ancy between the Loayza *et al.* (51) findings and those of Colgin *et al.* (52) along with ours. There are several possible explanations: we and Colgin *et al.* (52) altered whole expression levels of wild-type *POT1* with antisense oligonucleotides and expression vectors (52); thus, the discrepancy may be because of methodological differences. We did not perform any mutation analysis in the oligosaccharide/oligonucleotide-binding (OB) fold of Pot1 in the present study. This should be done in further study. Although telomere length may be regulated by the interaction between Pot1 and TRF1 complex, we did not examine TRF1 expression status in the present study. In yeast, the interaction between Cdc13 and telomerase has been previously described (14), but in human cells, the interaction between Pot1 and telomerase has not been examined. We presume that such investigation is most essential to understanding the function of Pot1.

Adequate telomere length, telomerase activity, and T-loop formation are required for maintenance of telomere function, and when only one mechanistic factor is compromised such as in a lack of functional telomerase or telomere shortening, the other components of the capping system can compensate (16). The association we observed between telomere length and telomerase activity indicates that Pot1 may play an important role in the maintenance of telomere function. Telomere dysfunction leads to genetic instability at the chromosome ends, and such instability is associated with the initiation of carcinogenesis (26, 30, 31, 34–39). We consider reduced levels of *POT1* expression to reflect telomere dysfunction and that they may serve as a useful screening tool for identifying individuals at greatest risk of carcinogenesis. Additional studies are needed to establish Pot1 as a clinical indicator of cancer risk.

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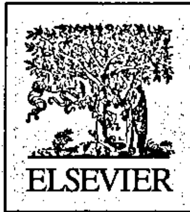


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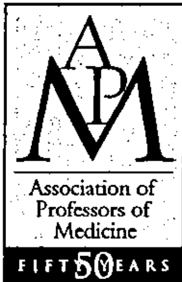


## Long-term effects of radiation dose on inflammatory markers in atomic bomb survivors

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BRIEF OBSERVATION

# Long-term effects of radiation dose on inflammatory markers in atomic bomb survivors

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Atomic bomb survivors have a persistently increased risk of cancer, hepatitis, and cardiovascular and autoimmune diseases.[1-4] There is no clear explanation for these late effects of radiation exposure. One hypothesis is that radiation causes chronic low-grade inflammation, with elevated circulating levels of cytokines. Proinflammatory cytokines, such as interleukin 6, tumor necrosis factor (TNF) $\alpha$, and interferon $\gamma$, and anti-inflammatory cytokines, such as interleukin 10, are synthesized predominantly by macrophages and lymphocytes, and regulate the inflammatory response.[5,6] Interleukin 6 in turn induces the synthesis of acute-phase plasma proteins, such as C-reactive protein.[7] Increased levels of inflammatory cytokines, even within the normal range, have been associated with an increased risk of cardiovascular disease.[8] Chronic low-grade inflammation may also influence the production of immunoglobulins by B cells.[9]

We therefore analyzed the effects of presumed radiation dose on inflammatory parameters in atomic bomb survivors.

## Methods

### Subjects

We studied subjects from Hiroshima who had participated in an epidemiological follow-up study of atomic bomb survivors, which collected health information from 2436 survivors during biennial medical examinations.[10] Peripheral blood samples were collected between March 1995 and April 1997. We obtained institutional approval from the human investigation committee and informed consent from participants. We excluded subjects with a history of cancer or diseases that have been associated with inflammation (e.g., current upper respiratory tract infection, chronic bronchitis, collagen disease, arthritis, or myocardial infarction). We classified the other participants into four radiation dose groups: nonexposed, low dose (0.005 to 0.7 Gy), medium dose (0.7 to 1.5 Gy), and high dose (>1.5 Gy). Estimated bone marrow doses were based on the 1986 Dosimetry System.[11] Doses were for whole-body exposure, mainly from gamma rays but with a small neutron component. We selected 180 subjects from the nonexposed group and 90 from each of the other

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**Table 1** Characteristics of the study subjects*

| Characteristic | Nonexposed (n = 179) | Radiation Exposure (Gy) 0.005–0.7 (n = 87) | 0.7–1.5 (n = 88) | >1.5 (n = 88) |
|---|---|---|---|---|
| | Number (%) or mean ± SD | | | |
| Radiation dose (Gy) | 0 | 0.3 ± 0.2 | 1.1 ± 0.2 | 2.1 ± 0.5 |
| Age (years) | 68 ± 11 | 69 ± 11 | 67 ± 10 | 68 ± 10 |
| Female sex | 96 (54) | 50 (58) | 52 (59) | 47 (53) |
| Body mass index (kg/m$^2$) | 23 ± 3 | 23 ± 3 | 22 ± 4 | 23 ± 4 |
| Current smokers | 44 (25) | 17 (20) | 23 (26) | 21 (24) |

*Among atomic bomb survivors from Hiroshima, Japan.

groups, such that the age and sex distributions were similar in the four groups. Data were missing for 8 subjects; these subjects were excluded from all analyses.

## Measurements

We measured plasma TNF-$\alpha$, interferon $\gamma$, and interleukin 10 levels in duplicate using a highly sensitive enzyme-linked immunosorbent assay kit (Quantikine HS; R&D Systems, Minneapolis, Minnesota). Mean values of duplicate measurements were reported for all assays. We quantitated immunoglobulin levels using standard kits (Bethyl Lab. Inc., Montgomery, Texas). The interassay and intra-assay coefficients of variations of these enzyme-linked immunosorbent assay kits were lower than 10%. The erythrocyte sedimentation rate was measured using standard methods.

## Statistical analysis

We estimated the effects of changes in several predictor variables (linear radiation dose, age, and sex), adjusted for current smoking and body mass index (in kg/m$^2$) using a multivariate linear regression model based on the log of the outcome variables (biological markers). We present results as percentage changes in the outcome variables with 95% confidence intervals. All analyses were performed using SAS software (Cary, North Carolina).

## Results

There were no significant differences in age, sex, body mass index, or current smoking among the four groups (Table 1). Interferon $\gamma$ levels and the erythrocyte sedimentation rate increased significantly with radiation dose (Figure). Tumor necrosis factor $\alpha$ and interleukin 10 levels also increased slightly but not significantly with radiation dose. The levels of immunoglobulin (Ig) A and IgM increased significantly with radiation dose, but those of IgG and IgE did not.

In multivariate models, the levels of TNF-$\alpha$, interferon $\gamma$, and interleukin 10, and the erythrocyte sedimentation rate, increased significantly with radiation dose, as did IgA, IgM, and total immunoglobulin levels (Table 2). The levels of TNF-$\alpha$, interleukin 10, IgG, IgA, and total immunoglobulins, and the erythrocyte sedimentation rate, increased significantly with age.

## Discussion

Tumor necrosis factor $\alpha$, interleukin 6, interferon $\gamma$, and interleukin 10 coordinate the inflammatory response. In the present study, plasma levels of inflammatory cytokines and biomarkers (TNF-$\alpha$ and the erythrocyte sedimentation rate) increased with radiation dose and with age. Plasma levels of other cytokines (interferon $\gamma$ and interleukin 10) and immunoglobulins (IgA and IgM) increased with radiation dose. Combined with previous results on other inflammatory signs, such as increased white blood cell counts, and sialic acid and C-reactive protein levels,[12,13] our results provide evidence of persistent inflammatory responses in atomic bomb survivors more than 50 years after radiation exposure.

In light of these studies, we hypothesized that radiation exposure accelerated aging. To test the hypothesis, we calculated radiation exposure as a function of age using inflammatory status as an index. We estimated that exposure to 1 Gy was equivalent to an increase in age of about 9.0 years. Noting that the mean exposure among atomic bomb survivors was about 0.2 Gy, we inferred that mean accelerated aging among atomic bomb survivors was about 2 years (range, 1 to 2.5 years). Others have reported that the decrease of CD4-expressing T cells was about 4% per 10 years and 2% per Gy, implying that the decrease per Gy is equivalent to about 5 years of aging.[14] Furthermore, interleukin 6 levels correlate

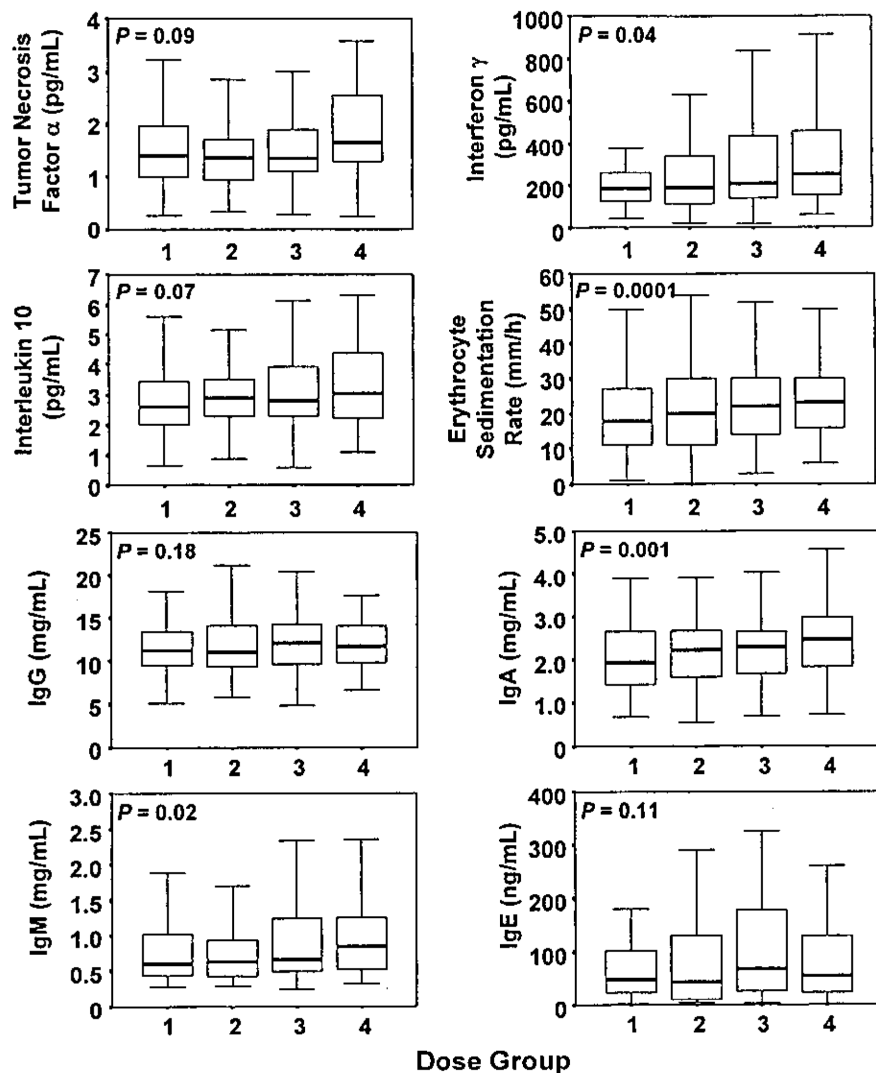


**Figure 1** Box plot of inflammatory biomarker levels and erythrocyte sedimentation rate among atomic bomb survivors. The horizontal line inside the box represents the median. Lower and upper boundaries represent the 25th and 75th percentiles. Whiskers represent the smallest and largest values that are less than 1.5 box-length from the 25th and 75th percentiles. 1 indicates nonexposed; 2 indicates radiation exposure of 0.005 to 0.7 Gy; 3 indicates exposure of 0.7 to 1.5 Gy; and 4 indicates exposure of >1.5 Gy. Ig = immunoglobulin.

negatively with the percentage of CD4 T cells.[13] Thus, acceleration of immunological aging may also be involved in radiation effects on the inflammatory status in humans.

Increased mortality and morbidity from cardiovascular disease has been observed in atomic bomb survivors,[10] and elevated plasma levels of inflammatory markers, including interleukin 6, have been associated with an increased risk of cardiovascular disease.[8] Indeed, C-reactive protein and complement are mediators of ischemic myocardial injury.[15] Further, the percentage of CD4 T cells in the blood is markedly lower among atomic bomb survivors who have a history of myocardial infarction.[16] We hypothesize that modification of cytokine production may be involved in the onset or progression of some of the conditions, such as hepatitis and cardiovascular disease, which are more common in atomic bomb survivors.

Several studies have shown that radiation causes short-term inflammatory effects, such as increased plasma levels of proinflammatory cytokines, among patients who received radiation therapy.[17-19] In addition, radiation for cancer or Hodgkin's disease leads to long-term depletion of naïve CD4 T cells,[20,21] and pathologic cardiac changes.[22] Our results suggest that radiation exposure may also produce long-term adverse effects by generating a persistent inflammatory status, manifested by cytokines and other inflammatory markers along with long-lasting impairment of CD4 T cells. Given the potential implication of our findings, follow-up of radiotherapy-treated patients is warranted to assess the asso-

**Table 2** Multivariate models of the effects of age, sex, and radiation dose on inflammatory biomarkers and immunoglobulins

| Variable | Tumor Necrosis Factor α | Interferon γ | Interleukin 10 | Erythrocyte Sedimentation Rate | Total Ig | IgG | IgA | IgM | IgE |
|---|---|---|---|---|---|---|---|---|---|
| | Percentage Increment (95% Confidence Interval) | | | | | | | | |
| Age per 10 years | 15 (9 to 20) | 4 (−4 to 12) | 8 (4 to 13) | 15 (9 to 20) | 3 (1 to 6) | 3 (1 to 6) | 5 (2 to 9) | −6 (−11 to 14) | 2 (−11 to 14) |
| Female sex* | 15 (2 to 30) | −8 (−23 to 10) | 6 (0 to 12) | 17 (9 to 24) | 5 (0 to 10) | 7 (1 to 13) | −9 (−17 to −1) | 14 (1 to 28) | −51 (−63 to −34) |
| Radiation dose per Gy | 7 (1 to 15) | 12 (2 to 23) | 6 (0 to 12) | 17 (9 to 24) | 3 (1 to 6) | 2 (−1 to 5) | 8 (3 to 13) | 9 (2 to 15) | 14 (−3 to 32) |

Ig = immunoglobulin.
*Compared with men.

ciation between inflammatory status and the occurrence of inflammation-associated diseases.

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# Single nucleotide polymorphisms in the *EXO1* gene and risk of colorectal cancer in a Japanese population

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**EXO1 is a member of the RAD2 nuclease family and functions in DNA replication, repair and recombination. We investigated the relationship of single nucleotide polymorphisms (SNPs) at exon 10 (T439M) and exon 13 (P757L) of the *EXO1* gene with development, progression and metastasis of colorectal cancer. For T439M, the *Thr/Met* genotype [odds ratio (OR) = 2.03, 95% confidence interval (CI) 1.04–3.98] and *Thr/Met* and *Met/Met* genotypes combined (OR = 2.37, 95% CI 1.23–4.56) demonstrated significant association with the development of colorectal cancer after adjusting for age, gender and smoking status. For P757L, patients with the *Leu/Leu* genotype showed a reduced risk of colorectal cancer (adjusted OR = 0.398, 95% CI 0.183–0.866) when the *Pro/Leu* and *Pro/Pro* genotypes were combined and used as the reference. The *Leu/Leu* genotype also had a reduced risk (adjusted OR = 0.373, 95% CI 0.164–0.850) when the *Pro/Leu* genotype was used as the reference. Individuals who carried both putative risk genotypes (*Thr/Met* and *Met/Met* for T439M and *Pro/Leu* for P757L) showed an adjusted OR of 4.95 (95% CI 1.56–15.7) compared with those who carried both low risk genotypes. Analysis of microsatellite instability (MSI) revealed that tumors from individuals who carried both putative risk genotypes tended to have a higher frequency of MSI positives than those from patients who carried both low risk genotypes, although a significant correlation was not found between *EXO1* genotype and MSI status. This is the first report to provide evidence for an association of *EXO1* gene polymorphisms with colorectal cancer risk. The *EXO1* genotypes were not associated with any clinicopathological characteristics in colorectal cancer patients.**

**Abbreviations:** CI, confidence interval; EXO1, exonuclease 1; HNPCC, hereditary non-polyposis colorectal cancer; MMR, mismatch repair; MSI, microsatellite instability; MSI-H, MSI high; MSI-L, MSI low; MSS, microsatellite stable; OR, odds ratio; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism; TNM, UICC Tumor-Node-Metastasis classification.

## Introduction

The exonuclease 1 (*EXO1*) gene, located at chromosome 1q42–q43, contains one untranslated exon followed by 13 coding exons and encodes an 846 amino acid protein (1,2). The gene product is a member of the RAD2 nuclease family and functions in DNA replication, repair and recombination (3–5). Recently it has been shown that it plays a role in both 5′→3′ and 3′→5′ mispair-dependent excision repair *in vitro* (6). Wei *et al.* (7) found that mammalian EXO1 functions in mutation avoidance and is essential for male and female meiosis. They also reported that EXO1 inactivation predisposes mice to the development of tumors late in life and specifically increases the risk of lymphoma. EXO1 can interact physically with the DNA mismatch repair (MMR) proteins MSH2 and MLH1 in both yeast and human cells and with MSH3 in human cells (2,8–12). Tishkoff *et al.* (13) reported that the expression of a 3.0 kb *EXO1* transcript was detected in significantly higher level in testis, thymus, colon and placenta. Elevated expression of the *EXO1* gene in the colon is intriguing because hereditary non-polyposis colorectal cancer (HNPCC), which is characterized primarily by the development of early onset colorectal cancer and a number of other epithelial malignancies, can be attributed to inherited defects in MSH2/MLH1-dependent mismatch repair (14). Wu *et al.* (15) proposed that the *EXO1* gene could be associated with HNPCC predisposition because EXO1 protein strongly interacts with MSH2 protein. More recently, the *EXO1* gene showed negative association with HNPCC, although it is involved in DNA MMR (16,17). However, we cannot exclude a role of *EXO1* as a low penetrance cancer susceptibility or modifying gene, because the studies performed to date have only focused on suspected HNPCC cases.

Genetic polymorphisms of DNA repair genes have been reported to determine susceptibility to several cancers, including lung, esophageal, bladder, nasopharyngeal and non-melanoma skin cancers (18–22). Although polymorphisms in some genes have been studied in relation to colorectal cancer (23–25), no study has been conducted on the association between polymorphisms in the *EXO1* gene and colorectal cancer risk. Wu *et al.* (15) identified 12 missense single nucleotide polymorphisms (SNPs) in the *EXO1* gene in exon 6 (D249N), exon 7 (G274R and N279S), exon 9 (R354H), exon 10 (T439M, V458M and V460L), exon 11 (K589E and G670E), exon 12 (C723R and S725S) and exon 13 (P757L), while the function of these polymorphisms remains unclear.

Microsatellite instability (MSI) is caused by a failure of the MMR system. Such MMR defects may be caused either by a germline MMR gene mutation, affecting mainly *MLH1* or *MSH2*, or by somatic MMR gene inactivation, most commonly through epigenetic silencing via methylation of the *MLH1* promoter. *EXO1* genotype may be associated with cancer incidence and with MSI status because EXO1 protein strongly interacts with the MSH2 and MLH1 proteins. In our present

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study we have investigated whether SNPs at T439M and P757L of the *EXO1* gene are associated with the risk of development, progression and metastasis of colorectal cancer. We also evaluated an association between *EXO1* SNPs and MSI status using five microsatellite markers (*BAT25*, *BAT26*, *D5S346*, *D2S123* and *D17S250*) that the National Cancer Institute Workshop on Hereditary Nonpolyposis Colorectal Cancer Syndrome proposed for MSI assessment in HNPCC, collectively known as the NCI panel (26).

## Materials and methods

### *Patients and control groups*

We analyzed 102 Japanese patients chosen from those who were histologically diagnosed as having primary colorectal cancer and underwent surgical operation at Okayama University Hospital in 1994–2003, which gathers patients from not only Okayama but also various regions, mainly the Chugoku and Shikoku Districts (around Okayama), in Japan. We confirmed microscopically that all colorectal cancer patients have primary colorectal carcinomas. Clinical stage and pathological grade in all colorectal cancer patients were confirmed by operation and pathology. The clinicopathological staging and histological classification were according to the criteria of the UICC Tumor–Node–Metastasis Classification of Malignant Tumours (TNM), 6th edition, 2002, colon and rectum (ICD-O C 18-C 20). The 110 controls were randomly selected from a prospective cohort study among a general Japanese population. Written informed consent was obtained from all colorectal cancer patients and controls. The characteristics of the 102 colorectal cancer patients and 110 controls are shown in Table I. There were no significant differences in gender and age at recruitment between the colorectal cancer patients and controls ($P > 0.05$). Pack-year equivalents ([cigarettes/day ÷ 20] × [smoking years]) were used for smoking status (we could not obtain smoking status for 3 of 102 patients). Patients who smoked for ≥20 years were less frequent than controls compared with never smokers ($P = 0.001$).

### *DNA extraction*

Genomic DNA of 102 patients was isolated from freshly frozen non-neoplastic colorectal mucosae using SDS/proteinase K treatment, phenol-chloroform extraction and ethanol precipitation. Tumor DNA was also isolated from freshly frozen neoplastic colorectal mucosae. Genomic DNA of 110 controls was extracted from peripheral lymphocytes.

### *Genotyping at T439M*

Genotype at T439M was analyzed by PCR-restriction fragment length polymorphism (RFLP). In brief, PCR was performed in a 20 μl reaction mixture with 20 ng genomic DNA, 2.5 mM each dNTP, 1× PCR buffer, 8 pmol each primer (forward primer, 5′-TCT CTA AGT ACA GGT GAA ACA AAG; reverse primer, 5′-GAG CTA TTT TTC TTG GTC TTC TAC) and 0.5 U rTaq DNA polymerase (Takara, Kyoto, Japan). Amplification conditions were 3 min of initial denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C, with a final extension of 7 min at 72°C. The 125 bp PCR products were digested overnight at 37°C with BsaAI (New England Biolabs, Beverly, MA). BsaAI digestion of the PCR product gives rise to 100 and 25 bp fragments for the *Thr* (ACG) allele and a single 125 bp fragment for the *Met* (ATG) allele. Digested fragments were subjected to electrophoresis on 3% agarose gel and visualized under UV light.

**Table I.** Characteristics of colorectal cancer patients and healthy controls

| | Patients ($n$ = 102) | Controls ($n$ = 110) |
|---|---|---|
| Gender[a] | | |
| Male | 62 (60.8%) | 79 (71.8%) |
| Female | 40 (39.2%) | 31 (28.2%) |
| Age (years ± SD) | 64.5 ± 11.5 | 66.4 ± 8.28 |
| Smoking status | | |
| Never | 44 (43.1%) | 26 (23.6%) |
| Current | 55 (53.9%) | 84 (76.4%) |
| <20 pack-years | 14 (25.5%) | 14 (16.7%) |
| ≥20 pack-years[b] | 41 (74.5%) | 70 (83.3%) |
| Unknown | 3 (2.9%) | 0 (0.0%) |

[a]$P > 0.05$ for differences between patients and controls.
[b]$P = 0.001$ compared with controls.

### *Genotyping at P757L*

Genotyping at P757L was also conducted by PCR-RFLP. PCR was performed with forward primer 5′-CAG AAT GGT CTT AAA ATG GGT GT and reverse primer 5′-TTC AGA ATA AGA AAC AAG GCA AC. Amplification conditions were 3 min initial denaturation at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 56°C and 30 s at 72°C, with a final extension of 7 min at 72°C. The 255 bp PCR products were digested overnight at 37°C with MnlI (New England Biolabs). MnlI digestion gives 153 and 102 bp fragments for the *Pro* (CCT) allele and a single 255 bp fragment for the *Leu* (CTT) allele.

### *Analysis of MSI*

Tumor and normal DNA was analyzed for MSI using the NCI panel of five microsatellite markers: *BAT25*, *BAT26*, *D5S346*, *D2S123* and *D17S250*. Oligonucleotide forward primers were fluorescently 5′-labeled. PCR was performed in a 20 μl reaction mixture with 20 ng genomic DNA, 2.5 mM each dNTP, 1× PCR buffer, 4 pmol each primer and 0.5 U rTaq DNA polymerase (Takara, Kyoto, Japan). Each primer sequence was as follows; *BAT25*, forward primer 5′-TCG CCT CCA AGA ATG TAA GT, reverse primer 5′-TCT GCA TTT TAA CTA TGG CTC; *BAT26*, forward primer 5′-TGA CTA CTT TTG ACT TCA GCC, reverse primer 5′-AAC CAT TCA ACA TTT TTA ACC C; *D5S346*, forward primer 5′-ACT CAC TCT AGT GAT AAA TCG G, reverse primer 5′-GTT TCC ATT GTA GCA TCT TGA C; *D2S123*, forward primer 5′-ACA TTG CTG GAA GTT CTG GC, reverse primer 5′-CCT TTC TGA CTT GGA TAC CA; *D17S250*, forward primer 5′-GCT GGC CAT ATA TAT ATT TAA ACC, reverse primer 5′-GGA AGA ATC AAA TAG ACA AT. Each amplification condition was as follows: *BAT25*, *BAT26* and *D2S123*, 3 min initial denaturation at 94°C, followed by 28 cycles of 30 s at 94°C, 1 min at 55°C and 1 min at 72°C, with a final extension of 7 min at 72°C; *D5S346* and *D17S250*, 3 min initial denaturation at 94°C, followed by 28 cycles of 30 s at 94°C, 1 min at 58°C and 1 min at 72°C, with a final extension of 7 min at 72°C. Then MSI was analyzed in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) using GeneScan Analysis 3.7 software (Applied Biosystems). MSI was indicated by the presence of novel peaks in the tumor tissue that were not seen in normal control tissue from the same patient or by a difference in microsatellite lengths between the two samples. Tumors exhibiting MSI at two or more markers were defined as MSI high (MSI-H). Tumors showing instability at only one marker were defined as MSI low (MSI-L). Tumors in which no markers exhibited MSI were considered to be microsatellite stable (MSS).

### *Statistical analysis*

All statistical analyses in this study were performed using SPSS software version 12.0 (SPSS Inc., Japan). Odds ratio (OR) and 95% confidence interval (CI) were adjusted for age, gender and smoking status using an unconditional logistic regression model. Accordance with the Hardy–Weinberg equilibrium was examined for colorectal cancer patients and controls using the $\chi^2$ test. In colorectal cancer patients the correlation between the genotype and clinicopathological characteristics was examined by $\chi^2$ and Fisher's exact probability tests. Values of $P < 0.05$ were considered significant.

## Results

### *Risk of colorectal cancer by genotype at T439M and P757L in the EXO1 gene*

Figure 1 shows the representative PCR-RFLP patterns of the T439M and P757L genotypes in the *EXO1* gene. Digestion of the PCR product (125 bp) with BsaAI at T439M resulted in a single fragment of 125 bp for the *Met* allele and two fragments of 100 and 25 bp for the *Thr* allele. Digestion of the PCR product (255 bp) with MnlI at P757L resulted in a single fragment of 255 bp for the *Leu* allele and two fragments of 153 and 102 bp for the *Pro* allele.

*EXO1* genotypes in colorectal cancer patients and healthy controls are shown in Table II. For T439M, the *Thr/Thr*, *Thr/Met* and *Met/Met* genotypes were found in 63 (63.6%), 30 (30.3%) and 6 (6.1%) of 102 colorectal cancer patients (the genotypes of three patients could not be identified because of unsuccessful PCR) and in 88 (80.0%), 22 (20.0%) and 0 (0%) of 110 controls, neither of which deviated from those expected

from the Hardy-Weinberg equilibrium ($P = 0.649$ and 0.507, respectively). *Met* allele frequencies were 21.2 and 10.0% in patients and controls, respectively ($P = 0.00148$). For P757L, the genotypes *Pro/Pro*, *Pro/Leu* and *Leu/Leu* were found in 35 (34.3%), 53 (52.0%) and 14 (13.7%) of 102 colorectal cancer patients and in 36 (32.7%), 47 (42.7%) and 27 (24.5%) of 110 controls. The distributions of genotypes at P757L in patients and controls also fitted the Hardy-Weinberg equilibrium ($P = 0.690$ and 0.342, respectively). *Leu* allele frequencies did not show a significant difference ($P = 0.197$) between patients and controls (39.7 and 45.9%, respectively). For T439M, the *Thr/Met* genotype (OR = 2.03, 95% CI 1.04-3.98) and *Thr/Met* and *Met/Met* genotypes combined (OR = 2.37, 95% CI 1.23-4.56) demonstrated a significant OR after adjusting for age, gender and smoking status when the *Thr/Thr* genotype (wild-type) was defined as the reference. An OR for the *Met/Met* genotype could not be calculated. For P757L, none of the *Pro/Leu* genotypes (OR = 1.17, 95% CI 0.610-2.24), *Leu/Leu* genotypes (OR = 0.436, 95% CI 0.183-1.04) or *Pro/Leu* and *Leu/Leu* genotypes combined (OR = 0.893, 95% CI 0.485-1.65) demonstrated a significant OR when the *Pro/Pro* genotype (wild-type) was defined as the reference. However, when the *Pro/Leu* and *Pro/Pro* genotypes were combined and used as the reference, the patients with the *Leu/Leu* genotype showed a reduced risk of colorectal cancer (adjusted OR = 0.398, 95% CI 0.183-0.866). The *Leu/Leu* genotype also had a reduced risk (adjusted OR = 0.373, 95% CI 0.164-0.850) when the *Pro/Leu* genotype was defined as the reference. Therefore, the relative risk of developing colorectal cancer did not show an allele-dose relationship for P757L.

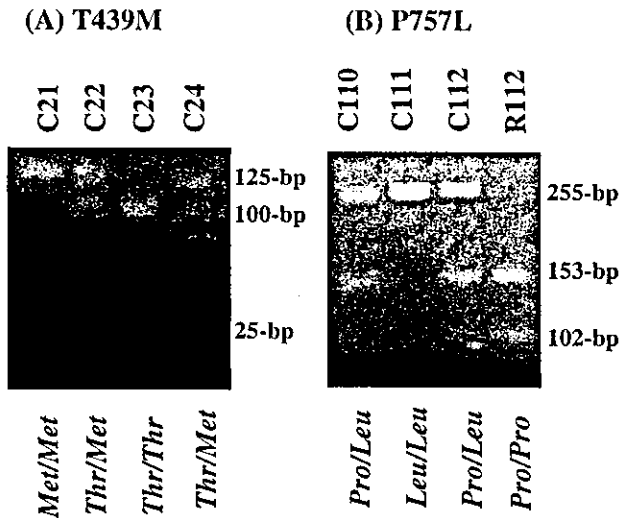


Fig. 1. Polymorphisms of *EXO1* exon 10 (T439M) and exon 13 (P757L). (A) T439M locus. (B) P757L locus. Numbers above the panel are case numbers. Genotypes are shown below each panel.

Table II. *EXO1* genotypes in colorectal cancer patients and healthy controls

| Amino acid | Genotype | Patients[a] ($n = 102$) | Controls[a] ($n = 110$) | $P^b$ | OR (95% CI) | |
|---|---|---|---|---|---|---|
| | | | | | Crude | Adjusted[c] |
| T439M | *Thr/Thr* | 63 (63.6%) | 88 (80.0%) | | 1 (Reference) | 1 (Reference) |
| | *Thr/Met* | 30 (30.3%) | 22 (20.0%) | 0.038 | 1.91 (1.01-3.61) | 2.03 (1.04-3.98) |
| | *Met/Met* | 6 (6.1%) | 0 (0%) | | Not calculated | Not calculated |
| | *Thr/Met* and *Met/Met* | 36 (36.4%) | 22 (20.0%) | 0.010 | 2.29 (1.23-4.25) | 2.37 (1.23-4.56) |
| | Unknown | 3 | 0 | | | |
| | Allele frequencies | | | | | |
| | *Thr* | 156 (78.8%) | 198 (90.0%) | 0.00148 | | |
| | *Met* | 42 (21.2%) | 22 (10.0%) | | | |
| P757L | *Pro/Leu* and *Pro/Pro* | 88 (86.3%) | 83 (75.5%) | | 1 (Reference) | 1 (Reference) |
| | *Leu/Leu* | 14 (13.7%) | 27 (24.5%) | 0.020 | 0.489 (0.240-0.996) | 0.398 (0.183-0.866) |
| | *Pro/Leu* | 53 (52.0%) | 47 (42.7%) | | 1 (Reference) | 1 (Reference) |
| | *Leu/Leu* | 14 (13.7%) | 27 (24.5%) | 0.019 | 0.460 (0.216-0.979) | 0.373 (0.164-0.850) |
| | Unknown | 0 | 0 | | | |
| | Allele frequencies | | | | | |
| | *Pro* | 123 (60.3%) | 119 (54.1%) | 0.197 | | |
| | *Leu* | 81 (39.7%) | 101 (45.9%) | | | |

[a]The observed genotype distributions of patients and controls were in agreement with the Hardy-Weinberg equilibrium.
[b]*P* values were for the difference in genotype frequencies between patients and controls.
[c]ORs were adjusted for age, gender and smoking status. Three patients whose smoking status was not known were omitted when adjusted ORs were calculated.

Table III. Joint effect of T439M and P757L and colorectal cancer risk

| T439M | P757L | Patients | Controls | $P^a$ | OR (95% CI) | | MSI (+)/cases |
|---|---|---|---|---|---|---|---|
| | | | | | Crude | Adjusted[b] | |
| *Thr/Thr* | *Leu/Leu* | 12 | 24 | | 1 (Reference) | 1 (Reference) | 1/12 (8.3%) |
| *Thr/Met* and *Met/Met* | *Leu/Leu* | 2 | 3 | 0.277 | 1.33 (0.196-9.08) | 0.168 (0.007-4.19) | 0/2 (0.0%) |
| *Thr/Thr* | *Pro/Leu* | 32 | 37 | 0.161 | 1.73 (0.747-4.00) | 1.88 (0.778-4.55) | 5/32 (15.6%) |
| *Thr/Met* and *Met/Met* | *Pro/Leu* | 21 | 10 | 0.007 | 4.20 (1.51-11.7) | 4.95 (1.56-15.7) | 4/21 (19.0%) |

[a]*P* values were for the difference in genotype frequencies between patients and controls.
[b]ORs were adjusted for age, gender and smoking status. Three patients whose smoking status was not known were omitted when adjusted ORs were calculated.

Table IV. Association between *EXO1* genotype and clinocopathological characteristics

| Characteristic | Factor[a] (+) ($n$ = 21) | Factor[a] (−) ($n$ = 78) | $P$ value |
|---|---|---|---|
| Histology | | | 0.912 |
| Well | 4 | 13 | |
| Moderate | 15 | 59 | |
| Poor | 1 | 2 | |
| Other | 1 | 4 | |
| T | | | 0.272 |
| Tis, T1, T2 | 1 | 12 | |
| T3, T4 | 20 | 66 | |
| N | | | 0.795 |
| N0 | 10 | 35 | |
| N1 | 6 | 29 | |
| N2 | 5 | 12 | |
| Unknown | 0 | 2 | |
| M | | | 0.180 |
| M0 | 13 | 54 | |
| M1 | 8 | 24 | |
| TNM stage | | | 0.260 |
| 0, I, II | 9 | 32 | |
| III, IV | 12 | 46 | |

T, primary tumor; N, lymph node metastasis; M, distant metastasis.
[a]Factor = *Thr/Met* and *Met/Met* (T439M) and *Pro/Leu* (P757L).

We also analyzed the joint effect of polymorphisms at T439M and P757L (Table III). There was no linkage disequilibrium between T439M and P757L polymorphisms. We considered carriers of the genotypes found to be at the lowest risk of disease (*Thr/Thr* for T439M and *Leu/Leu* for P757L) as the reference for this analysis. As shown in Table III, individuals who carried only one of the two polymorphisms associated with colorectal cancer risk (i.e. *Thr/Met* and *Met/Met* for T439M or *Pro/Leu* for P757L) were not at significant risk for development of colorectal cancer, whereas individuals who carried both putative risk genotypes showed an adjusted OR of 4.95 (95% CI 1.56–15.7). Adjustment for age, gender and smoking status was not significantly effective in all these estimates.

### *Association between EXO1 genotypes and MSI status*

Among tumors from the 102 colorectal cancer patients analyzed, 6 (5.9%) were MSI-H, 11 (10.8%) were MSI-L and the remaining 85 were MSS. We evaluated association between *EXO1* SNPs and MSI status (Table III). As shown in Table III, individuals who carried both putative risk genotypes tended to have a higher frequency of MSI-positive (MSI-H and MSI-L) tumors than those who carried both low risk genotypes, although no significant correlation was found between *EXO1* genotype and MSI status ($P$ = 0.422).

### *Association between EXO1 genotype and clinicopathological characteristics*

We analyzed the association between the *EXO1* genotype and clinicopathological characteristics in colorectal cancer patients (Table IV). We compared the patients with *Thr/Met* and *Met/Met* for T439M and *Pro/Leu* for P757L with the other patients. However, there were no significant differences between these two groups.

## Discussion

The *EXO1* gene has been investigated in association with HNPCC (14–17,27). Wu *et al.* (15) found several *EXO1* mutants in HNPCC patients who were shown to be negative for germline mutations in the *MLH1*, *MSH2* and *MSH6* genes. Among a total of 30 alterations in the *EXO1* exons, 14 were unique to HNPCC patients. However, the other 16 were thought to be polymorphisms because they occurred at similar frequencies in both patients and controls. Thompson *et al.* (17) reported that the *EXO1* gene does not appear to be associated with HNPCC, but did not refer to these polymorphisms. Therefore, we investigated T439M and P757L among these polymorphisms.

In the present study we have examined whether SNPs at T439M and P757L in the *EXO1* gene are associated with the risk for development, progression and metastasis of colorectal cancer. We found significant differences in genotype distribution of the *EXO1* gene between colorectal cancer patients and controls. We recognize that this population of colorectal cancer patients does not seriously deviate from the general Japanese population because Japan is an almost racially homogeneous nation and Okayama has had population influxes from other areas such as Tokyo and Osaka (urban cities representing Japan) and the Chugoku and Shikoku Districts (around Okayama). For T439M of *EXO1*, an OR of 2.03 was observed among individuals with the *Thr/Met* genotype and an OR of 2.37 was observed among individuals with the *Thr/Met* and *Met/Met* genotypes combined. For P757L of *EXO1*, patients with the *Leu/Leu* genotype had a reduced risk of colorectal cancer when the *Pro/Leu* and *Pro/Pro* genotypes combined or *Pro/Leu* genotype was defined as the reference (OR = 0.398 and 0.373, respectively). Interestingly, individuals with putative risk genotypes for both T439M (*Thr/Met* and *Met/Met*) and P757L (*Pro/Leu*) were at a 5-fold higher risk of colorectal cancer. As for an association between *EXO1* SNPs and MSI, individuals who carried both putative risk genotypes tended to have higher frequency of MSI-positive tumors than those who carried both low risk genotypes, although no significant correlation was found. This fact may support the proposal that high risk genotypes of the *EXO1* gene cause colorectal cancer through incomplete MMR. Our findings are the first to suggest an association between polymorphisms in the *EXO1* gene and risk of colorectal cancer. No other reports have found an association between other cancers and *EXO1* SNPs. Although intriguing, however, these joint effects should be interpreted with caution, given the modest size of the present study to evaluate joint effects. The *EXO1* genotype was not significantly associated with clinicopathological characteristics. Therefore, the *EXO1* gene does not appear to be associated with progression or metastasis of colorectal cancer.

As functional motifs of EXO1 protein, an EXO1 nuclease domain was identified in the N-terminal 391 amino acid residues (27) and a protein–protein interaction domain with MMR proteins was identified on the basis of sequence alignment and functional domain analysis (2,9–12,28) (Figure 2). T439M is located in the MLH1 interaction domain and P757L is located within the region required for interaction with MSH2. It is known that mutations of MSH2 and MLH1 are associated with typical HNPCC, resulting in a total loss of MMR function (29). In the case of T439M, a polar amino acid (Thr) is replaced by a non-polar amino acid (Met). Considering that the genotype *Met/Met* were not found in the control subjects in T439M, it is possible that substitution of *Thr* by *Met* may strongly affect interaction with other MMR proteins. As for P757L, this substitution may influence protein–protein interaction because

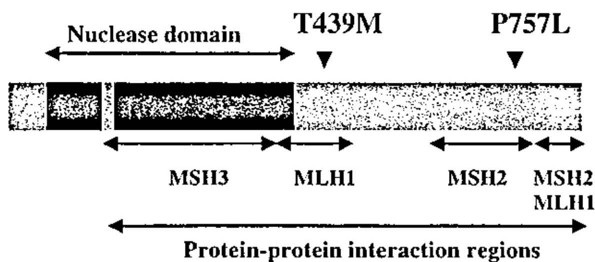


Fig. 2. Functional motifs of *EXO1* and the locations of polymorphisms. Nuclease domains and protein-protein interaction regions are shown by arrows. SNPs examined in the present study (T439M and P757L) are indicated above the motif by inverted triangles.

prolines tend to destabilize (kink) α-helices due to the lack of a backbone hydrogen bond and steric constraints (30–32). It is notable that the MMR proteins MSH2, MLH1 and MSH6 are constituents of BASC, the BRCA1-associated genome surveillance complex (33). If EXO1 is also involved in this complex through MMR proteins, the protein may play a more general role(s) in the protection of DNA in addition to MMR.

In conclusion, our data provide evidence for an association between the *EXO1* gene polymorphisms at T439M and P757L and the risk of development of colorectal cancer. It is possible that these polymorphisms may influence susceptibility to colorectal cancer through incomplete DNA repair. The association was more prominent for individuals who carried both of the two putative risk genotypes. Further study with sufficiently larger populations and functional analysis of these polymorphisms will be needed to clarify the unsolved issues.

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# Relationship between *CDX2* gene methylation and dietary factors in gastric cancer patients

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**Epigenetic gene silencing through DNA methylation is one of the important steps in the mechanism underlying tumorigenesis, including in the stomach. Past lifestyle factors of cancer patients, such as intake of vegetables, are very important in affecting gastric carcinogenesis. However, the relationship between DNA methylation and past dietary habits in cancer patients remains largely unknown. The CDX2 homeobox transcription factor plays a key role in intestinal development, but CDX2 is also expressed in most of the intestinal metaplasia and part of the carcinomas of the stomach. We analyzed the methylation status of the *CDX2* 5′ CpG island in gastric cancer cell lines by methylation-specific PCR (MSP), and then *CDX2* mRNA was found to be activated after 5-aza-2′-deoxycytidine treatment of the methylation-positive cells. We further examined the methylation status of *CDX2* in primary gastric carcinomas by MSP and compared it with the past lifestyle of the patients, including dietary habits. Methylation of *CDX2* was found in 20 (34.5%) of the 58 male patients and one (6.7%) of the 15 female patients. Since the methylation frequency was low in the female patients, the analysis was performed only on the male cases. *CDX2* methylation was correlated with the decreased intake of green tea and cruciferous vegetables, and also with full or overeating habits. These findings are consistent with epidemiological observations on gastric cancer. We also analyzed the methylation status of *p16/INK4a* and *hMLH1*, but their frequencies were not associated with dietary factors or other lifestyle factors. Thus, diet could be an important factor determining the methylation status of genes such as *CDX2* and the resultant aberrant expression of genes involved in carcinogenesis.**

Abbreviations: MSP, methylation-specific PCR; RT-PCR, reverse transcription-polymerase chain reaction.

## Introduction

Gastric cancer is the second most frequent cause of death from cancer in both sexes in the world (1). The precise mechanism underlying gastric carcinogenesis is not fully understood yet. However, several environmental factors, such as *Helicobacter pylori* infection, excessive intake of salt and low intake of vegetables and fruits, have been linked with gastric carcinogenesis (2–4).

Since alterations of gene functions in cancer cannot be explained by only the mutational rate, there should be a non-structural mechanism. Some cancers show hypermethylation of CpG islands in gene promoters, resulting in loss of gene function. Patterns of DNA methylation can be inherited when cells divide. This epigenetic process, as an alternative to mutations, inhibits tumor suppressor gene function (5,6).

Dietary factors are important determinants of cancer risk, including that of gastric cancer (2). Certain dietary factors and other lifestyle factors are associated with variations in DNA methylation, and these variations might underlie gastric carcinogenesis. For example, the incidence of hypermethylation of *p16/INK4a* (hereafter *p16*) in lung cancer is significantly higher in cigarette smokers than in those who have never smoked (7). Consequently, *p16* expression is silenced, resulting in the progression of carcinogenesis. The prevalence of promoter hypermethylation of six genes, such as *APC*, $p14^{ARF}$, *p16* and *hMLH1*, was higher in colorectal cancers derived from patients with a low folate/high alcohol intake than in ones with a high folate/low alcohol intake, but the differences were not statistically significant (8).

Several genes are aberrantly methylated in human primary cancers, including that of the stomach (9). *p16* and *hMLH1* have been extensively examined in gastric cancer (10–12). *p16* inhibits $G_1$ cyclin-dependent kinases and hence induces cell-cycle arrest, and has a tumor-suppressor gene function (13,14). Epigenetic inactivation of *hMLH1* due to promoter methylation is strongly associated with microsatellite instability and seems to be a significant event in the development of gastric cancer (10–12).

Human *CDX2* is a member of the *caudal*-related homeobox gene family (15,16). The expression of the rodent *Cdx2* homeobox gene is intestine-specific, and occurs from the early embryo to the adult stage (17), and thus it is likely that Cdx2 plays roles in both the establishment and maintenance of the intestinal epithelial phenotype. On the other hand, the ectopic expression of CDX2 has been related to intestinal metaplasia formation in the stomach. First, the CDX2 protein is not expressed in the normal stomach, but is highly expressed in nearly all of the intestinal metaplasia of the stomach (18–20). Secondly, when Cdx2 expression was directed to the gastric mucosae in transgenic mice using *cis*-regulatory elements of gastric mucosa-specific genes, ectopic Cdx2 expression induced gastric intestinal metaplasia in the mice (21,22). Gastric cancer is histologically classified into two

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main types, intestinal and diffuse (23). Intestinal type gastric cancers are thought to develop from intestinal metaplasia, while diffuse type ones may mainly develop from the normal mucosae (16). CDX2 expression is stronger in intestinal type than in diffuse type gastric cancers (18).

We analyzed here the methylation status of *p16* and *hMLH1* in gastric cancers. Furthermore, since CDX2 expression is lower in gastric cancers than in intestinal metaplasia (18), we also analyzed methylation of the *CDX2* 5′ CpG island, and compared them with the dietary habits, specifically those which have previously been reported as risk or preventive factors of gastric cancer in epidemiological observation.

## Materials and methods

### *Cell culture and drug treatment*

Human gastric cancer cell lines GT3TKB and MKN74 were grown in Dulbecco's modified minimum essential medium and RPMI1640 medium, respectively, supplemented with 10% fetal bovine serum. A human colon cancer cell line, RKO, was cultured in Eagle's minimum essential medium containing 10% fetal bovine serum. For demethylation studies, cells were treated daily with 5 μM 5-aza-2′-deoxycytidine (Sigma, St Louis, MO) for 3 days.

### *RNA extraction and reverse transcription–polymerase chain reaction (RT–PCR)*

Total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA). To synthesize the RT–PCR template, we used 2 μg of total RNA and reverse-transcribed it using a Superscript kit (Invitrogen). The primers used for *CDX2* amplification were described previously (24). We amplified with multiple cycle numbers (28–35 cycles) to obtain semi-quantitative differences in the expression level. As an internal control for RT–PCR analysis, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) transcripts were amplified for 19 cycles from the same cDNA samples (24).

### *Methylation analyses by the methylation-specific PCR (MSP) procedure and bisulfite DNA sequencing*

We extracted genomic DNA from cultured cells or paraffin-embedded tissues by the phenol–chloroform method, and then carried out bisulfite modification and the MSP procedure as described previously (25). The primer sequences of *CDX2* for the unmethylated reaction were 5′-GAAGTTGTTGGTTTGGGGT-TTTGTAT-3′ (sense) and 5′-CCCACAATACTCCACTAACTCCTCACA-3′ (antisense), and for the methylated reaction 5′-CGTCGGTTTGGGGTTTC-GTAC-3′ (sense) and 5′-GATACTCCGCTAACTCCTCGCG-3′ (antisense), according to the GenBank sequence (AL591024). The PCR reaction for *CDX2* was performed for 35 cycles in a 25-μl mixture containing bisulfite-modified DNA (~50 ng), 2.5 μl of 10× PCR buffer, 1.25 μl of 25 mM dNTP, 10 pmol of each primer and 1 U of JumpStart Red *Taq* polymerase (Sigma). Each PCR cycle consisted of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s, followed by final extension at 72°C for 5 min. The MSP primers and conditions used for *p16* and *hMLH1* were described previously (25,26). The PCR products were electrophoresed in 2.5% agarose gels. All the MSP procedures were repeated more than twice.

Bisulfite DNA sequencing of the *CDX2* 5′ CpG island was performed as described previously (27). The primer sequences for amplification were 5′-GAAGTTTTTAATTATTGGTGTTTGTGTT-3′ (sense) and 5′-AAACCT-CACCATACTACCTAAAAACC-3′ (antisense).

### *Immunohistochemistry*

Immunohistochemical analysis of the CDX2 protein was performed as described previously (18). Monoclonal antibody to the CDX2 protein (Bio Genex, San Ramon, CA) was diluted at 1:100.

### *Study population*

Cancer tissue specimens were collected from 73 consecutive patients with primary gastric carcinoma in an affiliated hospital of the Tokyo Medical and Dental University, and Saitama Cancer Center Hospital during 2000–2002. Informed consent was obtained from all patients, and the study was approved by the appropriate institutional review committee. A self-administered questionnaire was used in this study to assess their lifestyle before cancer onset, covering disease history, familial history of cancer, medication, cigarette smoking, alcohol consumption, physical activity, intake frequencies of selected food groups and food items, daily consumption of tea (green tea, oolong tea and black tea), regularity of sleep and meals, eating quantity, bowel motion, height and body weight. Food groups were beef, pork, chicken, ham/sausage/bacon, grilled meat, all meat, grilled fish, salted/dried/other processed fish products, pickled vegetables, green leaf vegetables, yellow color vegetables, cruciferous vegetables, all vegetables, fruits and probiotics-fermented milk. Intake frequencies of these food groups were categorized into not eating, 1–2 times/month, 1–2 times/week, 3–4 times/week, almost every day and almost every meal. Eating quantity was categorized as full or over-eating, eating in moderation and consciously under-eating. Most lifestyle factors in this questionnaire were selected from those which have previously been reported as risk or preventive factors of gastric and colon cancers in epidemiological observation.

Tumors were reviewed by a pathologist and microdissected prior to DNA extraction. Histological classification was performed according to the general rules established by the Japanese Gastric Cancer Association (28) and the Laurén's classification (23).

### *Statistical analysis*

The promoter methylation status of specific genes, clinicopathological parameters and lifestyle variables in the patients were computed. Differences in frequency by methylation status were tested using Fisher's exact test, and differences in mean values were tested using *t* test. Association between the methylation status and dietary variables was also analyzed by non-parametric test (Mann–Whitney *U* test). We further studied association by categorical regression analysis with optimal scaling using alternating least squares (29). In this analysis, intake frequencies of food groups and eating quantity were dichotomous: ≤twice/week versus ≥3 times/week for vegetable groups, ≤twice/month versus ≥once/week for meat and fish groups, ≤4 times/week versus ≥5 times/week for fruits and ≤6 cups/day versus ≥7 cups/day for green tea and full/over versus moderate/light for eating quantity. The statistical software used was SPSS software (version 11.0).

## Results

### *Epigenetic silencing of CDX2 in a gastric cancer cell line*

With a semi-quantitative RT–PCR assay to measure the *CDX2* mRNA level, GT3TKB did not express any *CDX2* mRNA, whereas MKN74 expressed it abundantly (Figure 1A). We then used the demethylating agent 5-aza-2′-deoxycytidine to study the epigenetic status of *CDX2* in these cell lines. *CDX2* was re-expressed in GT3TKB cells with this treatment (Figure 1A).

We have identified CpG islands associated with the *CDX2 5′* region (Figure 1B) and thus the methylation status of *CDX2* was studied by MSP and sodium bisulfite DNA sequencing. GT3TKB cells only exhibit a methylation signal, but MKN74 cells do not exhibit any (Figure 1C), consistent with the *CDX2* expression levels in these cells. The sodium bisulfite DNA sequencing of *CDX2* in these cell lines confirmed the methylation status, i.e. *CDX2* expression-negative GT3TKB cells show densely methylated clones, while expression-positive MKN74 cells and normal gastric mucosa have unmethylated clones (Figure 1D).

### *Methylation status of CDX2, p16 and hMLH1 in primary gastric carcinomas*

Methylation of *CDX2*, as determined by MSP, was frequent in primary gastric carcinomas, i.e. there were 21 positive cases among 73 (28.8%) total cases. Representative examples of gel analysis of MSP are shown in Figure 2. CDX2 protein expression was also analyzed in gastric carcinomas by immunohistochemistry (Figure 3 and Table I). As shown in Table I, negative or partial CDX2 expression was more frequently observed in diffuse type (28/32, 87.5%) than in intestinal type (20/35, 57.1%) gastric cancers ($P < 0.01$), which is consistent with the previous data (18). The *CDX2* methylation frequencies in gastric cancers with negative or partial expression were similar between the intestinal (40%) and diffuse types (35.7%). On the other hand, the overall methylation frequency in cancers with negative or partial CDX2 expression

**Fig. 1.** *CDX2* expression and methylation status in gastric cancer cell lines. (A) The *CDX2* expression level was examined by RT-PCR in two cell lines with (lanes +) or without (lanes −) treatment with 5-aza-2′-deoxycytidine. *GAPDH* expression was used as an internal loading control for the RT-PCR and $H_2O$ (no cDNA added). (B) Schematic representation of the *CDX2* gene promoter region. A box indicates exon 1, including coding (black) and non-coding (white) regions. Vertical bars show CpG sites. Arrows indicate the regions analyzed by MSP and bisulfite sequencing (BS). (C) MSP analysis of the CpG island of *CDX2* in two gastric cancer cell lines and peripheral blood lymphocytes (lane PBL). PCR products recognizing unmethylated (lanes U) and methylated (lanes M) CpG sites were analyzed in 2.5% agarose gels and stained with ethidium bromide. (D) Sodium bisulfite DNA sequencing of the *CDX2* CpG island in gastric cancer cell lines and normal stomach mucosa. Each horizontal row of squares represents analysis, in a single clone of bisulfite-treated DNA, of 24 CpG sites contained in the region shown. Solid and open squares represent methylated and unmethylated CpG sites, respectively.

(18/48, 37.5%) was significantly higher than that with positive expression (2/19, 10.5%) ($P = 0.03$), suggesting that methylation of the *CDX2* gene is important for gene silencing in primary gastric cancers. When we analyzed the *CDX2* methylation status by MSP in five normal and five intestinal metaplastic tissues of the stomach from independent patients, we found no methylation in any samples (Table I). Since the normal gastric mucosae did not reveal *CDX2* gene methylation, strong CDX2 expression in intestinal metaplasia compared with the normal mucosae is not attributable to demethylation but possibly to aberrant transcriptional activation.

The methylation status of the *p16* and *hMLH1* genes was also examined by MSP (Figure 2). Nine of 66 (13.6%) and seven of 67 (10.4%) gastric cancers exhibited MSP methylation signals, respectively.

**Fig. 2.** Representative examples of MSP analyses of *CDX2*, *p16* and *hMLH1* in primary gastric cancer tissues (Ca), normal gastric mucosae (N), and peripheral blood lymphocytes (PBL). A colorectal cancer cell line, RKO, was used as a methylation-positive control. Lanes: U, unmethylated alleles; M, methylated alleles; $H_2O$, no DNA added.

**Fig. 3.** Immunohistochemical staining of CDX2 in representative cancers. (A) A methylation-negative intestinal type gastric carcinoma showed nuclear CDX2 staining. (B) A diffuse type gastric carcinoma did not express the CDX2 protein, while adjacent intestinal metaplastic cells (right side) expressed CDX2. The microdissected cancer portion (Ca2) of this sample exhibited *CDX2* gene methylation (Figure 2). Original magnification ×200.

**Table I.** Protein expression and methylation status of the *CDX2* gene in gastric lesions

| Carcinoma | | | | | | Intestinal metaplasia | | Normal mucosa | |
|---|---|---|---|---|---|---|---|---|---|
| Histological type | Total | − or partial expression | | + expression | | Expression | Methylation | Expression | Methylation |
| | | No. | Methylated (%)[a] | No. | Methylated (%)[a] | | | | |
| Intestinal | 35 | 20[b] | 8 (40) | 15 | 1 (6.7) | Positive[c] | 0/5[d] | Negative[c] | 0/5[d] |
| Diffuse | 32 | 28[b] | 10 (35.7) | 4 | 1 (25) | | | | |
| Total | 67[e] | 48 | 18 (37.5) | 19 | 2 (10.5) | | | | |

[a]% of no. of methylated cases/no. of cases.
[b]The frequencies of cancers with negative or partial CDX2 expression were significantly higher in the diffuse type (28/32, 87.5%) than the intestinal type (20/35, 57.1%) ($P < 0.01$).
[c]Combined our data with references 18–20.
[d]No. of methylated/no. of cases examined.
[e]Of 73 cases examined for methylation, six cases were not informative for CDX2 expression.

**Table II.** Clinicopathological characteristics of study patients according to the methylation status of *CDX2*, *p16* and *hMLH1*

| | *CDX2* ($n$ = 73) | | | *p16* ($n$ = 66) | | | *hMLH1* ($n$ = 67) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Methylated ($n$ = 21) | Unmethylated ($n$ = 52) | $P$ value | Methylated ($n$ = 9) | Unmethylated ($n$ = 57) | $P$ value | Methylated ($n$ = 7) | Unmethylated ($n$ = 60) | $P$ value |
| Age (mean ± SD) | 66.6 ± 10.5 | 64.8 ± 9.6 | 0.76 | 59.1 ± 10.8 | 66.1 ± 9.2 | 0.04 | 69.8 ± 7.9 | 64.6 ± 10.1 | 0.20 |
| Sex | | | | | | | | | |
| Male | 20 | 38 | | 8 | 45 | | 5 | 50 | |
| Female | 1 | 14 | 0.03 | 1 | 12 | 0.43 | 2 | 10 | 0.37 |
| Size (cm, mean ± SD) | 5.7 ± 4.1 | 5.5 ± 3.5 | 0.85 | 8.1 ± 3.9 | 5.4 ± 3.6 | 0.04 | 6.2 ± 5.1 | 5.6 ± 3.6 | 0.65 |
| Histology | | | | | | | | | |
| Intestinal | 9 | 27 | | 1 | 30 | | 3 | 29 | |
| Diffuse | 12 | 25 | 0.33 | 8 | 27 | 0.02 | 4 | 31 | 0.55 |
| Depth of tumor invasion | | | | | | | | | |
| m, sm | 11 | 24 | | 2 | 28 | | 4 | 28 | |
| mp ~ si | 10 | 28 | 0.41 | 7 | 29 | 0.13 | 3 | 32 | 0.45 |
| Lymph node metastasis | | | | | | | | | |
| + | 9 | 26 | | 7 | 27 | | 4 | 28 | |
| − | 12 | 26 | 0.39 | 2 | 30 | 0.09 | 3 | 32 | 0.45 |

*The relationship between methylation frequencies of CDX2, p16 and hMLH1 and clinicopathological parameters*

Clinicopathological characteristics of study patients by the methylation status of *CDX2*, *p16* and *hMLH1* are shown in Table II. The methylation of *CDX2* was significantly more frequent in males (20/58, 34.5%) than in females (1/15, 6.7%) ($P = 0.03$). *p16* methylation was more frequently found in younger patients and larger cancers ($P = 0.04$), and was more common in diffuse type than in intestinal type gastric carcinomas ($P = 0.02$). In contrast, there was no statistically significant correlation between *hMLH1* methylation and clinicopathological parameters (Table II).

*The relationship between methylation frequencies of CDX2, p16 and hMLH1, and epidemiological parameters in male patients*

Since *CDX2* methylation was found only in one female cancer case, the following epidemiological analyses were only performed for male patients. Methylation of *CDX2*, *p16* and *hMLH1* was found in 20/58 (34.5%), 8/53 (15.1%) and 5/55 (9.1%) male patients, respectively; those revealing methylation in any of these genes were 23/53 (43.4%). None of the epidemiological variables revealed statistical significance in relation to the methylation status of *CDX2*, *p16*, *hMLH1* and 'any of the three genes', except beef intake and *p16* methylation (Table III). Because the methylation frequency of *hMLH1* was low and was not associated with epidemiological variables, its data are not shown in Table III. Since dietary factors are closely interrelated, we further conducted categorical regression analyses of clinical and epidemiological variables, and methylation in male gastric cancer patients. A significant association was found between eating quantity or the intake of green tea and methylation of *CDX2*, and between eating quantity and methylation of 'any of the three genes' (Table III). Increased methylation frequency of *CDX2* was significantly associated with full or overeating habits, adjusting for confounding variables ($P = 0.02$). On the other hand, increased daily consumption of green tea (7 or more cups/day) showed a significant association with decreased methylation frequency of *CDX2* after adjustment ($P = 0.02$). These epidemiological factors also revealed a close association with methylation frequency of any of *CDX2*, *p16* and *hMLH1* genes ($P = 0.02$ and 0.06 for eating quantity and intake of green tea, respectively).

When we analyzed the association between the methylation status and dietary variables by non-parametric test, increased intake of cruciferous vegetables was significantly associated

**Table III.** Relationship between the methylation status and dietary factors in male patients

| | *CDX2* ($n$ = 58) | | | | | *p16* ($n$ = 53) | | | | | Any of *CDX2*, *p16* and *hMLH1* ($n$ = 53) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Methylated ($n$ = 20) | Unmethylated ($n$ = 38) | Univariate[a] *P* value | Multivariate[b] | | Methylated ($n$ = 8) | Unmethylated ($n$ = 45) | Univariate[a] *P* value | Multivariate[b] | | Methylated ($n$ = 23) | Unmethylated ($n$ = 30) | Univariate[a] *P* value | Multivariate[b] | |
| | | | | β[c] | *P* value | | | | β[c] | *P* value | | | | β[c] | *P* value |
| Eating quantity | | | | | | | | | | | | | | | |
| moderate/light | 10 | 25 | | | | 4 | 28 | | | | 11 | 21 | | | |
| full/over | 10 | 12 | 0.16 | −0.37 | 0.02 | 4 | 17 | 0.39 | 0.06 | 0.73 | 12 | 9 | 0.09 | −0.39 | 0.02 |
| Green tea | | | | | | | | | | | | | | | |
| ⩽6 cups/day | 17 | 26 | | | | 6 | 34 | | | | 19 | 21 | | | |
| ⩾7 cups/day | 2 | 12 | 0.08 | 0.31 | 0.02 | 2 | 10 | 0.60 | −0.01 | 0.95 | 3 | 9 | 0.15 | 0.28 | 0.06 |
| Cruciferous vegetables | | | | | | | | | | | | | | | |
| ⩽twice/week | 14 | 18 | | | | 4 | 27 | | | | 15 | 16 | | | |
| ⩾3 times/week | 6 | 20 | 0.09 | 0.25 | 0.09 | 4 | 18 | 0.44 | −0.20 | 0.23 | 8 | 14 | 0.28 | 0.15 | 0.34 |
| Fruits | | | | | | | | | | | | | | | |
| ⩽4 times/week | 13 | 15 | | | | 5 | 20 | | | | 13 | 12 | | | |
| ⩾5 times/week | 6 | 21 | 0.05 | 0.14 | 0.36 | 3 | 22 | 0.35 | 0.04 | 0.81 | 9 | 16 | 0.20 | 0.03 | 0.88 |
| Beef | | | | | | | | | | | | | | | |
| ⩽twice/month | 13 | 16 | | | | 7 | 21 | | | | 15 | 13 | | | |
| ⩾once/week | 6 | 22 | 0.06 | 0.01 | 0.93 | 1 | 23 | 0.04 | 0.29 | 0.10 | 7 | 17 | 0.07 | 0.02 | 0.93 |

[a]Used by Fisher's exact test.
[b]Adjusted for age, body mass index (BMI), histological classification, tumor size, smoking, alcohol drinking, eating quantity, and intake frequencies of green tea, cruciferous vegetables, fruits, and beef, using the categorical regression analyses.
[c]Regression coefficients in an optimal linear regression equation.

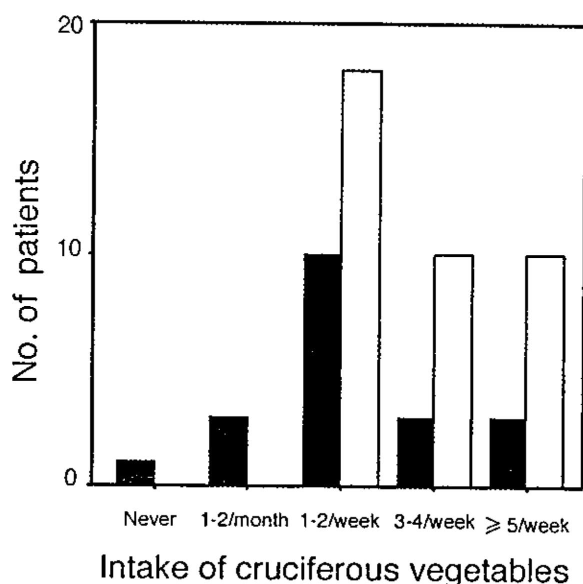


**Fig. 4.** Frequencies of the presence (closed bars) or absence (open bars) of *CDX2* methylation in gastric cancers stratified as to intake of cruciferous vegetables.

**Table IV.** *CDX2* methylation and combination of green tea and cruciferous vegetables

| | *CDX2* | | |
|---|---|---|---|
| | Methylated ($n$ = 19) | Unmethylated ($n$ = 38) | *P* value |
| Green tea ≤6 cups/day and cruciferous vegetables ≤twice/week | 12 (52.2%) | 11 (47.8%) | |
| Green tea ≥7 cups/day or cruciferous vegetables ≥3 times/week | 7 (20.6%) | 27 (79.4%) | 0.01 |

with decreased frequency of *CDX2* methylation ($P$ = 0.03). Distinct distribution of patients with the methylated and unmethylated *CDX2* is demonstrated for the intake of cruciferous vegetables (Figure 4).

Both green tea and cruciferous vegetables were inversely associated with *CDX2* methylation. Then we further analyzed the relationship between *CDX2* methylation and combination of these two factors. We found a stronger association in the combination (Table IV), i.e. the patients drinking more green tea or consuming cruciferous vegetables more often showed a lower methylation frequency than the patients drinking less green tea and consuming cruciferous vegetables less frequently.

## Discussion

We reported here that cultured gastric cancer cells exhibiting no CDX2 expression showed methylation of the *CDX2* CpG island, while cultured gastric cancer cells exhibiting high CDX2 expression did not show methylation. Moreover, treatment of cultured cells with the demethylating agent 5-aza-2′-deoxycytidine activated *CDX2* expression. The data indicate that *CDX2* expression is silenced on methylation of the CpG island associated with the *CDX2* gene promoter region.

*CDX2* gene methylation was found in 21 of the 73 (28.8%) primary gastric cancers. The methylation frequency in cancers with negative or partial CDX2 expression was significantly higher than that with positive expression, and five CDX2 expression-positive intestinal metaplastic tissues revealed no *CDX2* gene methylation as shown in Table I. Therefore, CDX2 expression may be silenced with gene methylation in primary gastric cancers, even though there may be also other unknown mechanisms. Because intestinal type gastric cancers are thought to develop through intestinal metaplasia (CDX2 expression-positive) (16,18–20), there may be more CDX2 expression-positive cancers in this type. On the contrary, many diffuse type gastric cancers may develop from the normal mucosa (CDX2 expression-negative) (16), and thus it is reasonable that there were more CDX2 expression-negative or -partial cancers in the diffuse type. The *CDX2* methylation frequencies were similar between the intestinal and diffuse type gastric cancers even in cancers with negative or partial expression. One reason might be that some gastric cancers develop as the intestinal type and then progress to the diffuse type with time (30).

The methylation frequency of *CDX2* did not show any significant relationship to pathologic characteristics, which may cause a bias in further analyses. We then analyzed the association between *CDX2* methylation and selected lifestyle factors known to be risky or preventive for gastric and colon cancers. The epidemiological analyses were carried out only on males, because *CDX2* methylation was found in only one female case. Univariate analysis revealed that none of the lifestyle variables was significantly associated with the methylation frequencies of *CDX2*, *p16*, *hMLH1* and any of the three genes except for beef intake and *p16*. However, multivariate analysis revealed significant differences between eating quantity and the methylation frequency of *CDX2* or any of the three genes, and also between the intake of green tea and *CDX2* methylation.

Full or overeating was associated with an increased frequency of methylation in *CDX2* and any of the three genes ($P$ = 0.02 and $P$ = 0.02, respectively). Although a few epidemiological studies have investigated eating quantity and gastric cancer, full eating is a consistent risk factor in the etiology of gastric cancer (31–33). One plausible interpretation may be that the gastric mucosa is physically damaged by repeated compulsory expansion of the gastric lining upon full eating, possibly resulting in an increased sensitivity of the cells to various exogenous compounds in food, which may include those promoting DNA methylation.

Among the 15 food groups and selected food items, an increased intake of green tea was independently and significantly associated with the *CDX2* methylation frequency ($P$ = 0.02) and methylation in any of *CDX2*, *p16* and *hMLH1* (borderline significance, $P$ = 0.06), after adjusting for confounding lifestyle and clinical variables. The effects of green tea seem to be dose-dependent: the *CDX2* methylation frequencies, 10/25 (40%), 7/18 (39%), 2/8 (25%), and 0/6 (0%) in three or less, four to six, seven to nine and ten cups or more a day, respectively.

Green tea contains several polyphenolic compounds, such as epigallocatechin gallate (EGCG). Significant inhibitory effects of EGCG or green tea extracts on carcinogenesis of rodents in various organs including the stomach have been demonstrated in many studies (34,35). Most epidemiological studies in Japan revealed cancer-preventive effects of drinking green tea

(36–39). Furthermore, it was reported recently that EGCG dose-dependently inhibited DNA methyltransferase activity in several cancer cells, resulting in the reactivation of methylation-silenced genes (*p16*, *retinoic acid receptor β* and *hMLH1*) (40). Taken together, our findings imply a novel mechanism of green tea in cancer prevention, i.e. inhibition of methylation of selected genes involved in gastric carcinogenesis.

Although we analyzed the association between dietary factors and DNA methylation using the categorical regression model, this analysis may overlook some factors due to a small number of study subjects. Therefore, we further reinvestigated the association by examining overall differences in the distribution of patients with methylated or unmethylated *CDX2* on the intake of food groups, using the non-parametric test, and found that the intake frequency of cruciferous vegetables in patients with unmethylated *CDX2* was distributed in higher categories than that in patients with methylated *CDX2* ($P = 0.03$, Figure 4). Cruciferous vegetables have been reported to be anticarcinogenic in a number of epidemiological and laboratory studies. Particularly, the active compounds in cruciferous vegetables, such as arylalkyl isothiocyanates (and their glucosinolate precursors) and indole-3-carbinol, have been extensively investigated, and the roles of isothiocyanates were presumed to suppress activating enzymes and induce detoxifying ones of carcinogens (41,42). There were several reports suggesting other cancer-preventive mechanisms of isothiocyanates, for example, dose-dependent inhibition of DNA methylation in nitrosomethylbenzylamine-induced esophageal tumorigenesis of rats (43), and inhibition of *Helicobacter pylori* and benzo[*a*]pyrene-induced stomach tumors in mice (44). Our findings provide evidence in humans supporting the cancer-preventive effects of cruciferous vegetables through the inhibition of DNA methylation.

We observed large differences in the methylation frequencies of genes examined, particularly *CDX2*, between male and female patients. Unexpectedly, gender comparison in dietary factors including eating quantity, and intake of green tea and cruciferous vegetables did not reveal any significant difference. However, male patients included more cases with advanced age at diagnosis (>60 years) than female ones ($P = 0.06$), and the intestinal type was more frequently found in male patients than in female ones ($P < 0.05$), implying a gender difference in the etiology of gastric cancer. These are in consistence with previous reports on gender difference in age at diagnosis and frequencies of gastric carcinoma and intestinal metaplasia (45,46). Thus, the gender difference in methylation frequency might be ascribed to gender-specific host factors, such as estrogens, not to lifestyle factors.

In this study, the methylation of *CDX2* and other genes involved in gastric carcinogenesis was investigated in relation to the clinicopathological and selected lifestyle factors of gastric cancer patients. We therein hypothesized that some of the lifestyle factors, particularly dietary ones, which have been reported to be risky or preventive for gastric cancer in epidemiological observation, may influence the development of gastric cancer through methylation of the selected genes. We for the first time found the inverse association of *CDX2* methylation with the intake of green tea and cruciferous vegetables, which have previously been suggested only by *in vitro* or animal studies, although a further study with an increased number of study patients should be required. Our findings may thus advance the chemoprevention of gastric cancer from a view of inhibiting gene methylation.

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