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ORIGINAL PAPER

Common polymorphisms in methylenetetrahydrofolate reductase gene are associated with risks of cervical intraepithelial neoplasia and cervical cancer in women with low serum folate and vitamin B12

Seo-yun Tong · Mi Kyung Kim · Jae Kwan Lee · Jong Min Lee · Sang Woon Choi · Simonetta Friso · Eun-Seop Song · Kwang Beom Lee · Jung Pil Lee

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Abstract
Objective We evaluated associations between folate, vitamin B12, and the methylenetetrahydrofolate reductase (*MTHFR*) gene, and risk of cervical intraepithelial neoplasia (CIN) and cervical cancer.
Methods This multicenter case–control study enrolled 927 Korean women (440 controls, 165 patients with CIN 1, 167 patients with CIN 2/3, and 155 patients with cervical cancer, aged 20–75 years).
Results Patients with cervical cancer had significantly lower median serum folate and vitamin B12 concentrations vs. controls. Higher serum folate was significantly associated with lower cervical cancer risk (p for linear trend = 0.0058) with a trend for a lower CIN risk after multivariate adjustment. Low folate and the *MTHFR 677* $C > T$ variant were associated with a higher risk for CIN2/3 and cervical cancer vs. wild-type or heterozygous genotypes with high folate [OR, 2.39 (1.18–4.85) and 3.19 (1.43–7.13)]. Low vitamin B12 and the *MTHFR 677* $C > T$ variant also were associated with a higher risk for CIN 2/3 and cervical cancers [OR, 2.52 (1.17–5.42) and 2.40 (1–5.73)] vs. wild-type or heterozygous status with high vitamin levels.
Conclusion Serum folate concentration is inversely associated with the risk of cervical cancer, and the *MTHFR* variant genotype may increase CIN and cervical cancer risk in women with low folate or vitamin B12 status.

Keywords Folate · Vitamin B12 · Genetic polymorphisms · Methylenetetrahydrofolate reductase · Cervical intraepithelial neoplasia · Cervical cancer

S. Tong · J. M. Lee
Department of Obstetrics and Gynecology, East–West Neo Medical Center, Kyunghee University College of Medicine, Seoul, Korea

M. K. Kim (✉)
Cancer Epidemiology Branch, National Cancer Center, 111, Jungbalsan-ro, Madu-dong, Ilsandong-gu, Goyang-si, Gyeonggi-do 411-769, Korea
e-mail: alrud@ncc.re.kr

J. K. Lee
College of Medicine, Korea University, Seoul, Korea

S. W. Choi
Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA, USA

S. Friso
University of Verona School of Medicine, Verona, Italy

E.-S. Song
The Inha University, Incheon, Korea

K. B. Lee
Gil Medical Center, Gachon University of Medicine and Science, Inchon, Korea

J. P. Lee
Ajou University School of Medicine, Suwon, Korea

Introduction

Cervical cancer is the second most common cancer in women worldwide, and it is estimated that there are over 471,000 new cases and 233,000 deaths globally each year [1]. In 2002, cervical cancer was the fourth most common malignant disease in Korean women, accounting for 9.8% of all new cancer cases [2].

Epidemiologic and experimental observations have implicated persistent infection with high-risk strains of human papillomavirus (HPV) as a cause of cervical cancer

[3]. However, because only a small portion of HPV-infected women develop cervical cancer, HPV alone cannot be entirely to blame. It appears that genetic or lifestyle factors may play an important role in the persistence of HPV infection and in the malignant conversion of cervical epithelial cells [4, 5].

Nutritional factors have been suggested to affect the persistence of HPV infection and thereby influence progression of early precancerous lesions to invasive cancer [6]. For example, higher folate status in subjects with high-risk HPV reduces the prevalence of positive HPV tests and may also reduce the risk of cervical cancer [7]. Although the results of epidemiologic studies are not consistent, they do suggest that adequate folate and vitamin B12 status may play a protective role in cervical carcinogenesis [8–11]. On the other hand, folate deficiency may increase the risk of cervical cancer in individuals infected with high-risk HPV, potentially through multiple candidate mechanisms: (1) by inducing megaloblastic changes in the cervicovaginal epithelium; (2) by reducing immunocompetence, given that folate deficiency reduces the proportion of circulating T lymphocytes and their proliferation in response to mitogen activation, which in turn decreases resistance to infections; (3) by promoting the integration of high-risk HPV into cervical cells, thus introducing chromosomal instability in the affected cells; and/or (4) by altering DNA methylation [11–13]. However, some other observational studies do not support a role for low folate or vitamin B12 in the development of cervical intraepithelial neoplasia (CIN) lesions or cervical cancer [14–17].

Genetic variations in folate metabolism can affect normal patterns of DNA synthesis and methylation. Methylenetetrahydrofolate reductase (MTHFR) is a critical enzyme in determining the proportions of folate coenzymes for DNA synthesis or DNA methylation [18, 19], the latter of which is a major epigenetic modification affecting gene expression and integrity [20, 21]. Two common functional polymorphisms in *MTHFR* are known. The most common is a *C*-to-*T* transition at nucleotide 677 (*677 C* $<$ *T*) in exon 4, resulting in an alanine-to-valine substitution that affects the catalytic domain of the enzyme, leading to reduced enzyme activity [22, 23]. Another common variant is an *A*-to-*C* transversion at position 1298 in exon 7 (*1298A* $>$ *C*), resulting in a substitution of glutamate with alanine at codon 429. This polymorphism also reduces enzyme activity, although to a lesser extent [24]. Several studies have evaluated associations between *MTHFR* genotypes and other cancers, but results have been inconsistent with respect to cancer sites and the combinatorial effects of other risk factors. For example, the *MTHFR 677 T* genotype is associated with a decreased risk of colorectal cancer [25–28]. However, in contrast to colorectal cancer, the effects of *MTHFR 677 C* $<$ *T* polymorphisms on the risks of cervical cancer and precancerous lesions are inconsistent and exhibit racial differences; the variant *T* genotype is a risk factor for CIN or carcinoma in the Americas [9, 29, 30], among Caucasians in the northern Netherlands [31], and among Koreans [32], but is not a risk factor for CIN among Greek women [33]. Henao et al. [34] reported that for African-American or Caucasian women in the United States, the *MTHFR* polymorphic genotype had a protective effect against CIN2 or 3. Furthermore, few studies have investigated genetic polymorphisms in conjunction with dietary intake or blood concentrations of B vitamins that may modify the genetic effect on the risk of cervical cancer.

To address this gap, in the present study, we assessed the associations of serum folate and vitamin B12 with the risk of CINs and cervical cancer and determined whether these associations can be modified by the *MTHFR* genotype.

Subjects and methods

Subject recruitment

This multi-institutional, hospital-based, case–control study was conducted at divisions of gynecologic oncology in five medical centers in the Republic of Korea from February 2006 to July 2007. Eligibility criteria for both cases and controls included not being pregnant at the time of recruitment and no prior history of cancer and/or cervical surgery. The age range was 20–75 years. Cases consisted of women who had abnormal Papanicolau (Pap) smear test results and a histopathological diagnosis of CIN or cervical cancer. The CIN lesions were divided into CIN1 and CIN2/3 according to the American Society for Colposcopy and Cervical Pathology (ASCCP) 2001 guidelines [35]. Case subjects included 165 CIN 1, 167 CIN 2/3, and 155 patients with cervical cancer. A total of 440 control participants who had a normal Pap smear on the day of recruitment without any history of abnormal Pap smears were enrolled from the participating hospitals during the study period. All participants filled out questionnaires about their lifestyle and dietary intake and provided blood samples. They all also gave informed consent after a full explanation of the study, which was approved by the institutional review boards of the Korea National Cancer Center and each study center.

Collection of epidemiological data and blood specimens

The women were interviewed by well-trained interviewers blinded to each subject's disease status, using both a non-dietary questionnaire and a 95-item, semi-quantitative food

frequency questionnaire designed for the Korean diet [36]. A wide range of information was collected on socio-demographic characteristics: body size, reproductive and menstrual history, exogenous hormone use, medical history, and family history of cervical and other cancers at the study enrollment, and before the onset of disease for the control and case subjects, respectively. Socio-demographic characteristics included education, occupation, cigarette smoking, alcohol consumption, and habitual exercise, with a detailed time-frame of exposures. Usual dietary factors detailing food intakes over the year prior to enrollment in the study were collected using the 95-item, semi-quantitative food frequency questionnaire. Immediately after the interview, a peripheral venous blood sample was obtained for laboratory assays before the initiation of any treatment/therapy.

Laboratory assays

Serum concentrations of folate and vitamin B12 were measured using a radioimmunoassay kit (SimulTRAC-SNB radioassay kit, ICN Diagnostics, Orangeburg, NY, USA) according to the manufacturer's instructions. The respective mean coefficients of variation for 22 pairs of replicate serum samples were 2.4% for folate and 6.8% for vitamin B12. Genotyping for the *MTHFR 677 C > T* (rs1801133) and *1298 A > C* (rs1801131) polymorphisms was determined by a 5′ exonuclease assay (Taqman) using the 7,900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). To ensure quality control, genotyping was performed blind to case or control status, a 10% masked, random sample of subjects was repeatedly tested, and the results were concordant for all masked duplicated sets. The observed genotype frequency was in agreement with the Hardy–Weinberg equilibrium (*MTHFR 677 C > T* = 0.91, MTHFR *1298 A > C* = 0.32). To identify the presence of high-risk HPV strains, cervical smears were tested with the Hybrid Capture II assay (Digene Corporation, Gaithersburg, MD, USA) that can detect high-risk HPV subtypes.

Statistical analysis

The χ^2 test was used to compare differences in proportions of covariates as categorical variables between cases and control subjects. The mean values and standard deviations (interquartile ranges) were calculated for continuous demographic variables, with the mean differences tested by ANOVA or ANCOVA (age-adjusted). Medians (5th–95th percentiles) were calculated for serum concentrations of folate and vitamin B12, and ANCOVA was used to compare the mean values of serum concentrations of folate and vitamin B12 after log transformation [37] with adjustments for age.

Unconditional logistic regression models were used to estimate crude and multivariate odds ratios (ORs) and corresponding 95% confidence intervals (CIs) and to assess whether serum concentrations of folate and vitamin B12 are associated with the risk of CINs and cervical cancer, respectively [38]. Risk estimates were computed with multivariate adjustments for age, menopausal status (pre-menopause vs. postmenopause), parity (one vs. two vs. more than three), oral contraceptive use, smoking habit (ever vs. never), alcohol consumption status (ever vs. never), and HPV infection status.

Serum concentrations of folate and vitamin B12 were categorized into quartiles based on the distribution of serum concentrations of these vitamins in the control participants. The test for linear trends was calculated using the median values for quartiles of serum vitamins as a continuous variable. Log-transformed values were considered as continuous variables and analyzed in the same models described above. In these models, the ORs (95% CIs) for serum concentrations of folate and vitamin B12 were then calculated as the risk for a change in the serum concentration by 1 standard deviation.

The relationship between the two polymorphisms of *MTHFR* (*677C < T* and *1298 A < C*) and CINs and cervical cancer were assessed using unconditional logistic regression. The risk estimates were calculated with the wild-type as the reference category. Tests for Hardy–Weinberg equilibrium among the controls were conducted using the observed genotype frequencies and a χ^2 test with 1 degree of freedom. A linear trend test was used to summarize the effect of each polymorphism. For further analysis of *MTHFR* polymorphisms, we modeled genotypes as dichotomous variables (*MTHFR 677 CC/CT* vs. *TT* and *MTHFR 1298 AA* vs. *AC/CC*).

Gene–nutrient interactions were evaluated using unconditional logistic regression models by joint categories of polymorphisms of *MTHFR* (*677 C > T* and *1298 A < C*) with serum folate and vitamin B12. We evaluated whether these associations of folate and vitamin B12 with the risk of CINs and cervical cancer could be modified by genotypes of *MTHFR*. Variables for joint effects were coded using *MTHFR 677 CC/CT* and *MTHFR 1298 AA* genotypes with the higher category of serum folate and vitamin B12 as a reference group. Tests of interaction were performed by entering multiplicative interaction terms of the ordinal score for each genotype and serum measurements into the model. Interactions were tested by using the log-likelihood ratio test, in which the model that includes the interaction term was compared with that without the term. All *p* values are two-sided, and all analyses were

performed using SAS 8.0 software (SAS Institute, Inc., Cary, NC, USA) [37].

Results

The 440 controls and 487 cases (CIN 1 = 165; CIN2/3 = 167; cancer = 155) were compared with regard to lifestyle factors (Table 1). Case subjects had significantly lower median concentrations of serum folate and vitamin B12 compared to controls. Cases also had a lower educational level and household income, a higher proportion of smokers and drinkers, and less multivitamin consumption.

Higher serum folate was associated with a lower risk of CINs and cancer after adjustment for high-risk HPV and other confounders such as age, menopausal status, parity, use of oral contraceptive, smoking habit, and drinking (Table 2). A significant inverse association was particularly strong with cervical cancer [p for linear trend across quartiles = 0.0058, 0.63 (95% CI, 0.50–0.79) per the log-transformed SD]. There was a borderline significant association with CIN 1 [p for linear trend across quartiles = 0.06, 0.77 (95% CI, 0.60–0.98) per the log-transformed SD] and CIN2/3 [p for linear trend across quartiles = 0.06, 0.78 (95% CI, 0.63–0.96) per the log-transformed SD]. Serum vitamin B12 had no significant association with either CINs or cervical cancer risk across quartiles or for overall trend. However, a significant inverse trend was found between serum vitamin B12 and risks of CINs and cervical cancer as the continuous variable (per SD); the odds ratios for CIN1, CIN2/3, and cervical cancer per SD of serum vitamin B12 were 0.83 (95% CI, 0.66–1.05), 0.85 (95% CI, 0.69–1.05), and 0.80 (95% CI, 0.65–0.998), respectively.

Table 3 shows the association between serum levels of folate or vitamin B12 and histologic grade of the cervix according to genotyping. Serum levels of folate were not significantly associated with the risk of CIN or cervical cancer according to *MTHFR A 1298C* or *MTHFR C677T* ($p > 0.05$), and neither were serum levels of vitamin B12 ($p > 0.05$).

Table 4 shows the frequencies of the *MTHFR* genotypes and the association between *MTHFR* genotypes and risks of CINs/cervical cancer. The *MTHFR 677 C > T* polymorphism was not significantly associated with the risk of CINs or cancer, while subjects carrying the *MTHFR 1298AC* or *CC* genotype had a significantly lower risk for CIN2/3 (OR = 0.64; 95% CI, 0.42–0.98) but not for CIN1 (OR = 0.88; 95% CI, 0.58–1.32) or cervical cancer (OR = 1.14; 95% CI, 0.76–1.71) compared to those with the *MTHFR 1298AA* genotype.

The interactions of folate or vitamin B12 with two common polymorphisms of *MTHFR* with the risks of CINs and cancer were not statistically significant, but the *MTHFR* polymorphisms still modified the associations of serum folate and vitamin B12 with risks of CINs and cancer (Tables 5 and 6). Compared with subjects who had serum folate concentrations higher than the median value (≥18.9 nmol/L) and the *MTHFR 677 CC/CT* genotypes, those with a lower serum folate (< 18.9 nmol/L) and the *MTHFR 677TT* genotype had a significantly increased risk of CIN2/3 (OR = 2.39; 95% CI, 1.18–4.85) and cervical

Table 1 General characteristics of the study subjects

Variables	Control (n = 440)	CIN 1 (n = 165)	CIN 2,3 (n = 167)	Cervical cancer (n = 155)	p
Age (y), mean (Q1–Q3)	45.7 (38–52)	40.6 (32–48)	40.7 (33–46)	50.8 (42–60)	<0.0001[a]
Parity, mean (SD[b])	2.25 (0.91)	2.26 (0.82)	2.19 (0.85)	2.63 (1.25)	0.06[a]
Serum folate (nmol/L), median (5th–95th)	18.6 (7.63–39.7)	16.4 (5.76–37.6)	15.8 (4.06–38.3)	15.0 (4.95–36.7)	<0.0001[a,c]
Serum vit. B_{12} (pmol/L), median (5th–95th)	520 (259–1073)	453 (214–942)	445 (223–1068)	471 (197–1075)	0.02[a,c]
Education (≥ university), %	31.5	33.5	24.7	9.0	<0.0001[d]
Oral contraceptive use, %	15.5	20.0	21.6	20.7	0.22[d]
Household income ≥4,000,000 (Won), %	36.3	33.1	19.9	10.4	<0.0001[d]
Ever smoker, %	8.9	16.4	14.4	12.3	0.04[d]
Passive smoking, %	40.0	51.5	49.7	42.6	0.02[d]
Ever drinker, %	46.8	70.3	65.3	47.1	<0.0001[d]
Ever use multivitamins, %	38.8	29.3	20.1	17.8	<0.0001[d]

CIN: cervical intraepithelial neoplasia

[a] p values are from ANCOVA (age-adjusted) test for continuous variables

[b] SD: standard deviation

[c] p values comparing log-transformed serum vitamin levels between groups

[d] p values are from chi-square test for categorical variables

Table 2 Odds ratios (ORs) and 95% confidence intervals (CIs) for CINs and cervical cancer by quartiles and per standard deviation (SD) (log scale) of folate and vitamin B12

Serum	Control	CIN 1		CIN 2,3		Cervical cancer	
Vitamins	*n* (%)	*n* (%)	OR (95% CI)[a]	*n* (%)	OR (95% CI)[a]	*n* (%)	OR (95% CI)[a]
Folate (nmol/L)[b]							
Q1 (3.09–14.0)	85 (25)	42 (37)	1.0[c]	58 (39)	1.0[c]	53 (43)	1.0[c]
Q2 (14.0–18.9)	85 (25)	28 (25)	0.74 (0.40–1.35)	30 (20)	0.52 (0.29–0.90)	24 (19)	0.42 (0.23–0.77)
Q3 (18.9–26.2)	85 (25)	21 (19)	0.51 (0.27–0.97)	26 (17)	0.45 (0.25–0.80)	18 (15)	0.33 (0.18–0.63)
Q4 (26.2–45.3)	85 (25)	22 (19)	0.62 (0.32–1.17)	35 (24)	0.65 (0.37–1.12)	29 (23)	0.49 (0.27–0.87)
p for linear trend[d]			0.0633		0.0657		0.0058
Continuous (log scale)	340 (100)	113 (100)	0.62 (0.39–0.97)	149 (100)	0.63 (0.42–0.93)	124 (100)	0.42 (0.28–0.64)
Per SD (log scale)[e]	340 (100)	113 (100)	0.77 (0.60–0.98)	149 (100)	0.78 (0.63–0.96)	124 (100)	0.63 (0.50–0.79)
Vitamin B12 (pmol/L)[f]							
Q1 (167–363)	92 (25)	38 (31)	1.0[c]	42 (27)	1.0[c]	35 (29)	1.0[c]
Q2 (363–514)	92 (25)	37 (30)	0.97 (0.55–1.74)	49 (32)	1.25 (0.74–2.13)	34 (28)	0.91 (0.51–1.61)
Q3 (514–680)	92 (25)	24 (20)	0.71 (0.38–1.34)	28 (18)	0.71 (0.40–1.28)	23 (19)	0.61 (0.33–1.14)
Q4 (680–1435)	92 (25)	24 (19)	0.77 (0.41–1.45)	34 (23)	0.98 (0.56–1.73)	30 (24)	0.76 (0.42–1.38)
p for linear trend[d]			0.2824		0.5027		0.2144
Continuous (log scale)	368 (100)	123 (100)	0.67 (0.41–1.10)	153 (100)	0.70 (0.45–1.10)	122 (100)	0.62 (0.39–0.995)
Per SD (log scale)[e]	368 (100)	123 (100)	0.83 (0.66–1.05)	153 (100)	0.85 (0.69–1.05)	122 (100)	0.80 (0.65–0.998)

[a] ORs and 95% CIs calculated by unconditional logistic regression, adjusted for age, menopausal status (premenopause vs. postmenopause), parity (one vs. two vs. more than three), oral contraceptive use, alcohol consumption status (ever vs. never), and HPV infection status

[b] Excluding 101 cases and 100 controls with missing or unreliable data (<2.27 or >45.3 nmol/L for folate)

[c] Lowest quartile is the reference category

[d] Linear trends across quartiles of serum folate and vitamin B_{12} concentrations, respectively, were tested in the logistic regression models by using the median level for each quartile as an ordinal variable

[e] OR (95%) were calculated as the risk for a change in the serum level by one SD

[f] Excluding 89 cases and 72 controls with missing or unreliable data (<73.8 or >1,476 pmol/L for vitamin B12)

Table 3 Folate and vitamin B12 status according to genotype

	MTHFR A1298C				MTHFR C677T			
	AA	AC	CC	*p*[a]	CC	CT	TT	*p*a
Folate, median (Q1–Q3)								
Normal	8.41 (5.91–12.0)	8.02 (6.35–10.9)	8.33 (7.76–14.9)	0.625	8.37 (6.38–12.0)	8.23 (6.08–11.7)	7.49 (5.00–10.7)	0.2502
CIN 1	6.75 (4.50–9.88)	7.64 (5.13–11.0)	10.7 (7.70–12.0)	0.2599	7.66 (5.76–10.8)	7.30 (4.97–10.7)	4.48 (2.73–9.79)	0.1388
CIN 2,3	6.88 (5.15–11.4)	7.22 (4.89–10.5)	8.75 (6.60–10.4)	0.7428	8.92 (5.98–11.7)	6.22 (4.56–11.0)	6.06 (5.02–10.0)	0.1265
Cervical cancer	5.99 (3.66–9.31)	7.50 (5.13–10.8)	12.7 (8.86–16.5)	0.1495	6.72 (4.21–9.65)	6.46 (5.37–10.7)	4.80 (3.54–10.9)	0.4639
Total	7.32 (5.09–11.0)	7.76 (5.72–10.8)	9.27 (7.70–13.6)	0.0998	7.91 (5.85–11.0)	7.56 (5.56–11.1)	6.58 (4.03–10.3)	0.0091
Vitamin B12, median (Q1–Q3)								
Normal	664 (504–939)	739 (475–902)	638 (488–999)	0.8261	679 (477–881)	706 (513–950)	716 (511–941)	0.6998
CIN 1	585 (445–792)	589 (458–853)	406 (260–814)	0.5108	662 (457–853)	549 (439–764)	557 (409–803)	0.5324
CIN 2,3	595 (438–903)	633 (496–948)	794 (564–1262)	0.6622	632 (531–927)	618 (411–988)	542 (411–868)	0.5035
Cervical cancer	582 (391–844)	621 (484–922)	770 (770–770)	0.7887	569 (434–690)	680 (495–1043)	704 (393–841)	0.1905
Total	631 (464–903)	668 (475–905)	674 (406–854)	0.6861	637 (472–861)	649 (474–960)	627 (439–864)	0.4918

[a] *p* values are from Kruskal–Wallis tests

cancer (OR = 3.19; 95% CI, 1.43–7.13). Similarly, we observed a significantly increased risk among subjects with a lower serum vitamin B12 (<518 pmol/L) and the *MTHFR 677TT* genotype compared with those with higher serum vitamin B12 (≥518 pmol/L) and the *MTHFR 677 CC/CT* genotypes: for CIN2/3, OR = 2.52 (95% CI, 1.17–5.42);

Table 4 Odds ratios (ORs) and 95% confidence intervals (CIs) for the 677 C > T and 1298 A > C *MTHFR* polymorphisms with risk of CINs and cervical cancer

MTHFR[a] genotypes	Control	CIN 1		CIN 2,3		Cervical cancer	
	n (%)[a]	*n* (%)[a]	OR (95% CI)[b]	*n* (%)[a]	OR (95% CI)[b]	*n* (%)[a]	OR (95% CI)[b]
MTHFR C677T							
CC	152 (36)	52 (33)	1.0[c]	54 (34)	1.0[c]	53 (36)	1.0[c]
CT	198 (46)	82 (52)	1.19 (0.77–1.82)	74 (46)	1.01 (0.66–1.55)	65 (45)	0.97 (0.62–1.51)
TT	77 (18)	25 (15)	0.90 (0.50–1.61)	32 (20)	1.09 (0.64–1.87)	28 (19)	1.09 (0.62–1.91)
P for linear trend[d]			0.9280		0.7789		0.8346
CC or CT	350 (82)	134 (84)	1.0[c]	128 (80)	1.0[c]	118 (81)	1.0[c]
TT	77 (18)	25 (16)	0.81 (0.48–1.37)	32 (20)	1.09 (0.67–1.75)	28 (19)	1.11 (0.66–1.84)
MTHFR A1298C							
AA	278 (65)	107 (67)	1.0[c]	117 (73)	1.0[c]	89 (60)	1.0[c]
AC	132 (31)	46 (29)	0.86 (0.56–1.32)	39 (24)	0.66 (0.43–1.03)	57 (39)	1.26 (0.83–1.92)
CC	18 (4)	7 (4)	1.03 (0.40–2.66)	4 (3)	0.51 (0.16–1.57)	2 (1)	0.29 (0.06–1.36)
p for linear trend[d]			0.6422		0.0391		0.9601
AA	278 (65)	107 (67)	1.0[c]	117 (73)	1.0[c]	89 (60)	1.0[c]
CC or AC	150 (35)	53 (33)	0.88 (0.58–1.32)	43 (27)	0.64 (0.42–0.98)	59 (40)	1.14 (0.76–1.71)

[a] MTHFR: methylenetetrahydrofolate reductase. Excluding 35 subjects for C677T and 31 subjects for A1298C with missing or unreliable data

[b] ORs and 95% CIs calculated by unconditional logistic regression, adjusted for age, menopausal status (premenopause vs. postmenopause), parity (one vs. two vs. more than three), oral contraceptive use, smoking habit (ever vs. never), alcohol consumption status (ever vs. never), and HPV infection status

[c] Reference category

[d] Assigning values 1, 2, and 3, representing the CC, CT, and TT categories for *MTHFRC677T* and AA, AC, and CC for *MTHFR A1298C*, respectively

for cervical cancer, OR = 2.40 (95% CI, 1.00–5.73) (Table 5).

Similar associations were also observed between the *MTHFR 1298 A < C* genotype and serum folate or vitamin B12 (Table 6). Among subjects carrying the *MTHFR 1298AA* genotype, lower serum folate concentrations (<18.9 nmol/L) were significantly associated with an increased risk of cervical cancer (OR = 2.51; 95% CI, 1.35–5.67), even though interactions were not statistically significant. Compared with subjects who had higher serum folate (≥18.9 nmol/L) with the *MTHFR 1298AA* genotype, those with lower serum folate (<18.9 nmol/L) and the *MTHFR 1298 CC/AC* genotype had a significantly increased risk of cervical cancer (OR = 2.23; 95% CI, 1.13–4.39). When serum vitamin B12 concentrations were high (≥ 518 pmol/L), those with the *MTHFR 1298 CC/AC* genotype had a reduced risk of CIN 2/3 with a borderline significance (OR = 0.53; 95% CI, 0.26–1.05) compared to those with the *MTHFR 1298AA* genotype.

Discussion

In this multi-institutional, case–control study, we investigated the lifestyle risk factors for cervical cancer. Compared to controls, case subjects were significantly different in most of the known lifestyle risk factors such as age, parity, smoking, and alcohol consumption. Among these, the most important lifestyle factor that requires attention is the status of two B-vitamins, folate and vitamin B12. The median values of serum folate and vitamin B12 among Korean females in the control group were 18.5 nmol/L and 520 pmol/L, respectively. In recently reported work also with a Korean population, an adequate serum level of folate was defined as ≥11.3 nmol/L, that for vitamin B12 was defined as ≥185 pmol/L, and the geometric means for the serum folate and vitamin B12 concentrations in Korean women were 16 nmol/L and 296 pmol/L, respectively [39].

Similar to the results of previous case–control studies that examined the associations between folate or vitamin B12 and CIN or cervical cancer [8, 10, 12], subjects with cervical cancer and CINs in the present study had significantly lower concentrations of serum folate compared to controls. A higher serum folate concentration was significantly associated with a lower risk of cervical cancer and exhibited a trend to a lower risk of CINs after multivariate adjustment. The association between vitamin B12 and cervical cancer risk has been less investigated than the association with folate, even though vitamin B12 is an important cofactor for folate metabolism. Case subjects in this study also had decreased serum vitamin B12 concentrations compared with controls, but we found an inverse

Table 5 Joint association of B vitamin and *MTHFR* C677T genotype with risks of CINs and cervical cancer

	MTHFR C677T	CIN 1		CIN 2,3		Cervical cancer	
		CC/CT	TT	CC/CT	TT	CC/CT	TT
Folate (nmol/L)[a]							
High (≥18.9)	*n* (control/case)	141/35	21/5	141/49	21/9	141/32	21/9
	OR (95% CI)[b]	1.0[c]	1.12 (0.38–3.31)	1.0[c]	1.30 (0.53–3.18)	1.0[c]	1.85 (0.74–4.64)
Low (<18.9)	*n* (control/case)	135/56	27/12	135/61	27/22	135/56	27/15
	OR (95% CI)[b]	1.59 (0.94–2.68)	1.67 (0.71–3.92)	1.22 (0.76–1.96)	2.39 (1.18–4.85)	2.06 (1.22–3.48)	3.19 (1.43–7.13)
	p Interaction[d]	0.9300	0.4734	0.7703			
Vit. B12(pmol/L)[a]							
High (≥518)	*n* (control/case)	137/32	25/6	137/45	25/13	137/34	25/13
	OR (95% CI)[b]	1.0[c]	0.97 (0.35–2.66)	1.0[c]	1.51 (0.68–3.33)	1.0[c]	2.09 (0.93–4.70)
Low (<518)	*n* (control/case)	139/59	23/11	139/65	23/18	139/54	23/11
	OR (95% CI)[c]	1.49 (0.88–2.53)	1.91 (0.77–4.73)	1.27 (0.79–2.04)	2.52 (1.17–5.42)	1.69 (1.00–2.85)	2.40 (1.00–5.73)
	p Interaction[d]	0.6749	0.6183	0.5197			

Excluding 126 cases and 116 controls with missing or unreliable data

[a] High and low levels of serum folate and vitamin B12 based on the median values of the control group, respectively

[b] ORs and 95% CIs calculated by unconditional logistic regression, adjusted for age, menopausal status (premenopause vs. postmenopause), parity (one vs. two vs. more than three), oral contraceptive use, smoking habit (ever vs. never), alcohol consumption status (ever vs. never), and HPV infection status

[c] Reference category

[d] Based on the likelihood ratio test comparing models with and without the interaction term between serum folate and vitamin B12 and *MTHFR C677T* genotype, respectively

Table 6 Joint association of B vitamin and *MTHFR* A1298C genotype with risks of CINs and cervical cancer

	MTHFR A1298C	CIN 1		CIN 2,3		Cervical cancer	
		AA	CC/AC	AA	CC/AC	AA	CC/AC
Folate[a]							
High (≥18.9)	*n* (control/case)	105/25	57/15	105/43	57/15	105/22	57/19
	OR (95% CI)[b]	1.0[c]	0.92 (0.43–1.99)	1.0[c]	0.63 (0.31–1.29)	1.0[c]	1.55 (0.75–3.21)
Low (<18.9)	*n* (control/case)	98/47	64/21	98/59	64/24	98/43	64/28
	OR (95% CI)[b]	1.79 (0.97–3.29)	1.18 (0.58–2.39)	1.43 (0.85–2.39)	0.83 (0.44–1.55)	2.51(1.35–5.67)	2.23 (1.13–4.39)
	p Interaction[d]	0.5165	0.8512	0.2514			
Vit B12[a]							
High (≥518)	*n* (control/case)	96/25	66/13	96/42	66/16	96/28	66/19
	OR (95% CI)[b]	1.0[c]	0.73 (0.34–1.57)	1.0[c]	0.53 (0.26–1.05)	1.0[c]	1.00 (0.50–2.02)
Low (<518)	*n* (control/case)	107/47	55/23	107/60	55/23	107/37	55/28
	OR (95% CI)[b]	1.46 (0.80–2.68)	1.21 (0.59–2.48)	1.17 (0.70–1.96)	0.84 (0.44–1.62)	1.38 (0.76–2.52)	1.79 (0.92–3.48)
	p Interaction[d]	0.8050	0.5135	0.5997			

Excluding 126 cases and 116 controls with missing or unreliable data

[a] High and low levels of serum folate and vitamin B_{12} based on the median values of the control group, respectively

[b] ORs and 95% CIs calculated by unconditional logistic regression, adjusted for age, menopausal status (premenopause vs. postmenopause), parity (one vs. two vs. more than three), oral contraceptive use, smoking habit (ever vs. never), alcohol consumption status (ever vs. never), and HPV infection status

[c] Reference category

[d] Based on the likelihood ratio test comparing models with and without the interaction term between serum folate and vitamin B12 and the *MTHFR C677T* genotype, respectively

trend only for the association of vitamin B12 with CINs and cervical cancer risk. The results also indicate that vitamin B12 status is weakly associated with cervical carcinogenesis, as described in another study [16].

Folate may possess dual modulatory effects on carcinogenesis depending on the timing and dose of folate intervention [40]. Although increases in folate before the existence of preneoplastic lesions (such as polyps in the colon) can prevent tumor development, supplementation with synthetic folic acid may enhance progression once preneoplastic lesions are present [41]. Similarly, animal studies have shown that the dose and timing of folate intervention are critical in providing safe and effective chemoprevention; exceptionally high supplemental folate levels and folate intervention after microscopic neoplastic foci are established in the colorectal mucosa promote rather than suppress colorectal carcinogenesis [40]. A higher folate status may reduce the risk of postmenopausal breast cancer in women with very low folate intake, whereas very high folate intake, attributable to supplement use, may increase cancer risk, and there is a suggested hypothesized nonlinear relation between folate status and breast cancer risk [41]. Our data also indicated that the second and third quartiles of serum folate in women significantly lowered the ORs of cervical cancer and high-grade CIN compared to the highest quartile; this finding has not been previously reported for cervical lesions.

Previous case–control studies have examined the interrelationship of folate or vitamin B12 with *MTHFR* polymorphisms and the risks of CIN or cervical cancer [29–32, 42], with inconsistent results. These conflicting results in cervical carcinogenesis may be associated with variable sample sizes and racial variations. Only a few of these studies have reported an association among B-vitamin status, *MTHFR C677T* genotype, and the risk of cervical dysplasia [9]. Goodman et al. reported that reduced dietary folate and the presence of the *MTHFR 677T* allele may increase the risk for cervical dysplasia [9], even though they also reported no association between plasma folate and vitamin B12 concentrations and a risk of cervical dysplasia in an earlier study [17]. In the present study, we observed no association between the *MTHFR 677* $C < T$ genotype and the risk of CIN or cervical cancer. However, case subjects carrying the *MTHFR 1298AC* or *CC* genotype had a significantly lower risk for CIN2/3 compared to the *MTHFR 1298AA* genotype. We also observed no interactions between any polymorphism and the two B-vitamins and no associations between serum levels of folate or vitamin B12 and the histologic grade of the cervix according to genotyping. Nevertheless, we did find an increased risk of CIN2/3 and cervical cancer with low serum concentrations of both folate and vitamin B12 in *MTHFR 677TT* genotype subjects compared to those with high vitamin concentrations and the *MTHFR 677 CC/CT* genotype. Subjects with low serum folate in both the *MTHFR 1298 AA* and the *1298 CC/AC* groups showed an increased risk of cervical cancer compared to subjects with high folate and the *1298 AA* genotype.

Notably, no study has previously examined the association among nutritional factors, the *MTHFR A1298C* polymorphism, and the risk of cervical cancer. This study is, to the best of our knowledge, the first to examine associations among serum folate and vitamin B12, single nucleotide polymorphisms in *MTHFR*, and the risk of CINs and cancer, as well as potential interactions between both B-vitamins and *MTHFR* genotypes in a multi-institutional, case–control investigation.

We did consider the possible confounding effects of lifestyle factors associated with cervical carcinogenesis. First, oncogenic HPV infection, which has been regarded a common agent of cervical cancer, was adjusted as a confounder in the multivariate model [3]. The main potential sources of bias in this study could be exposure measurement error and confounding, which are both inherent to a retrospective case–control study. A one-time blood test for folate or vitamin B12 might not be sufficient as a marker of vitamin status. Considering the long duration of cancer development, blood measurements might not be a reliable index of long-term dietary intake [43], even though it has been reported that blood vitamins accurately reflect the consumption of total fruits and vegetables and are thus considered reasonable biomarkers of dietary intake of fruits and vegetables, as well as long-term vegetable intake [44]. The blood samples in this study were collected with participants in a non-fasting state, and the measurements thus could have been influenced by current food intake. We therefore asked subjects about recent changes in their dietary habits and confirmed that none of them had made any substantial dietary changes, indicating that the serum concentrations of folate or vitamin B12 in this study reflect each participant's usual dietary intake (data not shown). Accordingly, we cannot exclude the possibility of exposure misclassifications of serum biomarkers. However, any such errors in the measurement of these exposures are therefore likely to have been mainly non-differential with respect to the disease outcome, thereby tending to bias the effect estimates toward the null. A 3-year analysis of workload data on the number of serum/plasma folate and red cell folate assays has indicated that serum folate measurements provide information equivalent to red cell folate measurements when the goal is determining whether folate deficiency is present, with the authors suggesting that the serum folate assay should be the method of choice [45].

The small sample size may reduce the credibility of the study results by introducing imprecision into the measurements and limiting our ability to estimate the

association more precisely. Even though this study had a relatively larger sample size in total, the *MTHFR 677TT* genotype frequency of 18% (77 cases) found in controls was somewhat low for evaluating potential interactions statistically. A larger study with greater statistical power will be needed to detect associations of the magnitude observed in the present study, and this issue is particularly relevant to subgroup analyses for interaction tests in the current work, with its small strata sizes. However, despite the limited statistical power resulting from the small sample size, this study managed to achieve statistical significance with serum folate and the *MTHFR* genotypes. We consider that the admitted limitation of a small sample size did not substantially affect our conclusions; nevertheless, a study with a larger sample size is needed to confirm the possible interaction between the risk of cervical carcinogenesis and nutritional factors that the *MTHFR* genotype may modify.

In summary, our findings suggest that serum folate is inversely associated with the risk of CINs and cervical cancer and that the *MTHFR* genotype further modifies the inverse association of serum folate with the risk of CIN and cervical cancer. Vitamin B12 has a similar but weaker effect on the risk of CINs and cervical cancer. These results may support the hypothesis that nutritional factors such as folate and vitamin B12 play a significant role in cervical cancer and may be modified by genetic factors, such as genetic polymorphisms.

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