the gastric mucosa that ultimately lead to gastric cancer: superficial gastritis, atrophic gastritis, intestinal metaplasia, dysplasia, and carcinoma[37]. In the present study, we observed that *CagA*-positive *H. pylori*-infected individuals who consume a large quantity of nuts may increase the risk of *RUNX3* methylation by 4-fold. The risk of *RUNX3* methylation was higher in *CagA*-positive *H. pylori*-infected subjects than in *CagA*- or *H. pylori*-negative patients of the same age.

The association of certain vitamin deficiencies and cancer is well established and is usually associated with environmental conditions that affect all tissues. High intakes of folate and vitamins B6 and B12 may protect against DNA methylation in colorectal cancer[35]. In the present study, however, we did not find a protective effect of these vitamins. Instead, we found that high intakes of vitamin B1 and carbohydrates provide a protective effect against *RUNX3* methylation in gastric cancer, but this was not demonstrated in individuals with *CagA*-positive status. Vitamin B1 is essential for the body to utilize carbohydrates for energy and for metabolizing amino acids. The main biologically active vitamin B1 derivative is thiamine diphosphate, which is involved in universal metabolic pathways including glycolysis, the pentose phosphate pathway and the tricarboxylic acid cycle. Several studies

Table 4 Associations between micronutrients and mineral salts intake and *RUNX3* methylation risk, according to *CagA* status

	Total subjects		*CagA* (-) or *H. pylori* (-)		*CagA* (+)	
	Methylated/ unmethylated	OR (95%CI)[1]	Methylated/ unmethylated	OR (95%CI)[1]	Methylated/ unmethylated	OR (95%CI)[1]
Protein						
Low	54/39	1.0	35/26	1.0	19/13	1.0
High	52/39	0.90 (0.50-1.64)	37/27	0.95 (0.46-1.95)	15/12	0.86 (0.29-2.57)
Fat						
Low	60/39	1.0	38/23	1.0	22/16	1.0
High	46/39	0.74 (0.21-1.35)	34/30	0.66 (0.32-1.36)	12/9	0.98 (0.30-3.25)
Carbohydrate						
Low	71/39	1.0	53/28	1.0	18/11	1.0
High	35/39	0.48 (0.25-0.91)[3]	19/25	0.41 (0.19-0.90)[3]	16/14	0.53 (0.15-1.91)
Vitamin A						
Low	57/39	1.0	36/27	1.0	21/12	1.0
High	49/39	0.73 (0.40-1.35)	36/26	0.88 (0.42-1.86)	13/13	0.51 (0.17-1.56)
Vitamin B1						
Low	65/40	1.0	48/24	1.0	17/15	1.0
High	41/38	0.67 (0.36-1.22)	24/29	0.42 (0.20-0.89)[2,3]	17/10	2.04 (0.63-6.62)[2]
Vitamin B2						
Low	56/39	1.0	37/25	1.0	19/14	1.0
High	50/39	0.81 (0.44-1.48)	35/28	0.77 (0.37-1.62)	15/11	0.97 (0.33-2.85)
Vitamin C						
Low	66/39	1.0	50/31	1.0	16/8	1.0
High	40/39	0.56 (0.30-1.04)	22/22	0.59 (0.27-1.29)	18/17	0.38 (0.11-1.28)
Vitamin E						
Low	73/39	1.0	54/24	1.0	19/15	1.0
High	33/39	0.47 (0.26-0.88)[3]	18/29	0.29 (0.13-0.62)[2,3]	15/10	1.30 (0.42-4.05)[2]

[1]Adjusted for age, sex, total energy intake; [2]Odds ratio (OR) was significantly different according to CagA status in a homogeneity test; [3]*P* value < 0.05 statistically significant difference.

have reported that a high intake of carbohydrates protects against esophageal cancer and gastric cancer[38]. Our study suggests that carbohydrates may protect against *RUNX3* promoter CpG island hypermethylation.

Vitamin E is a lipid-soluble antioxidant found in cell membranes where it prevents lipid peroxidation of polyunsaturated fatty acids. An Italian study reported that individuals with a high intake of vitamin E reduce their risk of gastric cancer by 50%[39]. In the present study, the protective effect of high vitamin E intake on *RUNX3* methylation was observed in *CagA*-negative or *H. pylori*-negative gastric cancer patients but not in individuals with *CagA*-positive *H. pylori* infection. However, adverse effects of vitamin E have also been reported[40]. Because vitamin E is metabolized using the same pathway as xenobiotics and may induce drug-metabolizing enzymes in rodents, it is hypothesized that high doses of vitamin E may lead to bioactivation of carcinogens within the human body[41].

The association between high fruit consumption and protection from gastric cancer has been reported in many studies[42,43]. The European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST) has reported similar results: fresh fruit and citrus fruit consumption may protect against diffuse and cardia gastric cancer, respectively[44], but consistent results have yet to be achieved. Fruits contain vitamins C and E, which may protect cell membranes and DNA from oxidative damage. In our study, a protective effect of high fruit intake on *RUNX3* methylation was observed, as well as a marginal inverse association between high vitamin C intake and *RUNX3* methylation.

We used nested PCR methods with DNA from gastric cancer tissues to detect *H. pylori* infection and *CagA* status. Nested PCR has the advantages of high sensitivity and specificity, quick results, and the ability to type bacteria without the requirement for special transport conditions. In our study, 89% of the gastric cancer patients were found to be positive for *H. pylori* DNA, and 32% were positive for *CagA* DNA. A Japanese study in which PCR methods were used to detect *H. pylori* infection and *CagA* status reported a 31.6% *CagA*-positive rate in 57 gastric cancer tissues[27], which is very similar to our result. Several South Korean studies documented a higher prevalence of *CagA*-positive *H. pylori* infection[45,46]. However, one study used an immunoblot method and the other included a smaller sample size. Because of the potential for false-positive immunoblot test results and the fact that individuals who had the infection in the past, irrespective of current infection, were classified as *CagA*-positive by this test, the actual *CagA* prevalence was likely lower than reported (97%). The difference between the *CagA* positive rates in our study and the previous one could be explained by the different detection methods. Moreover, a majority of the previous studies only investigated the role of *RUNX3* promoter methylation or *H. pylori* infection and *CagA* status in gastric cancer. We demonstrate, for the first time, that the interaction between *CagA*-positive

H. pylori infection and a high methionine intake contributes to *RUNX3* methylation in gastric cancer.

This study has some limitations. Because non-cancerous mucosa from gastric cancer patients or normal mucosal tissues from healthy controls were not included in this study, we were unable to determine *RUNX3* hypermethylation status or *RUNX3* expression level in those tissues. In addition, because this study is retrospective, we could not determine the temporal sequence of *H. pylori* infection and *RUNX3* promoter methylation. Finally, our sample size was not large enough to allow us to draw firm conclusions, especially regarding the effects of dietary factors on *RUNX3* promoter methylation.

In summary, a high consumption of chicken, eggs, or nuts rich in methionine may increase the risk of *RUNX3* promoter methylation in gastric cancer patients, whereas diets rich in fruits, carbohydrates, and vitamins B1 and E may decrease that risk. However, these inverse associations were observed only in patients negative for *CagA* or *H. pylori*. Moreover, a high intake of nuts combined with *CagA*-positive *H. pylori* infection significantly increased the risk of *RUNX3* promoter methylation. The *CagA* status of the *H. pylori* infection may be an important modifier of *RUNX3* methylation in gastric cancer patients.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection is a major risk factor for gastric cancer, and the cytotoxin-associated gene A (*CagA*) is considered a marker for enhanced *H. pylori* virulence. Individuals infected with *CagA*-positive *H. pylori* strains have a higher risk of developing gastric cancer. *RUNX3* is a candidate tumor suppressor gene, and loss of *RUNX3* expression is considered a critical step in the genesis and progression of gastric cancer.

Research frontiers

Recent epidemiological studies have investigated the relationship between inactivation of *RUNX3* and the risk of cancer development in various organs. *RUNX3* inactivation has been reported to be frequently, but not always, found in gastric cancer tissues. One of the most important mechanisms for silencing *RUNX3* expression is *via* hypermethylation of the gene promoter, but little is known regarding the causative factors of RUNX3 promoter hypermethylation. The authors cannot rule out the possibility that other risk factors for gastric cancer, such as dietary factors and CagA-positive *H. pylori* infection, can induce promoter hypermethylation of the RUNX3 gene. In this study, the authors found that *H. pylori* infection, regardless of *CagA* status, is not associated with *RUNX3* methylation and that the ability of *RUNX3* to function as a tumor suppressor is not specific to *H. pylori*-related gastric cancer. In addition, the authors identified dietary factors that, in combination with *CagA*-positive *H. pylori* infection, modify the risk of *RUNX3* promoter methylation.

Innovations and breakthroughs

The authors determined the relationship between *CagA*-positive *H. pylori* infection and certain dietary factors with *RUNX3* promoter hypermethylation positive in gastric cancer patients. The authors adopted this case-case study design to maximize comparability and statistical power. To the best of our knowledge, this is the first study of the interaction between *CagA*-positive *H. pylori* infection and *RUNX3* methylation in gastric cancer tissues.

Applications

The results of this study suggest that the *CagA* status of *H. pylori* infection may modify dietary effects on *RUNX3* promoter hypermethylation in gastric cancer tissue. Further studies are needed to determine the mechanisms by which *CagA* and dietary factors interact and influence the development of gastric cancer.

Terminology

CagA, a 120-145 kDa protein encoded on the 40 kb *Cag* pathogenicity island, is a *Helicobacter pylori* virulence factor. RUNX3 is a member of the runt domain-containing family of transcription factors and is encoded by the *RUNX3* gene.

Peer review

In this study, the authors evaluated associations of *Helicobacter pylori* infection, CagA status, and dietary factors with RUNX3 promoter hypermethylation in 184 Korean patients with gastric cancer.

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