Lower serum folate is associated with development and invasiveness of gastric cancer

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AIM: To evaluate the associations of serum folate level with development, invasiveness and patient survival of gastric cancer.

METHODS: In this nested case-control study, patients with newly diagnosed gastric cancer undergoing gastrectomy were enrolled, and patients receiving chemotherapy prior to surgery, with other concurrent malignancy, or of the aboriginal and alien populations were excluded. In total, 155 gastric cancer patients and 149 healthy controls were enrolled for determination of serum folate levels and their correlation with gastric cancer. Using the median value of serum folate computed among the overall population as the cutoff value, the associations between serum folate and gastric cancer in all cases and different age and gender subgroups were analyzed by multivariate logistic regression analysis. In the patient cohort of gastric cancer, receiver-operating characteristic analyses were performed to calculate the best cutoff values of serum folate, and the associations between serum folate levels and clinicopathological features were further analyzed by multivariate regression analysis. Survival analyses were conducted using the Cox proportional hazards model.

RESULTS: The mean serum folate level was significantly lower in gastric cancer patients than that in controls (3.71 ± 0.30 ng/mL vs 8.00 ± 0.54 ng/mL, \(P < 0.01\)), and folate levels were consistently lower in gastric cancer patients regardless of age and gender (all \(P < 0.01\)). Using the median serum folate value as the cutoff value, low serum folate was significantly associated with gastric cancer risk in the whole population (OR = 19.77, 95% CI: 10.54-37.06, \(P < 0.001\)) and all strata (age < 60 years OR = 17.39, 95% CI: 7.28-41.54, \(P < 0.001\)), age \(\geq 60\) years (OR = 21.67, 95% CI: 8.27-56.80), and females (OR = 17.95, 95% CI: 7.93-40.62), and males (OR = 20.95, 95% CI: 7.66-57.31); all \(P < 0.001\). In the patient cohort of gastric cancer, the respective cutoff values showed that low serum folate levels were significantly associated with serosal invasion (OR = 2.54, 95% CI: 1.23-5.23), lymphatic invasion (OR = 2.23, 95% CI: 1.17-4.26), and liver metastasis (OR = 2.23, 95% CI: 1.17-4.26).
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6.67, 95%CI: 1.28-34.91) of gastric cancer (all P < 0.05). Serum folate level below 1.90 ng/mL was associated with poor patient survival (HR = 1.84, 95%CI: 1.04-3.27, P < 0.05) in univariate analysis.

CONCLUSION: Lower serum folate levels were significantly associated with gastric cancer development and invasive phenotypes. The role of folate depletion in gastric cancer invasion warrants further study.

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Key words: Folic acid; Folate; Plasma; Metastasis; Invasion

Core tip: Low folate status is involved in the development of gastric cancer, but the role of folate in invasiveness of gastric cancer remains unclear. In addition, although folate levels in blood may reflect the degree of folate depletion, an association between blood folate status and gastric cancer has not been established. In this case-control study, we found lower serum folate was significantly associated with gastric cancer development. Besides, in the patient cohort of gastric cancer, lower serum folate was significantly associated with invasive phenotypes such as serosal invasion, lymphatic invasion and liver metastasis. These findings warrant further study.

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INTRODUCTION

Gastric cancer remains the fourth most common cancer and the second leading cause of cancer mortality worldwide, and the median survival in patients with advanced (stages III-IV) gastric cancer is generally less than one year[10]. Due to poor treatment response in advanced gastric cancer, detection of early-stage gastric cancer can effectively improve outcomes[11]. However, early diagnosis remains a challenge in most countries due to invasive characteristic of gastroscopy and a lack of practical screening biomarkers, and most patients already suffer from advanced tumor when gastric cancers are newly diagnosed[12]. In addition, the optimal degree of lymph node dissection for gastric cancer is still matter of debate, and inaccurate pre-operative image studies of lymph node metastasis frequently result in over- or incomplete resection[13]. New biomarkers to screen for and predict the invasiveness of gastric cancer may provide novel ways to resolve the dilemma and are therefore urgently needed[14].

Folate is involved in biological methylation reactions and nucleotide biosynthesis, and depletion of folate can result in global DNA hypomethylation, DNA damage, impaired DNA repair and altered proto-oncogene/tumor suppressor gene expressions[15,16]. Folate supplementation was recently reported to significantly increase global DNA methylation and reduce mucosal inflammation and dysplasia in a Helicobacter pylori-infected mouse model, and gastric cancer may be prevented by high folate intake[17]. Although the results of studies on dietary folate intake and risk of gastric cancer were inconsistent[18-20], gene 677CT polymorphism of methylenetetrahydrofolate reductase (MTHFR), a key enzyme in the metabolism of folate, was associated with increased risk of gastric cancer for individuals with low folate status[11,16]. Moreover, folate concentrations were reported to be significantly lower in gastric cancer tissues than in gastritis tissues, and hypomethylation and overexpression of c-myc were correlated with lower folate concentrations in tissues[21]. Increasing evidence suggests low folate status is involved in the development of gastric cancer, but the role of folate in gastric cancer invasiveness remains unclear.

Although folate levels in blood may reflect the degree of folate depletion, an association between blood folate status and gastric cancer has not been established. In a study of advanced gastric cancer, plasma folic acid concentration was lower in patients with total genomic DNA hypomethylation than that in patients with normal methylation[22]. Tan et al[9] observed that the mean serum folate concentration of gastric cancer cases was significantly lower than that of controls. However, Vollset et al[23] reported a null association between plasma folate levels and gastric cancer risk, but a mean duration of 3.3 years between blood donation and cancer diagnosis could have biased the observed results. The serial changes of serum folate were not reported when patients developed gastric cancer, and further studies to verify lower blood folate in gastric cancer patients are needed.

To our knowledge, the associations between serum folate levels, invasive phenotypes and patient survival of gastric cancer have not been evaluated. Therefore, we conducted a study to determine whether serum folate level could be clinically associated with development, invasiveness and patient survival of gastric cancer.

MATERIALS AND METHODS

Study subjects
In this case-control study, we used blood samples collected from individuals participating in two national projects conducted from January 1998 to April 2006 for the investigation of risk factors of gastric cancer in Taiwan[24,25]. Patients with newly diagnosed gastric cancer undergoing gastrectomy were enrolled, and patients receiving chemotherapy prior to surgery, with other concurrent malignancy, or of the aboriginal and alien populations were excluded. Control blood samples were obtained from individuals who visited health examination clinics with minimal gastritis or normal appearance of the gastric...
mucosa on gastroscopic examination. Informed consents were obtained from all subjects and/or guardians on a voluntary basis, and all patient-derived specimens were collected under protocols approved by the Institutional Review Boards (IRBs) of the parent institutions (IRB No. C07199). All the study subjects who fulfilled the inclusion and exclusion criteria and signed the informed consents were recruited.

In total, we studied 155 patients with gastric cancer, for whom complete clinical data, including tumor stage, degree of tumor invasion and presence of metastasis, and a serum sample were available. All recruited patients had been followed up for at least 5 years. The controls had been followed up for at least 5 years. The controls were matched by age (within 5 years) and date of blood collection (± 3 mo), and 149 cases were recruited. In addition, status of *Helicobacter pylori* (*H. pylori*) infection was determined by Giemsa staining of gastric tissue biopsy or by serum *H. pylori* antibody test (INOVA Diagnostics, San Diego, CA).

### Measurement of serum folate levels

Blood samples were obtained after overnight fasting, and serum folate concentrations were measured by microbiological assay.[28] Cryopreserved *Lactobacillus casei* (NCIB 10463) culture were thawed into reconstituted assay medium in a water bath at 37 °C, and then added to bulk assay medium at a concentration of 200 µL/100 mL. Sodium ascorbate (0.5%) solution was used to dilute serum samples 1:20. Standard solution of folate (500 pg/mL) was made by diluting stock standards in sodium ascorbate. Assay microorganism was added, and the plates were sealed and incubated at 37 °C in the dark for 42 h after adding disinfectant. Then they were read at 570 nm and serum folate concentrations were calculated.

### Statistical analysis

The discrete variables are presented as number and percentage (%); continuous variables are presented as mean and standard error. The demographic data of patients and controls were compared using the χ² test and Student’s t-test. To avoid possible confounding effects of age and gender, multivariate logistic regression analyses were conducted to evaluate the associations between serum folate levels, gastric cancer risk, and clinicopathological features. Using the median value of serum folate computed among the overall population of cases and control as the cutoff value, the associations between serum folate and gastric cancer in all cases and different age and gender subgroups were analyzed. In the patient cohort of gastric cancer, we considered that the cutoff values in various clinicopathological features should be different, so nonparametric receiver-operating characteristics (ROC) analyses were plotted for determining the best cutoff values of serum folate in various clinicopathological features. Using the respective cutoff values, the associations between serum folate levels and clinicopathological features were analyzed. Furthermore, to determine whether serum folate concentration was associated with patients’ outcomes, survival analyses were conducted using the Cox proportional hazards model. Data were analyzed using SPSS, version 11.0 (SPSS Inc., Chicago, Illinois, United States). Nonparametric ROC analyses were performed via the STATA program, version 8.0 (Stata Corporation, College Station, Texas, United States).

## RESULTS

### Association between serum folate levels and gastric cancer

Demographic characteristics are summarized in Table 1. Control subjects were on average about 5 years younger than gastric cancer cases. There were no differences in gender distribution or *H. pylori* infection status between the two groups. The mean serum folate level was significantly lower in gastric cancer patients compared to that in controls (3.71 ± 0.30 ng/mL vs 8.00 ± 0.54 ng/mL, *P* < 0.01). Since gastric cancer patients were slightly older than controls, we further analyzed folate concentrations based on age and gender, and found folate levels were consistently lower in gastric cancer patients regardless of age and gender (all *P* < 0.01).

Using the median value of serum folate (4.38 ng/mL) computed among the overall population of cases and control as the cutoff value, the adjusted odds ratio (OR) for detecting the occurrence of gastric cancer was 19.77 (95%CI: 10.54–37.06) (Table 2). In addition, serum folate level lower than 4.38 ng/mL was found to be consistently associated with higher gastric cancer risk in all strata (age < 60 years, age ≥ 60 years, males, and females; all *P* < 0.001). No significant interaction of other variables was observed.

### Association between serum folate levels and invasiveness of gastric cancer

To further assess the value of using serum folate levels for detecting invasiveness among gastric cancer patients, we calculated the best cutoff values of various invasive phenotypes by ROC analyses. Using the cutoff values for multivariate regression analyses, the adjusted ORs for detecting the invasiveness of gastric cancer were obtained.
as shown in Table 3. Serum folate $\leq 2.61 \text{ ng/mL}$ was significantly associated with serosal invasion (OR = 2.54, 95%CI: 1.23-5.23) and lymphatic invasion (OR = 2.23, 95%CI: 1.17-4.26). Serum folate $\leq 1.90 \text{ ng/mL}$ was significantly correlated with liver metastasis (OR = 6.67, 95%CI: 1.28-34.91) (all $P < 0.05$).

### Association between serum folate levels and patient survival

The survival curves for patients with different serum folate levels are compared in Figure 1. A folate level lower than 1.90 ng/mL was associated with poor survival ($P = 0.03$). Cox proportional hazard models showed that advanced stage ($P < 0.001$), serosal invasion ($P < 0.001$), lymph node metastasis ($P < 0.001$), lymphatic invasion ($P < 0.001$), venous invasion ($P < 0.001$), liver metastasis ($P < 0.001$), and serum folate level lower than 1.90 ng/mL ($P = 0.036$) were associated with poor overall survival (Table 4). Multivariate analysis showed that only serosal invasion ($P < 0.001$) and liver metastasis ($P < 0.001$) were independent risk factors for poor overall survival.

### DISCUSSION

Previous studies have shown that low folate status is involved in the development of gastric cancer\cite{[15-17]}, but the role of folate in the invasiveness of gastric cancer is still unclear. To our knowledge, this is the first study to investigate whether serum folate could be clinically associated with invasiveness of gastric cancer. We found low serum folate levels were significantly associated with various invasive phenotypes such as serosal invasion, lymphatic invasion and liver metastasis. The findings of our study suggest that folate depletion may play a role in the development of gastric cancer, but the effect of folate depletion in gastric cancer invasion warrants further investigation.

Although folate supplementation has been shown to be effective in preventing global loss of methylation in a mouse model of gastric cancer development\cite{[24]}, further study will be required to determine whether folate depletion and subsequent perturbed hypomethylation directly contribute to tumor invasion and metastasis. In a recent study, Wang et al\cite{[31]} found that folate deficiency could enhance invasiveness of colon cancer cells by activation of Shh signaling through promoter hypomethylation and cross actions with the NF-kB pathway. However, the effect of folate deficiency on methyl group metabolism and methylation is highly complex, and is influenced by cell type, stage of transformation, and genetic variations at specific sites in the genome\cite{[25-28],[26-28]}. For example, in animal studies of colorectal cancer, although folate showed chemoprotective effects against carcinogenesis, it also appeared to promote progression of cancer once neoplastic foci had been established\cite{[20-31]}. The mechanisms of folate deficiency that contribute to the invasiveness of gastric cancer have yet to be fully elucidated.

Few studies have investigated the association between

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**Table 2** Association between serum folate level and occurrence of gastric cancer $n$ (%)

| Folate | Odds ratio$^1$ | 95%CI | $P$ value |
|--------|---------------|-------|-----------|
| $> 4.38 \text{ ng/mL}$ | $33 (21.3)$ | 122 (78.7) | 19.77 | $< 0.001$ |
| $\leq 4.38 \text{ ng/mL}$ | 30 (20.1) | (10.54-37.06) | |

**Table 3** Associations between serum folate levels and clinicopathological features of gastric cancer $n$ (%)

| Folate | Odds ratio$^1$ | 95%CI | $P$ value |
|--------|---------------|-------|-----------|
| $> 1.90 \text{ ng/mL}$ | 26 (60.5) | 17 (39.5) | 1 | (1.23-5.23) |
| $\leq 1.90 \text{ ng/mL}$ | 70 (62.5) | (0.60-3.14) | |

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$^1$Age and gender were adjusted by multivariate regression analysis.
blood folate status and gastric cancer development, and the usefulness of blood folate as a biomarker is still a subject of debate. A Chinese case-control study showed that gastric cancer patients had a significantly lower serum folate level than that of controls, but a European study found there was no significant difference in plasma folate level between pre-gastric cancer patients and the controls\(^{[19,20]}\). However, in that European study, the blood samples were collected 3.3 years (mean, with a range of 0.4-7.13 years) before diagnosis of gastric cancer, and this discrepancy could be biased by the time interval between blood donations and cancer diagnosis. In addition, other risk factors may be also involved in the discrepancy between Chinese and European populations. For example, in a recent meta-analysis\(^{[34,35]}\), the gene polymorphism in thymidylate synthase, an important enzyme involved in folate metabolism, was associated with an increased risk of gastroesophageal cancer among Asians, but not among Caucasians. However, the interaction between other risk factor and blood folate in different populations warrants further evaluation. In the present study of Chinese population, we confirmed that the serum folate level was significantly lower in patients than that in controls, and low serum folate was significantly associated with increased gastric cancer risk regardless of age and gender. According to the observations of previous studies and our results, decreased folate levels can be found after the development of gastric cancer. Further studies to confirm the causal relationship between folate depletion and gastric cancer evolution will be crucial.

The association between folate and outcomes of gastric cancer has rarely been investigated, although MTHFR 677TT carriers with low folate and vitamin B12 intakes were reported to have the lowest survival rate in a cohort study of gastric cancer\(^{[31]}\). The present investigation is the first to report that a low serum folate level was associated with poor patient survival in univariate analysis, but the association became insignificant in multivariate analysis. Although low serum folate level was not associated with prognosis in this study, outcome analysis could be biased by different strategies and clinical conditions in cancer treatment. Large-scale well-controlled prospective studies will be needed to determine the value of serum folate level as a prognostic biomarker.

Decreased serum folate levels may be caused by poor intake of folate-rich foods, impaired folate absorption, impaired folate metabolism or rapid folate consumption. Interestingly, the role of dietary folate intake in the development of gastric cancer has been reported in several studies, with controversial results\(^{[12-14]}\). Larsson et al\(^{[12]}\) conducted a meta-analysis and found significant heterogeneity, especially among different geographic regions. The estimated relative risks for subjects with the highest dietary folate intake relative to the lowest dietary folate intake were 0.68 (95%CI: 0.58-0.80) for studies conducted in the United States, 1.15 (95%CI: 0.91-1.45) for European studies, and 0.89 (95%CI: 0.40-1.96) for studies conducted elsewhere. The disparity in these results may be due to complex host-environment interactions. Moreover, a variety of conditions, including defects in the uptake system, gastrointestinal diseases, drug interaction, and hypochlorhydria may also affect the normal intestinal folate absorption process\(^{[34,35]}\). Gastric cancer arises by a multi-stage process, and severity of atrophic gastritis is correlated to the risk of progression to gastric cancer\(^{[36]}\). Although low serum folate in patients with gastric cancer may be caused by disturbed folate absorption due to severe underlying atrophic gastritis, the possible mechanisms involved in folate depletion may be complicated and should be further clarified.

The association between H. pylori infection and folate depletion has been evaluated in previous studies. In a recent meta-analysis\(^{[10]}\), no significant association between H. pylori infection and folate levels was observed, and eradication of H. pylori infection had no effect on serum folate levels. In this case-control study, the impact of H. pylori infection and folate depletion on gastric cancer risk needs further investigation.

### Table 4: Univariate analysis of mortality predictors in gastric cancer patients

| Predictor            | Hazard ratio | 95%CI       | P value
|----------------------|--------------|-------------|----------
| Age                  |              |             |          |
| > 60 vs ≤ 60         | 1.27         | 0.97-1.67   | NS       |
| Gender               |              |             |          |
| Male vs female       | 1.29         | 0.98-1.69   | NS       |
| Stage                |              |             |          |
| Advanced vs early    | 7.78         | 3.96-15.26  | <0.001   |
| Depth of invasion    |              |             |          |
| Serosal vs non-serosal invasion | 3.96 | 2.67-5.89  | <0.001   |
| Lymph node metastasis|              |             |          |
| Yes vs no            | 2.47         | 1.78-3.43   | <0.001   |
| Lymphatic invasion   |              |             |          |
| Yes vs no            | 2.17         | 1.53-3.07   | <0.001   |
| Venous invasion      |              |             |          |
| Yes vs no            | 2.45         | 1.73-3.46   | <0.001   |
| Liver metastasis     |              |             |          |
| Yes vs no            | 6.01         | 4.04-8.93   | <0.001   |
| Folate ≤ 1.90 vs > 1.90 ng/mL | 1.84 | 1.04-3.27  | 0.036    |

\(^{1}\)All P values ≥ 0.05 were considered to be statistically non-significant (NS).
Treatment response in advanced gastric cancer is usually poor, but detection of invasion warrants further study. Further prospective control studies are needed to clarify the effects of other risk factors.

The present study demonstrated several important findings: first, lower serum folate was observed in gastric cancer patients. Second, we found strong relationships between lower serum folate and various invasive phenotypes, including serosal invasion, lymphatic invasion and liver metastasis in gastric cancer. Third, serum folate level was demonstrated to be a potentially useful biomarker of gastric cancer progression in terms of occurrence, serosal invasion, lymphatic invasion and liver metastasis, but further studies are needed to confirm that serum folate is an important biomarker for gastric cancer.

In this case-control study, authors found lower serum folate was significantly associated with invasive phenotypes and patient survival of gastric cancer. Findings of this study suggest that folate depletion may play a role in the development and invasiveness of gastric cancer, but the effect of folate depletion in gastric cancer invasion warrants further investigation.

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