was 0.50 (95% CI 0.36–0.70; $P<0.001$), and the HR for severe neutropenia in comparison to absent neutropenia was 0.51 (95% CI 0.35–0.73; $P < 0.001$). The rightmost column of Table 2 also shows the results of multivariate regression analyses with neutropenia as a TVC. Neutropenia was still a highly statistically significant prognostic factor. The HR for mild neutropenia in comparison to absent neutropenia was 0.61 (95% CI 0.43–0.85; $P = 0.004$), and the HR for severe neutropenia in comparison to absent neutropenia was 0.61 (95% CI 0.41–0.88; $P = 0.009$). In subgroup analyses, both mild and severe neutropenia tended to be associated with improved prognosis in most subgroups (Figure 3). Among the patients in landmark cohorts, mild and severe neutropenia remained significant prognostic factors according to survival analyses (Table 3).

discussion

In this study, we found significantly improved survival in patients who experienced neutropenia during weekly paclitaxel administration as second-line chemotherapy for AGC. The

Table 3. Univariate and multivariate analysis with or without TVCs (landmark cohort)

Baseline and clinical features	Univariate analysis without TVC			Multivariate analysis without TVC			Multivariate analysis with TVC		
	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
Neutropenia									
Absent	1.00	–	–	1.00	–	–	1.00	–	–
Mild	0.68	0.49–0.98	*0.039*	0.54	0.36–0.82	*0.004*	0.60	0.41–0.88	*0.009*
Severe	0.71	0.46–1.06	0.13	0.61	0.39–0.95	*0.032*	0.65	0.44–0.98	*0.048*
Age (years)									
<65	1.00	–	–	1.00	–	–	1.00	–	–
≥65	1.15	0.84–1.58	0.39	–*	–*	–*	–*	–*	–*
Gender									
Male	1.00	–	–	1.00	–	–	1.00	–	–
Female	1.11	0.81–1.54	0.52	–*	–*	–*	–*	–*	–*
ECOG PS									
0–1	1.00	–	–	1.00	–	–	1.00	–	–
2	2.21	1.53–3.19	*<0.001*	2.25	1.51–3.38	*<0.001*	2.27	1.57–3.28	*<0.001*
Histological type									
Diffuse	1.00	–	–	1.00	–	–	1.00	–	–
Intestinal	0.90	0.63–1.29	0.54	0.72	0.49–1.09	0.12	0.74	0.50–1.11	0.13
Disease status									
Advanced	1.00	–	–	1.00	–	–	1.00	–	–
Recurrent	0.75	0.54–1.01	0.06	–*	–*	–*	–*	–*	–*
Prior gastrectomy									
No	1.00	–	–	1.00	–	–	1.00	–	–
Yes	0.85	0.62–1.16	0.31	–*	–*	–*	–*	–*	–*
Adjuvant chemotherapy									
No	1.00	–	–	1.00	–	–	1.00	–	–
Yes	0.67	0.46–0.98	*0.043*	0.60	0.38–0.91	*0.03*	0.66	0.45–0.97	*0.035*
Peritoneal metastasis									
No	1.00	–	–	1.00	–	–	1.00	–	–
Yes	1.36	0.97–1.89	0.07	–*	–*	–*	1.29	0.91–1.82	0.14
Liver metastasis									
No	1.00	–	–	1.00	–	–	1.00	–	–
Yes	1.08	0.76–1.53	0.67	–*	–*	–*	–*	–*	–*
No. of metastatic sites									
1	1.00	–	–	1.00	–	–	1.00	–	–
2 or more	1.90	1.38–2.61	*<0.001*	2.09	1.42–3.09	*<0.001*	1.91	1.39–2.62	*<0.001*
First-line chemotherapy									
Monotherapy	1.00	–	–	1.00	–	–	1.00	–	–
Combination	1.12	0.79–1.57	0.52	–*	–*	–*	–*	–*	–*
TTF of first line									
<Median	1.00	–	–	1.00	–	–	1.00	–	–
≥Median	0.83	0.60–1.12	0.21	1.28	0.90–1.81	0.16	1.21	0.89–1.83	0.19
Pretreatment neutrophil count									
<Median	1.00	–	–	1.00	–	–	1.00	–	–
≥Median	1.28	0.94–1.74	0.11	1.29	0.92–1.80	0.14	1.25	0.90–1.75	0.18

TVC, time-varying covariate; HR, hazard ratio; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status; TTF, time to treatment failure. –* indicates variable excluded from the model by stepwise method. Statistically significant values are in italics.

frequency of neutropenia in this study is comparable to previous clinical study reports on weekly paclitaxel regimens for AGC [13, 14]. Our results indicate that both mild and severe episodes of neutropenia occurring during chemotherapy have a significant impact on the risk of death, after adjustment for baseline prognostic factors. To the best of our knowledge, this is the first evidence of this phenomenon in pretreated advanced cancer patients. From our results, we speculate that neutropenia, an indication of bone marrow suppression caused by a particular dose of chemotherapeutic agent, may also be a surrogate marker that indicates that the same dose is adequate in providing an antitumor effect. In other words, lack of neutropenia indicates an absent or weak biological effect of chemotherapy, which is possibly due to administering too low a dose to an individual patient. The causes of this interpatient variation are unclear, but genetic polymorphisms involved with drug metabolism or elimination may be among them.

Since neutropenia does not exist before the initiation of chemotherapy, a false association between neutropenia and patient outcomes might have been observed because of a higher incidence of neutropenia with increasing cycles of chemotherapy in patients with better prognosis. Therefore, to answer our *a priori* hypothesis, analyses using the Cox proportional hazards model were carried out to remove confounding factors and neutropenia as TVCs, which is one of the strengths of this study. We further evaluated the impact of neutropenia in patients who survived >2.5 months and found that neutropenia consistently showed improved survival. Additionally, 74.4% of patients with neutropenia experienced their highest grade within 4 weeks, and those without neutropenia during the first 4 weeks rarely experienced severe late-onset neutropenia. These observations were similar to results in our previous report on patients with metastatic colorectal cancer who received FOLFOX as first-line chemotherapy [9]. These results indicate that the possibility of false-positive association by lead-time bias is low. In addition, the feasibility of a weekly paclitaxel regimen is also confirmed in our study since the frequencies of toxic effects other than neutropenia are low. These findings provide some insight for future evaluations of dose escalation in patients without neutropenia during the early course of treatment to prolong the survival. Neutropenia in earlier cycles can be used as a surrogate marker for adequate paclitaxel dose intensity, and future evaluation of this procedure is strongly warranted.

There are several methodological issues. This was a retrospective cohort study evaluating the association between neutropenia and overall survival. There are several reports that high neutrophil or leukocyte counts before treatment might be poor prognostic factors and that these patients might be less likely to experience neutropenia during treatment; however, our multivariate analysis, which included the pretreatment neutrophil counts, showed that neutropenia during chemotherapy was independently associated with prognosis. Since the median number of chemotherapy cycles was higher in patients with neutropenia compared with those without neutropenia. There might be the possibility that duration of chemotherapy itself might affect the treatment results. However, since most patients discontinued treatment due to disease progression, there might be the possibility that treatment was discontinued earlier by tumor progression due to insufficient dose. Therefore, it is questionable that duration of chemotherapy itself might affect the treatment results. The moderate sample size in this study may be a limitation, indicating that this analysis should be duplicated in another independent cohort.

In conclusion, neutropenia occurring during weekly paclitaxel treatment administered as second-line treatment to AGC patients is strongly associated with better prognosis. This may indicate that neutropenia is a surrogate marker for adequate antitumor doses of chemotherapeutic agents. An additional well-defined prospective trial that evaluates dose escalation in patients without neutropenia during the early course of treatment is warranted.

disclosure

The authors declare no conflicts of interest.

references

1. Gurney H. How to calculate the dose of chemotherapy. Br J Cancer 2002; 86: 1297–1302.
2. Poikonen P, Saarto T, Lundin J et al. Leucocyte nadir as a marker for chemotherapy efficacy in node-positive breast cancer treated with adjuvant CMF. Br J Cancer 1999; 80: 1763–1766.
3. Samson MK, Rivkin SE, Jones SE et al. Dose-response and dose-survival advantage for high versus low-dose cisplatin combined with vinblastine and bleomycin in disseminated testicular cancer. A Southwest Oncology Group study. Cancer 1984; 53: 1029–1035.
4. Rankin EM, Mill L, Kaye SB et al. A randomised study comparing standard dose carboplatin with chlorambucil and carboplatin in advanced ovarian cancer. Br J Cancer 1992; 65: 275–281.
5. Hovgaard D, Nissen NI. A phase I/II study of dose and administration of non-glycosylated bacterially synthesized G-M CSF in chemotherapy-induced neutropenia in patients with non-Hodgkin's lymphomas. Leuk Lymphoma 1992; 7: 217–224.
6. Di Maio M, Gridelli C, Gallo C et al. Chemotherapy-induced neutropenia and treatment efficacy in advanced non-small-cell lung cancer: a pooled analysis of three randomised trials. Lancet Oncol 2005; 6: 669–677.
7. Kishida Y, Kawahara M, Teramukai S et al. Chemotherapy-induced neutropenia as a prognostic factor in advanced non-small-cell lung cancer: results from Japan Multinational Trial Organization LC00-03. Br J Cancer 2009; 101(9): 1537–1542.
8. Yamanaka T, Matsumoto S, Teramukai S et al. Predictive value of chemotherapy-induced neutropenia for the efficacy of oral fluoropyrimidine S-1 in advanced gastric carcinoma. Br J Cancer 2007; 97: 37–42.
9. Shitara K, Matsuo K, Takahari D et al. Neutropenia as a prognostic factor in metastatic colorectal cancer patients undergoing chemotherapy with first-line FOLFOX. Eur J Cancer 2009; 45: 1757–1763.
10. Koizumi W, Narahara H, Hara T et al. S-1 plus cisplatin versus S-1 alone for first-line treatment of advanced gastric cancer (SPIRITS trial): a phase III trial. Lancet Oncol 2008; 9: 215–221.
11. Sparano JA, Wang M, Martino S et al. Weekly paclitaxel in the adjuvant treatment of breast cancer. N Engl J Med 2008; 358: 1663–1671.
12. Katsumata N, Yasuda M, Takahashi F et al. Dose-dense paclitaxel once a week in combination with carboplatin every 3 weeks for advanced ovarian cancer: a phase 3, open-label, randomised controlled trial. Lancet 2009; 374(9698): 1331–1338.
13. Hironaka S, Zenda S, Boku N et al. Weekly paclitaxel as second-line chemotherapy for advanced or recurrent gastric cancer. Gastric Cancer 2006; 9(1): 14–18.
14. Kodera Y, Ito S, Mochizuki Y et al. A phase II study of weekly paclitaxel as second-line chemotherapy for advanced gastric cancer (CCOG0302 study). Anticancer Res 2007; 27(4C): 2667–2671.

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Research Article

Cancer Epidemiology, Biomarkers & Prevention

Folate Intake along with Genetic Polymorphisms in Methylenetetrahydrofolate Reductase and Thymidylate Synthase in Patients with Advanced Gastric Cancer

Kohei Shitara[1], Kei Muro[1], Seiji Ito[2], Akira Sawaki[3], Masahiro Tajika[3], Hiroki Kawai[3], Tomoya Yokota[1], Daisuke Takahari[1], Takashi Shibata[1], Takashi Ura[1], Hidemi Ito[4], Satoyo Hosono[4], Takakazu Kawase[4], Miki Watanabe[4], Kazuo Tajima[5], Yasushi Yatabe[6], Hideo Tanaka[4,7], and Keitaro Matsuo[4,7]

Abstract

Background: A relationship between dietary folate intake and efficacy of fluorouracil (FU) is supported by preclinical data. Furthermore, there are several reports that evaluated genetic polymorphisms of *MTHFR* (methylenetetrahydrofolate reductase) or *TYMS* (thymidylate synthase) and efficacy of FU. However, to our knowledge, there are no reports that evaluate simultaneously the effects of folate intake and genetic polymorphisms on clinical outcome of gastric cancer patients.

Methods: We retrospectively analyzed the survival impact of estimated folate intake by a food frequency questionnaire and *MTHFR* and *TYMS* polymorphisms in 132 patients with advanced gastric cancer who were treated with first-line FU-based chemotherapy.

Results: Median overall survival was 11.3 months (95% confidence interval, 9.4-13.4 mo) and median progression-free survival was 5.2 months (95% confidence interval, 4.1-6.3 mo). Patients with folate intake of >260 μg/day (n = 88) showed longer overall survival compared with low folate intake (n = 44; overall survival, 12.2 versus 8.4 mo). In a multivariate Cox model, patients who had folate intake of >260 μg/day, *MTHFR* 677 TT polymorphism, and *TYMS*-3′ untranslated region 6-bp insertion were associated with better survival. Similar tendency was observed in progression-free survival. No interaction was observed between folate intake and favorable genotypes.

Conclusion: Folate intake and genetic polymorphisms of *MTHFR* and *TYMS* were associated with better clinical outcome by FU-based chemotherapy in advanced gastric cancer.

Impact: Our results suggested folate intake and folate-related genetic polymorphisms may play an important role in efficacy of FU-based chemotherapy in advanced gastric cancer. *Cancer Epidemiol Biomarkers Prev; 19(5); 1311–9. ©2010 AACR.*

Introduction

Fluorouracil (FU) is the most widely used drug for advanced gastric cancer. Oral fluoropyrimidines, such as capecitabine or S-1, which contain the prodrug of FU, show similar efficacy to FU (1-3). FU is converted to 5-fluorodUMP, which forms a ternary complex with thymidylate synthase (TYMS) and 5-10-methylene tetrahydrofolate (4). Formation of this ternary complex results in sustained inhibition of TYMS and further DNA synthesis, which is thought to be the predominant mechanism of the antitumor effect of FU or fluoropyrimidines (4).

Folate metabolism is an important pathway for the antitumor effect of FU because antitumor activity is dependent on an interaction with folate metabolism (4). Increased 5-10-methylene tetrahydrofolate may produce tighter ternary complexes and improved antitumor efficacy of FU. 5,10-Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in folate metabolism, which catalyzes the irreversible conversion of 5-10-methylene tetrahydrofolate to 5-methyltetrahydrofolate (5). Because decreased activity of MTHFR may result in accumulation of 5-10-methylene tetrahydrofolate and improve the antitumor efficacy of FU, several studies evaluated genetic polymorphisms of *MTHFR*, with or without genetic polymorphisms of *TYMS* in patients with advanced gastric cancer, although the clinical data are still controversial (6-10).

Authors' Affiliations: Departments of [1]Clinical Oncology, [2]Surgery, [3]Gastroenterology, [4]Division of Epidemiology and Prevention, [5]Director, Aichi Cancer Center Research Institute, [6]Department of Pathology and Molecular Diagnostics, Aichi Cancer Center Hospital, [7]Department of Epidemiology, Nagoya University Graduate School of Medicine, Nagoya, Aichi, Japan

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The Ministry of Education, Science, Sports, Culture and Technology of Japan was not involved in the study design, subject enrollment, study analysis or interpretation, and submission of the manuscript for this study.

Corresponding Author: Kohei Shitara, Department of Clinical Oncology, Aichi Cancer Center Hospital, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Aichi, Japan. Phone: 81-52-762-6111; Fax: 81-52-752-8390. E-mail: Kouheis0824@yahoo.co.jp

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In preclinical data, a substantial effect of dietary folate intake on the efficacy and safety of FU was suggested (11, 12), although only two clinical studies in colorectal cancer evaluated this relationship (13, 14). In addition, combined analysis between folate intake and genetic polymorphisms of *MTHFR* or *TYMS* is reported to be important when investigating gastric cancer risk (15-17). However, there are no reports that evaluate the effect of folate intake and genetic polymorphisms simultaneously on clinical outcome of advanced gastric cancer.

To address this issue, we did a retrospective cohort study using data from the Hospital-Based Epidemiologic Research Program at Aichi Cancer Center (HERPACC) combined with clinical data from Aichi Cancer Center Hospital, Japan.

Materials and Methods

Patients. Cases were selected from the database of the HERPACC conducted at Aichi Cancer Center Hospital. Details of the HERPACC have been described elsewhere (18, 19). In brief, 23,408 HERPACC-enrolled, first-visit outpatients treated between January 2001 and November 2005 were asked to provide blood samples in addition to information on lifestyle factors. Of those who participated, 22,727 (97.1%) subjects completed the questionnaire satisfactorily and were enrolled in the HERPACC. The study was approved by the Institutional Ethical Committee of Aichi Cancer Center Hospital.

In the present study, cases of newly diagnosed advanced gastric cancer who participated in the HERPACC with the following criteria were included: (*a*) presence of histologically or cytologically proven, inoperable gastric cancer, (*b*) treated with first-line chemotherapy with FU, (*c*) performance status according to the Eastern Cooperative Oncology Group criteria of 0 to 2, (*d*) sufficient oral intake possible (no use of i.v. hyperalimentation, (*e*) written informed consent before chemotherapy, and (*f*) blood samples available for the analysis. During the same period of HERPACC, overall 267 patients with advanced gastric cancer were treated by chemotherapy in our hospital. Among them, 212 patients completed the questionnaire of HERPACC (79.4%) and 132 patients met the criteria and were included as subjects in this study.

Estimation of folate intake. Folate intake was estimated through responses in the HERPACC questionnaire. The HERPACC questionnaire included items on demographic characteristics, family and individual medical history, height and weight, exercise, smoking and drinking habits, vitamin use, and consumption of selected foods and beverages. All dietary exposures were determined by a food frequency questionnaire (20, 21), a self-administered questionnaire given to patients at their first visit to Aichi Cancer Center Hospital before any diagnostic procedures were conducted. Briefly, the food frequency questionnaire consisted of 47 single food items with frequencies in eight categories. We estimated the average daily intake of nutrients by multiplying the food intake (in grams) or serving size by the nutrient content per 100 g of food as listed in the standard tables of food composition. Consumption of nutrients from supplements was not considered in total vitamin consumption because the questionnaire for multivitamins was not quantitative (22). However, this might affect the results of folate intake; therefore, we included the variable use of vitamin supplement (yes, 1; no, 2) in the multivariate analysis. Energy-adjusted intakes of food groups and nutrients were calculated using the residual method (23). The food frequency questionnaire was validated by referring to a 3-day weighted dietary record as a standard, which showed validity (20) and reproducibility to be satisfactory. The deattenuated *r*'s for energy-adjusted

Table 1. Patient characteristics and genetic polymorphisms

Characteristics		*n* (%; *N* = 132)
Age	Median (range)	58 (30-80)
Gender	Male	91 (69)
	Female	41 (31)
ECOG PS	0-1	111 (84)
	2	21 (16)
Disease status	Advanced	98 (74)
	Recurrent	34 (26)
Pathologic type	Diffuse	103 (78)
	Intestinal	29 (22)
Previous gastrectomy	Yes	59 (45)
	No	73 (55)
Adjuvant	Yes	8 (6)
	No	124 (94)
Metastatic place	1	73 (55)
	≥2	59 (45)
Ascites	Yes	28 (21)
	No	104 (79)
Folate intake*	Low	44 (33)
	Medium	44 (33)
	High	44 (33)
MTHFR 677	C/C	53 (40)
	C/T	59 (45)
	T/T	20 (15)
TYMS-5'UTR	2R/2R	3 (2)
	2R/3R	41 (31)
	3R/3R or 3R/other	88 (67)
TYMS-3'UTR	+6 bp/+6 bp	19 (14)
	+6 bp/−6 bp	68 (52)
	−6 bp/−6 bp	45 (34)

Abbreviations: ECOG, Eastern Cooperative Oncology Group; PS, performance status.
*Folate intake was divided in three groups: low (≤260 μg/d), medium (>260 and <340 μg/d), and high (≥340 μg/d).

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Table 2. Univariate and multivariate analysis of overall survival

Variant	Genotype/classification	*n*	Univariate analysis		Multivariate analysis	
			HR (95% CI)	*P*	HR (95% CI)	*P*
Folate intake	Low (≤260 μg/d)	44	1.00		1.00	
	Medium/high (>260 μg/d)	88	0.60 (0.41-0.89)	0.013	0.65 (0.44-0.96)	0.030
MTHFR 677	C/C or C/T	112	1.00		1.00	
	T/T	20	0.78 (0.48-1.2)	0.300	0.57 (0.33-0.97)	0.039
TYMS-5′UTR	2R/2R or 2R/3R	44	1.00		1.00	
	3R/3R or 3R/other	88	0.87 (0.59-1.28)	0.508	0.78 (0.51-1.18)	0.220
TYMS-3′UTR	−6 bp/−6 bp or +6 bp/−6 bp	113	1.00		1.00	
	+6 bp/+6 bp	19	0.45 (0.25-0.81)	0.008	0.41 (0.22-0.76)	0.005

NOTE: Adjusted by age, performance status, pathologic type, disease status, previous gastrectomy, adjuvant, ascites, metastatic location, regimens, vitamin use, and calorie intake (less than median or more than or equal to median).
Abbreviation: HR, hazard ratio.

intakes of folate were 0.36 [95% confidence interval (95% CI), 0.12-0.58] in men and 0.38 (95% CI, 0.25-0.62) in women, respectively.

Evaluation of genetic polymorphisms. DNA of each subject was extracted from the buffy coat fraction with the DNA Blood mini Kit (Qiagen K.K.). Genotyping for the *MTHFR* C677T [a database of Single Nucleotide Polymorphism (dbSNP) ID, rs1801133] was based upon Taqman Assays (Applied Biosystems). The *TYMS* 28-bp variable number of tandem repeat polymorphism (dbSNP ID, rs45445694) was defined by PCR using 5′-CGTGGCTCCTGCGTTTCC-3′ and 5′-GAGCCGGCCACAGGCAT-3′ primers. The *TYMS* 6-bp insertion/deletion (6/6) in the 3′ untranslated region (UTR) polymorphism (dbSNP ID, rs16430) was determined by PCR using 5′-CAAATCTGAGGGAGCTGAGT-3′ and 5′-CAGATAAGTGGCAGTACAGA-3′ primers followed by digestion with the restriction enzyme *Dra*I (New England BioLabs). Five percent of the samples were examined in duplicate for consistency, and 100% agreement was observed.

Evaluation of treatment and statistical methods. The primary purpose of this study was to evaluate the association between estimated folate intake, genetic polymorphisms, and overall survival, which was defined as the interval between the date of initial chemotherapy to the date of death or last follow-up using the Kaplan-Meier method. Progression-free survival associated with first-line chemotherapy was also measured from the beginning of treatment to the date of disease progression, which was evaluated by each physician. Vital status or disease status was confirmed by checking medical record at the last date of follow-up visit. In the case of lost to follow-up, vital status was confirmed by census registration conducted annually. Association between genetic polymorphisms, folate intake, and progression-free survival was also evaluated. To evaluate the effect of genetic polymorphisms and folate intake on overall survival and progression-free survival, univariate and multivariate Cox proportional hazards modeling was applied. Therefore, a measure of association in this study was the hazard ratio along with a 95% CI. Forward and backward stepwise methods were used for model building using threshold *P* values 0.10 for inclusion and 0.20 for exclusion. Toxicity during first-line chemotherapy was also evaluated and graded according to the National Cancer Institute Common Toxicity Criteria version 3.0. Distribution of subject characteristics was assessed by the χ^2 test or the Fisher's exact test as appropriate. Statistical analyses were done using STATA ver. 10 (StataCorp LP). All tests were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Patient characteristics and survival. Detailed characteristics of 132 patients are shown in Table 1. Although vitamin supplement was not reported in detail, any vitamin supplement was used in 30 (22.7%) of 132 patients. First-line chemotherapy was administered as follows: S-1 alone ($n = 92$), S-1 combination ($n = 19$; S-1 + cisplatin in 16 patients, S-1 + docetaxel in two patients, S-1 + irinotecan in one patient), and other FU combinations ($n = 21$; FU + cisplatin in 13 patients, FU + methotrexate in eight patients), indicating fewer patients received combination chemotherapy ($n = 40$; 30%) compared to monotherapy ($n = 92$; 70%). Detailed schedule of each chemotherapy was shown in Supplementary Table S1 and in reference (3, 24-28). Median duration of first-line chemotherapy was 4.8 months. One hundred twenty-three patients experienced disease progression with a median progression-free survival of 5.2 months (95% CI, 4.1-6.3 mo). Among the patients who experienced disease progression, second-line chemotherapy was applied in 90 patients (73%). At the time of analysis, 118 (89%) patients had died, with a median follow-up of 61 months since initiation of

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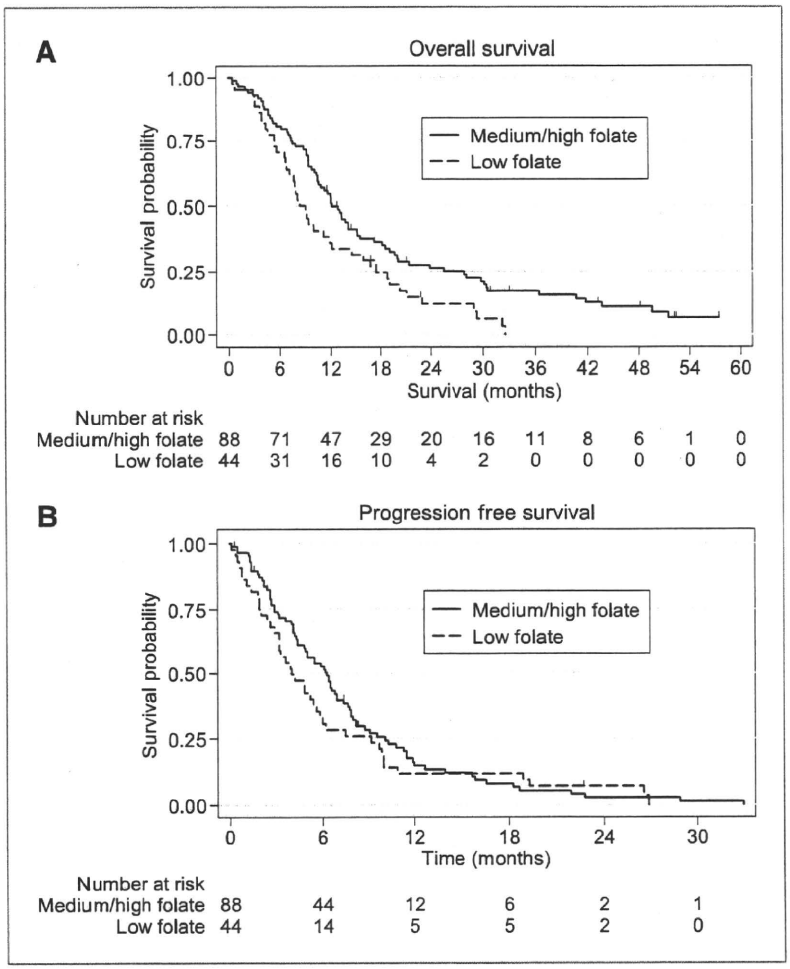

Figure 1. A, Kaplan-Meier survival curves of overall survival. Patients with intermediate or high folate intake (n = 88) were significantly associated with better survival than patients with low folate intake (n = 44; overall survival, 12.2 versus 8.2 mo; hazard ratio, 0.65; 95% CI, 0.44-0.96; P = 0.03). B, Kaplan-Meier survival curves of progression-free survival. Patients with intermediate or high folate tended to be associated with longer progression-free survival without statistical significance (progression-free survival, 6.3 versus 4.0 mo; hazard ratio, 0.74; 95% CI, 0.50-1.07; P = 0.094; Fig. 2).

first-line chemotherapy. Median overall survival for all patients was 11.3 months (95% CI, 9.4-13.4 mo), which was almost similar to the patients with advanced gastric cancer who were treated in our hospital and were not included in this analysis (n = 135; 11.5 mo; 95% CI, 9.8-12.6 mo).

***Results of folate intake and genetic polymorphisms of* MTHFR *and* TYMS.** Estimated folate intake was divided into three groups: low (≤260 μg/d; lowest tertile), medium (>260 and ≤340 μg/d; middle tertile), and high (>340 μg/d; highest tertile), and the number of patients in these groups was 44, 44, and 44, respectively. The estimated median total calorie intakes in each folate group were as follows: 1,580 kcal (range, 1,131-2,498), 1,600 kcal (range, 910-2,667), and 1,703 kcal (range, 965-2,467).

The frequencies and types of the *MTHFR* C677T polymorphisms, polymorphic variable number of tandem repeat in the *TYMS*-5′UTR region, and the 6+/6− polymorphism in the *TYMS*-3′UTR were shown in Table 1. Genotype distributions of all the polymorphisms were in accordance with Hardy-Weinberg equilibrium. We also confirmed genotyping of insertion/deletion for by sequencing of 10% of samples with completed accordance.

Overall survival according to folate intake and genetic polymorphisms. Table 2 shows univariate and multivariate analyses of folate intake and genetic polymorphisms as prognostic factors for a better overall survival. In the multivariate analysis, patients with intermediate or high folate intake (n = 88) were significantly associated with better survival than patients with low folate intake (n = 44; overall survival, 12.2 versus 8.2 mo; hazard ratio, 0.65; 95% CI, 0.44-0.96; P = 0.03; Fig. 1A). Hazard ratios for medium and high groups were 0.65 (95% CI, 0.41-1.03) and 0.62 (95% CI, 0.38-1.01), respectively. Therefore, we decided to conduct analyses by dichotomization by medium/high (>260 μg/d) versus low (≤260 μg/d) folate intake. In addition, when we stratified other clinical factors, vitamin use, or estimated total calorie, medium/high folate intake tended to be associated with improved prognosis in almost all subgroups (Fig. 1B).

Patients who had *MTHFR* TT also had significantly better survival compared with patients with CT or

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CC (hazard ratio, 0.57; 95% CI, 0.33-0.97; $P = 0.039$). Hazard ratios for CT and TT compared with CC were 1.05 (95% CI, 0.70-1.65) and 0.59 (95% CI, 0.32-1.06), respectively. Therefore, we used the recessive model (CC/CT versus TT).

Patients who had +6/+6 in *TYMS*-3′UTR had also significantly better survival compared with −6/−6 or −6/+6 (hazard ratio, 0.41; 95% CI, 0.22-0.76; $P = 0.01$). In contrast, no association was seen between presence of *TYMS*-5′UTR and survival (hazard ratio, 0.78; 95% CI, 0.51-1.18; $P = 0.220$). We did not conduct the haplotype analysis because linkage disequilibrium between repeat polymorphism and insertion/deletion polymorphism was very low ($R^2 = 0.03$; $D' = 0.3$).

Progression-free survival according to folate intake and genetic polymorphisms. According to the multivariate analysis for progression-free survival, patients with intermediate or high folate tended to be associated with longer progression-free survival with marginal statistical significance (progression-free survival, 6.3 versus 4.0 mo; hazard ratio, 0.74; 95% CI, 0.50-1.07; $P = 0.094$; Table 3; Fig. 2). Presence of +6/+6 in *TYMS*-3′UTR or *MTHFR* TT was significantly associated with longer progression-free survival (Table 3), similar to what was seen in overall survival.

Interaction of genetic polymorphisms and folate intake. The interaction of favorable genotypes and folate intake is shown in Table 4. When we stratified patients according to genotypes, medium or high folate intake was associated with better survival regardless of any genotypes, and no significant interaction was observed between folate intake and genetic polymorphisms on either overall survival or progression-free survival.

Relationships between toxicity and folate intake or genetic polymorphisms. Hematologic toxicity (grade 3-4) was observed in 34 patients (20.3%), and nonhematologic toxicity (grade 3-4) was seen in 21 patients (15.9%). The frequency of hematologic and nonhematologic grade 3 to 4 toxicity was significantly higher in patients with medium/high folate intake than low folate intake after it was adjusted by age, performance status, gender, and regimens (Table 5). Although favorable genotypes did not correlate to grade3 to 4 toxicity, *MTHFR* TT tend to be associated with higher frequency of hematologic toxicity with borderline significance ($P = 0.072$; Table 5).

Discussion

In this study, we found that medium or high amount of folate intake and genetic polymorphisms in *TYMS* and *MTHFR* were associated with significantly better survival in advanced gastric cancer patients treated with FU-based chemotherapy. Similar tendencies were observed in progression-free survival, thus inferring that folate intake and these two polymorphisms have predictive values for FU-based chemotherapy in advanced gastric cancer. However, folate intake and genetic polymorphisms were independent, and no significant interaction was observed. In addition, folate intake was associated with increased frequency of toxicity.

To our knowledge, there are only two studies that directly evaluated treatment outcome of FU-based chemotherapy and folate (13, 14). In one study, Canadian patients receiving adjuvant FU and leucovorin were prospectively assessed for biomarkers of folate metabolism (13). Multivariate analyses identified baseline serum folate as an independent positive predictor of grade 3 and/or 4 toxic effect. Similar results were found in a study on capecitabine monotherapy in Australia (14), in which patients with higher baseline levels of serum folate had a significantly increased incidence of toxic events. These two reports suggested importance of folate in patients with treated with FU, although they did not evaluate the efficacy of treatment or the genetic polymorphisms. In contrast, we evaluated efficacy (overall survival and progression-free survival) and toxicity in this study. In addition, we evaluate folate and genetic polymorphisms simultaneously. As a result, increased

Table 3. Univariate and multivariate analysis of progression-free survival

Variant	Genotype/classification	*n*	Univariate analysis		Multivariate analysis	
			HR (95% CI)	*P*	HR (95% CI)	*P*
Folate intake	Low (≤260 μg/d)	44	1.00		1.00	
	Medium/high (>260 μg/d)	88	0.81 (0.55-1.18)	0.280	0.74 (0.50-1.07)	0.094
MTHFR 677	C/C or C/T	112	1.00		1.00	
	T/T	20	0.66 (0.38-1.15)	0.140	0.53 (0.29-0.99)	0.046
TYMS-5′UTR	2R/2R or 2R/3R	44	1.00		1.00	
	3R/3R or other	88	1.10 (0.77-1.69)	0.500	0.94 (0.62-1.43)	0.780
TYMS-3′UTR	−6 bp/−6 bp or +6 bp/−6 bp	113	1.00		1.00	
	+6 bp/+6 bp	19	0.56 (0.31-0.99)	0.049	0.49 (0.25-0.9)	0.029

NOTE: Adjusted by age, performance status, pathologic type, disease status, previous gastrectomy, adjuvant, ascites, metastatic location, regimens, vitamin use, and calorie intake (less than median or more than or equal to median).

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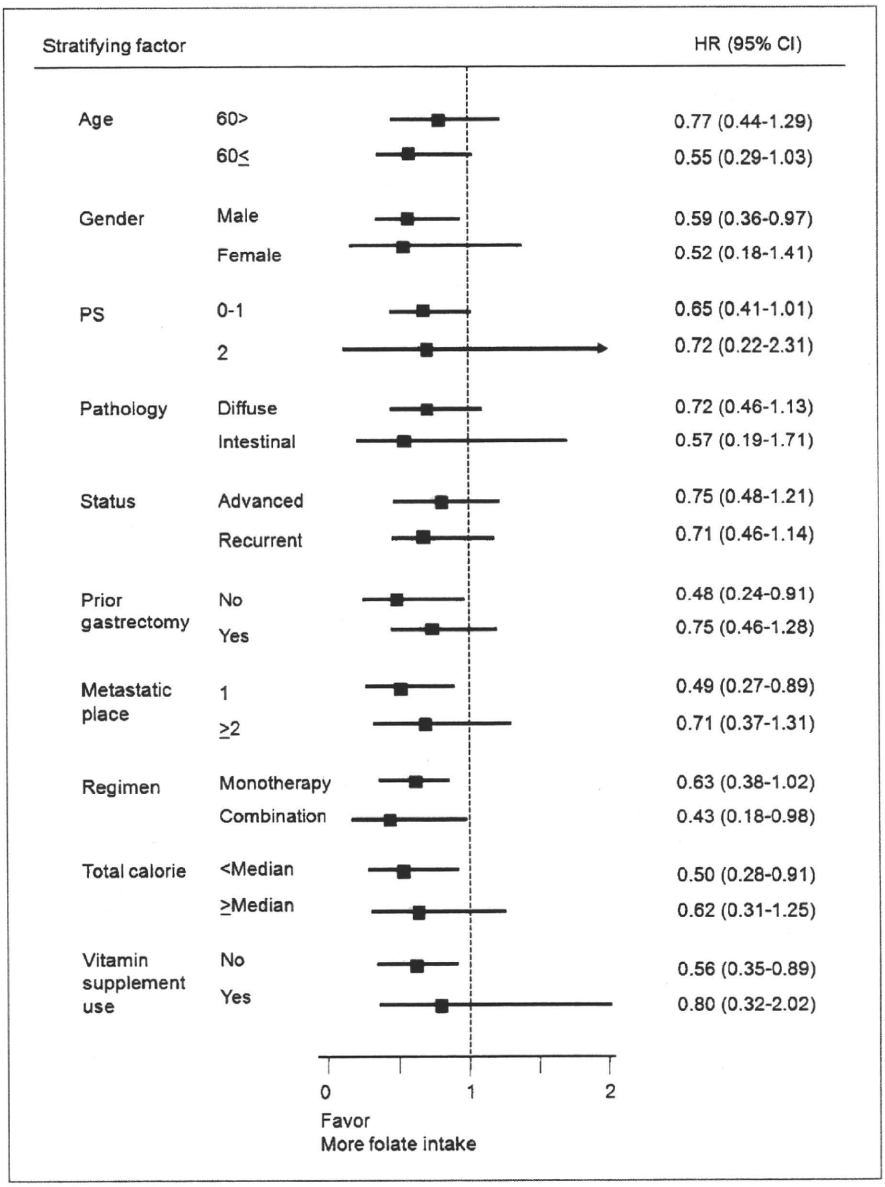

Figure 2. Hazard ratios for death and 95% CIs. HR, hazard ratio. In subgroup analyses, medium/high folate intake tended to be associated with improved prognosis in almost all subgroups. PS, performance status.

folate intake was associated with better survival of advanced gastric cancer in this study. Although the cause of this association is unclear, one may suggest that sufficient folate intake might be important for FU to exert its antitumor effect. Supporting this hypothesis, folinic acid, which increases the 5-10-methylene tetrahydrofolate levels in cancer cells, is reported to result in a tighter ternary complex of TS, 5-10-methylene tetrahydrofolate, and 5-fluoro-dUMP (4) and showed increased antitumor effects of FU in gastric cancer (29). Therefore, in addition to folinic acid, daily supplementation of folate may be an important factor having an impact on the efficacy of FU. Toxicity is also higher in patients with medium/or high folate intake patients, which suggests increased cytotoxicity by folate intake not only in cancer cells but also in normal tissue. Although methodology was different in our study (estimated folate intake) and previous studies (serum folate), these results might suggest importance of folate in FU-based chemotherapy. Because the impact of folate intake was more intense in overall survival than progression-free survival in our study, other mechanisms than through FU may exist that explain the better survival of advanced gastric cancer patients with higher folate intake.

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Table 4. Association of folate intake and survival stratification by genotype or other clinical factors

		Folate intake*	*n*	Multivariate analysis for OS		Multivariate analysis for PFS	
				HR (95% CI)	*P*†	HR (95% CI)	*P*†
MTHFR 677	C/C or C/T	Low	36	1.00	0.55	1.00	0.83
		Medium/high	76	0.59 (0.37-0.92)		0.73 (0.48-1.11)	
	T/T	Low	8	1.00		1.00	
		Medium/high	12	1.19 (0.22-6.3)		3.1 (0.52-19)	
TYMS-5'UTR	2R/2R or 2R/3R	Low	17	1.00	0.45	1.00	0.12
		Medium/high	27	0.44 (0.21-0.92)		0.51 (0.24-1.07)	
	3R/3R or other	Low	27	1.00		1.00	
		Medium/high	61	0.69 (0.43-1.15)		1.02 (0.61-1.71)	
TYMS-3'UTR	−6 bp/−6 bp or	Low	37	1.00	0.27	1.00	0.98
	+6 bp/−6 bp	Medium/high	76	0.66 (0.43-1.01)		0.71 (0.47-1.08)	
	+6 bp/+6 bp	Low	7	1.00		1.00	
		Medium/high	12	0.10 (0.01-0.46)		0.33 (0.03-3.9)	

NOTE: Adjusted by age, performance status, pathologic type, disease status, previous gastrectomy, adjuvant, metastatic location, regimens, vitamin supplement use, and calorie intake (less than median or more than or equal to median).
Abbreviations: OS, overall survival; PFS, progression-free survival.
*Folate intake was divided as follows: low (≤260 μg/d) and medium/high (>260 μg/d).
†For interaction.

The *MTHFR* 677TT genotype was strongly associated with better clinical outcome according to our multivariate survival analysis. *MTHFR* 677C/T results in an alanine-to-valine substitution that induces a thermolabile variant of the enzyme with reduced activity (5). This may result in accumulation of 5-10-methylene tetrahydrofolate and improved efficacy of FU. Not only better survival but also relatively higher toxicity was seen in TT type in this study, which may support increased cytotoxicity in TT type. In addition, no gene-folate interaction was seen in this study; *MTHFR* 677TT and folate intake may complementarily increase levels of 5-fluoro-dUMP and therefore increase the effect of FU.

In our study, patients homozygous for the insertion (+6 bp/+6 bp) polymorphism *TYMS*-3UTR had significantly better survival than those homozygous for the deletion or those that were heterozygous (+6 bp/−6 bp). Given the controversies on the biological significance of the *TYMS*-3UTR insertion/deletion polymorphism (30, 31), further studies are necessary to evaluate several genetic

Table 5. The frequency of toxicity according to genetic polymorphisms or folate intake

Variant		Toxicity					
		Hematologic (grade 3-4)			Nonhematologic (grade 3-4)		
		n (%)	OR (95% CI)	*P*	*n* (%)	OR (95% CI)	*P*
Folate intake*	Low (*n* = 44)	4 (9)	1.00		5 (11)	1.00	
	Medium/high (*n* = 88)	23 (26)	3.91 (1.21-12.6)	0.022	16 (18)	1.81 (0.60-5.34)	0.29
MTHFR 677	C/C or C/T (*n* = 112)	20 (17)	1.00		16 (14)	1.00	
	T/T (*n* = 20)	7 (35)	2.87 (0.90-8.91)	0.072	5 (25)	2.00 (0.59-6.75)	0.24
TYMS-5'UTR	2R/2R or 2R/3R (*n* = 44)	9 (20)	1.00		7 (15)	1.00	
	3R/3R or 3R/other (*n* = 88)	18 (20)	1.14 (0.42-3.12)	0.81	14 (15)	1.23 (0.42-3.59)	0.71
TYMS-3'UTR	−6 bp/−6 bp or +6 bp/−6 bp (*n* = 113)	23 (20)	1.00		17 (19)	1.00	
	+6 bp/+6 bp (*n* = 19)	4 (21)	1.07 (0.28-4.11)	0.92	4 (21)	1.66 (0.42-6.41)	0.47

NOTE: Adjusted by age, performance status, gender, regimens, vitamin supplement use, and calorie intake (less than median or more than or equal to median).
Abbreviation: OR, odds ratio.
*Folate intake was divided as follows: low (≤260 μg/d) and medium/high (>260 μg/d).

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polymorphisms of TYMS in terms of not only clinical outcome but also resulting TYMS activity in blood and tumor tissue simultaneously.

An additional *TYMS* polymorphism consisting of two or three 28-bp repeated sequences in the *TYMS* 5′UTR locus did not show any impact on clinical outcome in our study. Several studies on TYMS expression and this polymorphism were reported especially in colorectal cancer, with some clinical studies showing favorable results in 2R/2R (32-34). However, two recent studies in advanced gastric cancer did not show any significant impact of this polymorphism as seen with our results (7, 9). Although further studies may be necessary, the clinical significance of this polymorphism may be limited.

Our study had several methodologic strengths. First, exposure of interest, folate intake, and polymorphisms were measured before the treatment; therefore, chronological relation between exposure and outcome was in order. Moreover, because clinicians associated with cases in this study did not know the exposure status until the study, it is less likely to introduce the view of researchers as a bias. Secondly, potential confounders such as performance status and disease status were considered in the analyses; therefore, associations that we observed were theoretically independent of confounders, although we cannot completely rule out effect of residual confounding by unevaluated factors. Lastly, given that our allele frequencies were comparable to those previously reported in public databases, bias in the distribution of selected polymorphisms was negligible.

There are several methodologic issues in this study. The food frequency questionnaire was quite short, and validity and reliability of folate intake were modest. There is a possibility of misclassification of exposure and high folate intake being a marker for other behaviors. However, we tried to include other possible factor such as performance status or disease site to exclude this bias. In addition, when we evaluated other estimated nutrients (Supplementary Table S2), none was considered to be significant other than folate intake. In addition, we did not conduct validation between estimated nutrients and biomarkers such as serum folate concentration. In addition, we did not evaluate behavior change after the diagnosis. These points are also limitations in this study.

A small portion of patients received combination chemotherapy because monotherapy of FU or S-1 was the standard chemotherapy regimen at the time of this study. Current standard treatment for advanced gastric cancer in Japan and other countries is FU plus platinum if tolerable, so our patient population does not directly reflect current clinical practice. However, combined agents may also make the impact of genetic polymorphism on the effect of FU itself more obscure. Therefore, this study may be more suggestive in evaluating the impact of polymorphisms on FU itself compared with other studies that evaluate patients receiving combined chemotherapy. In our study, when we limited the cohort to patients who received S-1 alone as first-line chemotherapy (n = 91), almost similar results were obtained (Supplementary Table S3). FU or fluoropyrimidine alone is considered to be optimal for frail patients or those receiving adjuvant chemotherapy, making our results useful to predict patients who may benefit from FU-based chemotherapy. As for genetic polymorphisms, we did not genotype the SNP located within the promoter repeat of TS, which might be confounding the results. In addition, the small sample size used may be a study limitation, which may contribute to lack of statistical power to show the interaction between folate intake and genetic polymorphisms. Therefore, further study is required to duplicate this work in a larger cohort.

In conclusion, this is the first report that simultaneously evaluated the effects of folate intake and genetic polymorphisms on clinical outcome of FU-based chemotherapy in advanced gastric cancer. Our findings indicate that folate intake and genetic polymorphisms of *MTHFR* or *TYMS* may play an important role in FU-based chemotherapy in advanced gastric cancer. Further prospective evaluation is warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Cunningham D, Starling N, Rao S, et al. Capecitabine and oxaliplatin for advanced esophagogastric cancer. N Engl J Med 2008;358:36–46.
2. Kang YK, Kang WK, Shin DB, et al. Capecitabine/cisplatin versus 5-fluorouracil/cisplatin as first-line therapy in patients with advanced gastric cancer: a randomised phase III noninferiority trial. Ann Oncol 2009;20:666–73.
3. Boku N, Yamamoto S, Fukuda H, et al. Fluorouracil versus combination of irinotecan plus cisplatin versus S-1 in metastatic gastric cancer: a randomised phase 3 study. Lancet Oncol 2009;10:1063–9.
4. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer 2003;3:330–8.
5. De Mattia E, Toffoli G. C677T and A1298C MTHFR polymorphisms, a

DOI:10.1158/1055-9965.EPI-09-1257

challenge for antifolate and fluoropyrimidine-based therapy personalisation. Eur J Cancer 2009;45:1333–51.
6. Lee J, Jeong CK, Hong SP, et al. Clinical significance of thymidylate synthase and methylenetetrahydrofolate reductase gene polymorphism in Korean patients with gastric cancer. Korean J Gastroenterol 2005;46:32–8.
7. Ruzzo A, Graziano F, Kawakami K, et al. Pharmacogenetic profiling and clinical outcome of patients with advanced gastric cancer treated with palliative chemotherapy. J Clin Oncol 2006;24:1883–9.
8. Lu JW, Gao CM, Wu JZ, Cao HX, Tajima K, Feng JF. Polymorphism in the 3-untranslated region of the thymidylate synthase gene and sensitivity of stomach cancer to fluoropyrimidine based chemotherapy. J Hum Genet 2006;51:155–60.
9. Keam B, Im SA, Han SW, et al. Modified FOLFOX-6 chemotherapy in advanced gastric cancer: results of phase II study and comprehensive analysis of polymorphisms as a predictive and prognostic marker. BMC Cancer 2008;8:148.
10. Lu JW, Gao CM, Wu JZ, Sun XF, Wang L, Feng JF. Relationship of methylenetetrahydrofolate reductase C677T polymorphism and chemosensitivity to 5-fluorouracil in gastric carcinoma. Ai Zheng 2004; 23:958–62.
11. Branda RF, Nigels E, Lafayette AR, Hacker M. Nutritional folate status influences the efficacy and toxicity of chemotherapy in rats. Blood 1998;92:2471–6.
12. Branda RF, Chen Z, Brooks EM, Naud SJ, Trainer TD, McCormack JJ. Diet modulates the toxicity of cancer chemotherapy in rats. J Lab Clin Med 2002;140:358–68.
13. Ho C, Ng K, O'Reilly S, Gill S. Outcomes in elderly patients with advanced colorectal cancer treated with capecitabine: a population-based analysis. Clin Colorectal Cancer 2005;5:279–82.
14. Sharma R, Rivory L, Beale P, Ong S, Horvath L, Clarke SJ. Phase II study of fixed-dose capecitabine and assessment of predictors of toxicity in patients with advanced/metastatic colorectal cancer. Br J Cancer 2006;94:964–8.
15. Lin D, Li H, Tan W, Miao X, Wang L. Genetic polymorphisms in folate-metabolizing enzymes and risk of gastroesophageal cancers: a potential nutrient-gene interaction in cancer development. Forum Nutr 2007;60:140–5.
16. Tan W, Miao X, Wang L, et al. Significant increase in risk of gastroesophageal cancer is associated with interaction between promoter polymorphisms in thymidylate synthase and serum folate status. Carcinogenesis 2005;26:1430–5.
17. Galván-Portillo MV, Cantoral A, Oñate-Ocaña LF, et al. Gastric cancer in relation to the intake of nutrients involved in one-carbon metabolism among MTHFR 677 TT carriers. Eur J Nutr 2009;48:269–76.
18. Tajima K, Hirose K, Inoue M, Takezaki T, Hamajima N, Kuroishi T. A model of practical cancer prevention for out-patients visiting a hospital: the Hospital-based Epidemiologic Research Program at Aichi Cancer Center (HERPACC). Asian Pac J Cancer Prev 2000;1:35–47.
19. Hamajima N, Matsuo K, Saito T, et al. Gene-environment interactions and polymorphism studies of cancer risk in the Hospital-based Epidemiologic Research Program at Aichi Cancer Center II (HERPACC II). Asian Pac J Cancer Prev 2001;2:99–107.
20. Tokudome Y, Goto C, Imaeda N, et al. Relative validity of a short food frequency questionnaire for assessing nutrient intake versus three-day weighed diet records in middle-aged Japanese. J Epidemiol 2005;15:135–45.
21. Imaeda N, Goto C, Tokudome Y, Hirose K, Tajima K, Tokudome S. Reproducibility of a short food frequency questionnaire for Japanese general population. J Epidemiol 2007;17:100–7.
22. Imaeda N, Goto C, Tokudome Y, Hirose K, Tajima K, Tokudome S. Dietary supplement use by community-living population in Japan: data from the National Institute for Longevity Sciences Longitudinal Study of Aging (NILS-LSA). J Epidemiol 2006;16:249–60.
23. Willett W, Stampfer MJ. Total energy intake: implications for epidemiologic analyses. Am J Epidemiol 1986;124:17–27.
24. Koizumi W, Narahara H, Hara T, et al. S-1 plus cisplatin versus S-1 alone for first-line treatment of advanced gastric cancer (SPIRITS trial): a phase III trial. Lancet Oncol 2008;9:215–21.
25. Yoshida K, Ninomiya M, Takakura N, et al. Phase II study of docetaxel and S-1 combination therapy for advanced or recurrent gastric cancer. Clin Cancer Res 2006;12:3402–7.
26. Imamura H, Iishi H, Tsuburaya A, et al. Randomized phase III study of irinotecan plus S-1 (IRIS) versus S-1 alone as first-line treatment for advanced gastric cancer(GC0301/TOP-002). 2008 Gastrointestinal Cancer Symposium; Abstract #5, Orland, USA. 2008.
27. Ohtsu A, Shimada Y, Shirao K, et al. Randomized phase III trial of fluorouracil alone versus fluorouracil plus cisplatin versus uracil and tegafur plus mitomycin in patients with unresectable, advanced gastric cancer: the Japan Clinical Oncology Group Study (JCOG9205). J Clin Oncol 2003;21:54–9.
28. Yamao T, Shimada Y, Shirao K, et al. Phase II study of sequential methotrexate and 5-fluorouracil chemotherapy against peritoneally disseminated gastric cancer with malignant ascites: a report from the Gastrointestinal Oncology Study Group of the Japan Clinical Oncology Group, JCOG 9603 Trial. Jpn J Clin Oncol 2004;34: 316–22.
29. Lutz MP, Wilke H, Wagener DJ, et al. Weekly infusional high-dose fluorouracil (HD-FU), HD-FU plus folinic acid (HD-FU/FA), or HD-FU/FA plus biweekly cisplatin in advanced gastric cancer: randomized phase II trial 40953 of the European Organisation for Research and Treatment of Cancer Gastrointestinal Group and the Arbeitsgemeinschaft Internistische Onkologie. J Clin Oncol 2007;25:2580–5.
30. Kawakami K, Watanabe G. The association of thymidylate synthase mRNA expression with its three gene polymorphisms in colorectal cancer. Proc Am Assoc Cancer Res 2004;45:2104.
31. Mandola MV, Stoehlmacher J, Zhang W, et al. A 6 bp polymorphism in the thymidylate synthase gene causes message instability and is associated with decreased intratumoral TS mRNA levels. Pharmacogenetics 2004;15:319–27.
32. Marsh S, McKay JA, Cassidy J, McLeod HL. Polymorphism in the thymidylate synthase promoter enhancer region in colorectal cancer. Int J Oncol 2001;19:383–6.
33. Pullarkat ST, Stoehlmacher J, Ghaderi V, et al. Thymidylate synthase gene polymorphism determines response and toxicity of 5-FU chemotherapy. Pharmacogenomics J 2001;1:65–70.
34. Park DJ, Stoehlmacher J, Zhang W, Tsao-Wei D, Groshen S, Lenz HJ. Thymidylate synthase gene polymorphism predicts response to capecitabine in advanced colorectal cancer. Int J Colorectal Dis 2002;17:46–9.

Diagnostic utility of EUS-guided FNA in patients with gastric submucosal tumors (CME)

Mohamed A. Mekky, MD, Kenji Yamao, MD, Akira Sawaki, MD, Nobumasa Mizuno, MD, Kazuo Hara, MD, Mohamed A. Nafeh, MD, Ashraf M. Osman, MD, Takashi Koshikawa, MD, Yasushi Yatabe, MD, Vikram Bhatia, MD

Nagoya, Japan

Background: Submucosal tumors (SMTs) comprise both benign and malignant lesions, and most of the gastric lesions tend to be malignant. The addition of EUS-guided FNA (EUS-FNA) has the potential to improve this distinction, but published series are limited.

Objective: To evaluate the yield of EUS-FNA in gastric SMTs with referral to a criterion standard final diagnosis.

Design: Retrospective study.

Setting: Tertiary-care referral center.

Patients: This study involved 141 consecutive patients with gastric SMTs, who underwent EUS-FNA from January 2000 to December 2008. Immunohistochemical staining with c-kit, CD34, actin, and S-100 antibodies was done if a spindle cell tumor was found. Based on FNA sample adequacy, and whether a specific diagnosis could be established, EUS-FNA results were categorized as diagnostic, suggestive, or nondiagnostic. The criterion standards for final diagnosis were the surgical histopathological results or the follow-up course for malignant, inoperable cases.

Intervention: EUS-FNA.

Main Outcome Measurements: Diagnostic yield of EUS-FNA and factors related to sampling adequacy for cytological and immunohistochemical evaluation.

Results: A total of 141 patients (52% female, mean age 56.7 years) underwent EUS-FNA (range 1-5 passes). The overall results of EUS-FNA were diagnostic, suggestive, and nondiagnostic in 43.3%, 39%, and 17.7% of cases, respectively. Adequate specimens were obtained in 83% of cases, and 69 cases (48.9%) had a definitive final diagnosis. The most common gastric SMT was GI stromal tumor (59.5%). EUS-FNA results were 95.6% accurate (95% confidence interval [CI], 87.5%-99%) for the final diagnosis and 94.2% (95% CI, 85.6%-98.1%) accurate for differentiating potentially malignant lesions. A heterogeneous echo pattern was the only independent predictor for sampling adequacy (adjusted odds ratio 6.15; $P = .002$). There were no procedure-related complications.

Limitations: Possibility of selection bias.

Conclusion: EUS-FNA is an accurate method for diagnosis of gastric SMTs and for differentiating malignant lesions. (Gastrointest Endosc 2010;71:913-9.)

Abbreviations: EUS-FNA, EUS-guided FNA; GIST, GI stromal tumor; IHC, immunohistochemical; SMT, submucosal tumor.

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Current affiliations: Department of Gastroenterology (M.A.M., K.Y., A.S., N.M., K.H.), Aichi Cancer Center Hospital, Nagoya, Japan, Department of Tropical Medicine and Gastroenterology (M.A.M., M.A.N., A.M.O.), Assiut University, Egypt, Aichi Prefectural College of Nursing and Health (T.K.), Nagoya, Japan, Department of Pathology and Molecular Diagnostics (Y.Y.), Aichi Cancer Center Hospital, Nagoya, Japan, Department of Medical Hepatology (V.B.), Institute of Liver and Biliary Sciences, New Delhi, India.

Reprint requests: Mohamed A. Mekky, MD, Department of Gastroenterology, Aichi Cancer Center Hospital, 1-1 Kanokoden, Chikusa-ku, Nagoya, Aichi 464-8681 Japan.

If you would like to chat with an author of this article, you may contact Dr. Mekky at doc_mekky0000@yahoo.com.

Gastric submucosal tumors (SMTs) include a diverse array of benign, potentially malignant, and malignant lesions. These lesions are being increasingly recognized during routine endoscopies, with a reported frequency of 1 in every 100 to 300 gastroscopic examinations.[1,2] Polkowski[2] estimated that about 13% of GI SMTs were malignant, with the highest risk of malignancy in the stomach. EUS imaging features alone cannot substitute for a pathological diagnosis of SMT subtype, and EUS is an imperfect tool for assessing the malignancy risk for these lesions. Hence, EUS-assisted tissue sampling modalities have been increasingly incorporated for evaluation of SMTs.[2-11]

Cytomorphologically, spindle cell tumors are the most commonly encountered SMTs. The most common subtype is GI stromal tumor (GIST), which needs to be distinguished from its benign spindle cell counterparts like leiomyomas and schwannomas.[2-7] This distinction is difficult on cytology smears alone and requires immunohistochemical (IHC) staining and ultrastructure studies.[4-9] EUS-assisted sampling by both EUS-guided FNA (EUS-FNA) and EUS-guided Trucut Biopsy (EUS-TCB) can provide cytological material as well as tissue cores for histological evaluation.[4-5] The use of the latter has been limited because of its potential complications and difficulty of use.[2,10-12] On the other hand, EUS-FNA makes it possible to obtain an adequate cell block specimen, which then can be examined histologically and immunohistochemically. Most previous reports of EUS-FNA studies in SMTs have been limited to GISTs or mesenchymal tumors, rather than encompassing the entire spectrum of lesions encountered in practice.[4,11-16] The reported accuracy rates in these studies have varied widely between 19% and 100%, with most studies lacking a final surgical diagnosis for reference.[4,5,11-17] Hence, the aim of this study was to evaluate the diagnostic yield of EUS-FNA with the addition of IHC staining for gastric SMTs with reference to a criterion standard final diagnosis.

Capsule Summary

What is already known on this topic

- Variable accuracy rates have been reported with the use of EUS-guided FNA in submucosal tumors, a large proportion of which tend to be malignant.

What this study adds to our knowledge

- In a retrospective study of 141 consecutive patients with gastric submucosal tumors who underwent EUS-guided FNA, adequate tissue sampling was obtained in 83%, a concordant diagnosis was reached in 95.6%, and malignant lesions were diagnosed in 94.2%.

PATIENTS AND METHODS

Consecutive patients with gastric SMTs, who had undergone EUS-FNA at Aichi Cancer Center Hospital, Nagoya, Japan between January 2000 and December 2008, were retrospectively selected. Informed consent was given by each patient prior to the procedure as a part of their clinical management. Those patients who had undergone EUS-FNA in some other institution and patients in whom on-site cytological evaluation was unavailable during the EUS-FNA procedures were excluded. The objective and outcome measurements were the diagnostic yield of EUS-FNA and factors related to sampling adequacy for cytological and IHC evaluation.

Study procedures

All patients underwent an upper endoscopic examination prior to EUS-FNA. The procedures were performed with the patients under conscious sedation (using intravenous Pentzaocine 15mg; Pentagin, Daiichi-sankyo Corp., Tokyo, Japan, and intravenous midazolam 5-10mg; Dormicum, Astellas Corp., Tokyo, Japan). EUS-FNA was performed by using a convex array echoendoscope (GF–UCT240; Olympus Optical Corp Ltd, Tokyo, Japan) connected to a US scanning system (SSD 5500; Aloka, Tokyo, Japan). All FNA procedures were performed by using 22-gauge needles (eg, NA-10J-1, NA-10J-KB, NA-11J-KB, or NA-200H-8022; Olympus Medical System Corp Ltd, Tokyo, Japan). Patients were followed-up after the procedure for 48 hours for any procedure-related complications. Cytological samples were processed by the same experienced cytopathologist (T.K.). For all samples, one slide was fixed by air drying and then stained with modified Giemsa stain (Diff-Quik; Kokusai Shiyaku, International Reagents, Kobe, Japan) and reviewed immediately (on-site examination) by the cytopathologist (or cytotechnician) to ensure specimen adequacy. The other slides were fixed by immediate immersion in 95% alcohol and then stained with the Papanicolaou stain. The cell-block material was processed by fixation in 10% neutral buffered formalin solution and then embedded in paraffin to be handled as a routine tissue block. Thin sections from paraffin-embedded cell blocks were cut and then stained with hematoxylin and eosin. A provisional diagnosis was first assigned with the cytology smear, and then cell blocks were stained by IHC staining if indicated.

For lesions diagnosed with EUS-FNA cytology as spindle cell tumors, IHC stain preparations were assembled in cell-block specimens. The Avidin Biotin Complex (ABC; VECTASTAIN, Vector Laboratories Ltd, California, USA) was used with the following antibodies: c-kit (Dako Inc., California, USA), CD34 (Novocastra, Leica Microsystems Ltd., Newcastle Upon Tyne, UK), S-100 (Mie University laboratories, Aichi, Japan; noncommercial), and Actin (Nichirei Bioscience Inc., Tokyo, Japan). The results of IHC staining were described as positive or negative. Positive IHC staining was defined as staining of >50% of the tumor cells. Negative IHC staining was defined as either focal positivity or staining of <50% of the tumor cells. A

diagnosis of GIST was made by positive c-kit staining, with or without positive CD34 IHC staining. Leiomyoma and leiomyosarcoma were diagnosed by positive actin staining and schwannomas by positive S-100 staining.

Study definitions

For the overall diagnostic yield, the procedure results were categorized as the following: (1) diagnostic, if sufficient samples were obtained for cytology, cell-block preparation, and IHC staining, if needed, and a specific diagnosis could be established, (2) suggestive, if sufficient samples were obtained for cytology, and a suggestive primary diagnosis was assigned, but samples were inadequate for IHC staining, and/or a definitive final diagnosis was not achieved, and (3) nondiagnostic, if samples were primarily insufficient, and/or the results were discordant with the criterion standard.

The results of EUS-FNA and the final diagnosis were categorized into 2 groups: (1) malignant or potentially malignant group, including all GISTs, malignant lymphomas, and gastric wall carcinomas, and (2) benign group, including leiomyomas, schwannomas, gastric desmoid tumors, ectopic pancreatic tissues, benign inflammatory granulomas, glomus tumors, and lipomas. We considered all GISTs as potentially malignant, in accordance with the National Institutes of Health consensus statement.[8,9]

The criterion standard for final diagnosis was either the surgical histopathological results for resected specimens or the clinical management and follow-up course for malignant, inoperable cases.

Statistical analysis

Frequencies, percentages, and means were used, as appropriate, for descriptive analysis. Univariate and a multivariate logistic regression analysis were performed to assess the significant predictors of obtaining sufficient specimens (insufficient versus sufficient samples). All statistical analysis was conducted by using SPSS software for Windows, release 11 (SPSS Inc, Chicago, Ill). A P value of $< .05$ was considered significant.

RESULTS

A total of 141 consecutive patients with SMTs of the stomach, who fulfilled our inclusion criteria, were identified. Fifty-two percent were women, and the mean (± SD) age of the patients was 56.7 years ± 14.4 years. Over 87.8% of the patients were asymptomatic, and the SMTs were discovered incidentally. The mean (± SD) diameter of the SMTs was 29.9 mm (± 16.0 mm; range 6-90 mm). The characteristics of the 141 gastric SMTs, including their locations, endoscopic characteristics, sizes, layers of origin, and echo patterns, are summarized in Table 1.

Among the 141 cases, 69 (48.9%) had a definitive final diagnosis (67 cases were surgically resected, and 2 cases were proved on follow-up to be malignant lymphomas).

TABLE 1. Endoscopic and EUS characteristics of gastric submucosal lesions (n = 141)

Characteristic	No. (%)
Location within the stomach	
Cardia	30 (21.3)
Fundus	45 (31.9)
Body	29 (20.6)
Antrum	31 (22)
Pyloric canal	6 (4.3)
Endoscopic characteristics	
Smooth mucosa	92 (65.2)
Mucosal ulceration	11 (7.8)
Umbilication	10 (7.1)
Multinodular lesion	24 (17)
Multiple lesions*	4 (2.8)
Size	
<20 mm	34 (24.1)
20-50 mm	90 (63.8)
>50 mm	17 (12.1)
EUS layer of origin	
Third layer (submucosa)	21 (14.9)
Fourth layer (muscle)	108 (76.6)
Extragastric	5 (3.5)
Undetermined	7 (5)
Echo pattern	
Homogeneous-hypoechoic	65 (46.1)
Homogeneous-hyperechoic	3 (2.1)
Heterogeneous	73 (51.8)
Other characteristics	
Presence of cystic spaces	20 (14.1)
Adjacent lymphadenopathy	5 (3.5)
Irregular border	31 (22)

*Only the largest lesion was included in the analysis.

Of the remaining cases, 63 (44.6%) were followed-up without surgical resection for at least 12 months, and 9 cases (6.5%) were lost to follow-up. The mean number of FNA passes was 2.5 (SD:0.7; range 1-5). The overall rate of sample adequacy was 83% (117 cases). Adequate samples were obtained in 67.6% of lesions with size <20 mm, 86.6% of lesions with size between 20 and 50 mm, and 94.1% of lesions with size >50 mm ($P = .01$). IHC staining

TABLE 2. Diagnostic yield of EUS-FNA and its presumptive pathological diagnosis in patients with gastric submucosal tumors (n = 141)

	Sufficient samples (n = 117; 83%)					Insufficient samples (n = 24; 17%)	
	IHC stained (n = 64)			IHC not stained (n = 53)			
Diagnostic category	GIST	Leiomyoma	Schwannoma	Spindle cell tumor	Misc	Unknown	Total, no. (%)
Diagnostic	37	9	2	0	13	0	61 (43.3)
Suggestive	9	7	0	29	10	0	55 (39)
Nondiagnostic	0	0	0	0	1	24	25 (17.7)
Total, no. (%)	46 (32.6)	16 (11.3)	2 (1.4)	29 (20.6)	24 (17)	24 (17)	141 (100)

EUS-FNA, EUS-guided FNA; *IHC*, immunohistochemical; *GIST*, GI stromal tumor; *misc*, miscellaneous tumor; *CI*, confidence interval.
EUS-FNA was classified as *diagnostic* in 61 cases (43.3%; 95% CI, 35%-51%), *suggestive* in 55 cases (39%; 95% CI, 31%-47%), and *nondiagnostic* in 25 cases (17.7%; 95% CI, 12.5%-25%).

with c-kit, CD34, actin, and S-100 antibodies were done on cell-block samples in 64 of 141 cases (45.6%) in which cytological evaluation showed a spindle cell tumor. There were no serious procedure-related complications.

Diagnostic yield of EUS-FNA

The EUS-FNA diagnosis was classified as *nondiagnostic*, *suggestive*, or *diagnostic* in 25 (17.7%), 55 (39%), and 61 (43.3%) cases, respectively.

Of the 25 cases classified as *nondiagnostic*, EUS-FNA failed to provide adequate samples in 24 cases (1 each of lipoma and desmoid tumor, which were resected, 13 that were followed-up, and 9 that were lost to follow-up). One GIST case, which was eventually resected, was misdiagnosed as benign inflammatory granuloma by EUS-FNA.

All of the 55 cases classified as *suggestive* had adequate FNA sampling but a lack of criterion standard diagnosis for final reference. In 16 of the 55 cases, the specimens were adequate for IHC staining and were given a diagnosis of GIST (9 cases) and leiomyoma (7 cases) after EUS-FNA. However, because these patients did not undergo surgical resection, we conservatively classified them as *suggestive*. In the remaining 39 cases, IHC staining was not done. In 29 of these cases, spindle cell tumor was found on cytology, but the cell-block specimens were inadequate for IHC staining. Three of these 29 lesions were resected because of an increase in size on follow-up and were found to be GISTs. The remaining 10 of the 39 cases were presumptively diagnosed after EUS-FNA as 6 ectopic pancreatic tissues, 3 benign epithelioid cells suggestive of glomus or carcinoid tumors (2 of them were resected), and 1 inflammatory granuloma.

For the remaining 61 cases, EUS-FNA specimens were adequate, and a definitive final diagnosis was achieved either by surgery (59 cases) or the follow-up course for malignant, inoperable cases (2 cases of lymphoma), and the EUS-FNA results were classified as *diagnostic*. The detailed summary is shown in Table 2.

Performance characteristics of EUS-FNA

Of the 69 cases with a definite final diagnosis, 41 (59.4%) were GISTs, 9 (13%) were leiomyomas, 7 were gastric wall carcinomas, 6 were extragastric lesions (3 pancreatic tumors, 2 abdominal lymph nodes, and 1 peritoneal desmoid tumor), 2 were glomus tumors, 2 were schwannomas, 1 was a gastric inflammatory granuloma, and 1 was a lipoma. Fifty-three of the 69 SMTs (76.8%) were proven finally to be malignant lesions or potentially malignant lesions.

EUS-FNA results were concordant with the final diagnosis in 66 of 69 lesions (accuracy rate 95.6%; 95% CI, 87.5%-99%), as shown in Table 3. For the differentiation of benign from potentially malignant lesions, EUS-FNA had a sensitivity of 92.4% (95% CI, 82%-98%), specificity of 100% (95% CI, 79%-100%), positive predictive value of 100% (95% CI, 92.5%-100%), negative predictive value of 80% (95% CI, 56.3%-94%), and accuracy rate of 94.2% (95% CI, 85.6%-98.1%) (Table 4).

Factors related to sampling adequacy

Logistic regression analysis showed that a heterogeneous echo pattern of the lesion was the only independent predictor for obtaining a sufficient sample by EUS-FNA (adjusted odds ratio 0.1; 95% CI, 0.02-0.4; $P = .002$). Other factors such as the size of the mass, the long axis location within the stomach, the number of needle passes, and EUS layer of origin were not significant (Table 5).

DISCUSSION

Gastric SMT is an umbrella term that encompasses both neoplastic and nonneoplastic lesions.[1-3] Once the lesions are viewed endoscopically, the main challenge is to distinguish the potentially malignant SMTs from their benign counterparts.[3,8] The reported yield of EUS-FNA cytology for the diagnosis of SMTs is less than that

TABLE 3. Comparison of EUS-FNA diagnosis with the final diagnosis of gastric submucosal tumors (n = 69)

	EUS-FNA diagnosis no. (%)	Final diagnosis no. (%)
GIST*	37 (53.6)	41 (59.4)
Leiomyoma	9 (13)	9 (13)
Spindle cell tumor	3 (4.3)	0
Schwannoma	2 (2.9)	2 (2.9)
Gastric carcinoma	7	7
Pancreatic tumor	3	3
Desmoid tumor	0	1
Extragastric lymphoma	2	2
Glomus tumor†	2	2
Lipoma	0	1
Inflammatory granuloma	2	1
Unknown (insufficient)‡	2	0

EUS-FNA, EUS-guided FNA; *GIST*, GI stromal tumor.
The final diagnosis was achieved by surgery (67 cases) and follow-up for inoperable cases (2 extragastric malignant lymphomas). The diagnosis was concordant in 66 lesions or 95.6%. Sixty-one were diagnostic, and 5 were suggestive diagnoses. The diagnosis was discordant (nondiagnostic) in 3 lesions or 4.3%.
*GIST cases (n = 41): 37 correctly diagnosed; 1 lesion was a primary inflammatory granuloma (nondiagnostic), and 3 were benign spindle cell tumors (suggestive).
†Two glomus tumors were suspected in FNA specimens as benign vascular epithelioid cell tumors (suggestive).
‡There were 2 nondiagnostic (insufficient) FNA specimens; 1 was a lipoma and the other a desmoid tumor.

TABLE 4. Performance characteristics of EUS-FNA for differentiating benign from malignant (or potentially malignant) gastric submucosal tumors

	Final diagnosis	
EUS-FNA diagnosis	**Benign**	**Malignant**
Benign	16 (TN)*	4 (FN)†
Malignant	0 (FP)	49 (TP)

EUS-FNA, EUS-guided FNA; *TN*, true negative; *FN*, false negative; *FP*, false positive; *TP*, true positive.
*The 2 insufficient cases and the 2 cases of glomus tumor were treated as TN cases.
†Of the 4 FN cases, the EUS-FNA diagnosis was spindle cell tumor in 3 cases and benign inflammatory granuloma in 1 case; all proved to be GI stromal tumors after resection.

for other targets, and previous studies have been limited by small patient numbers, lack of a defined criterion standard, and a limited spectrum of lesions.[2,5-7,11-18] Ando et al[4] examined 49 submucosal tumors, with 91.8% adequate samples. Twenty-three lesions were surgically resected (20 were GISTs), and their accuracy rate was 95%. Arantes et al[17] studied 10 SMTs with 80% sampling adequacy, and GIST was suggested in 6 cases (60%). Vander Noot et al[18] also reported a 94.4% sampling adequacy rate with 18 GISTs, but it was unclear whether or not these results were confirmed surgically. Recently, Hoda et al[16] described the yield of EUS-FNA in 112 upper GI SMTs as diagnostic, suspicious (spindle cells), and nondiagnostic in 61.6%, 22.3%, and 16.1% of cases, respectively, with an overall accuracy rate of 84%. However, their study also lacked the final criterion standard reference. Accordingly, it may be difficult to calculate a weighted average accuracy for EUS-FNA in these studies because of their varied inclusions and designs. Some reviews mentioned a weighted average accuracy rate of EUS-FNA as 60% to 80%, but their pooled studies were concerned mainly with GISTs.[2,4,12]

In our study, we reported an accuracy rate of 95.6% in achievement of a concordant diagnosis and 94% in detecting malignant lesions, with a sensitivity and specificity of 92.4% and 100%, respectively. On-site cytological analysis as well as our recruiting design may have contributed to these relatively high figures. We not only reported a high rate of sampling adequacy (83%), with a mean number of 2.5 passes, but also we demonstrated that both cytology and cell-block processing were possible with the use of standard, 22-gauge, FNA needles. As expected, there was an increase in sampling adequacy with increasing size of the SMT, with a 95% yield with lesion size of >50 mm. Similar findings were reported by Akahoshi et al,[15] who had a 100% yield of EUS-FNA with lesion size of >40 mm. For SMTs, a 22-gauge, FNA needle is thought to be enough to obtain sufficient samples for cell-block preparations and then IHC staining, which is very useful for diagnosing the SMT subtypes and, hence, should become routine practice in sampling these lesions.

A large proportion of our gastric SMTs were found to be malignant (76.8%). This high percentage may be an overrepresentation, likely because our design may have led to a selection bias of higher-risk cases referred for surgery. In agreement with numerous previous reports, we found that GISTs were the most common SMT in the stomach, and only 40% of the gastric SMTs were not GISTs.[2-5,11-19] GISTs have a wide spectrum of risk behavior—from small, indolent tumors to overt sarcomas.[2,3,7-9] Nevertheless, it is this unpredictable behavior that leads many experts to recommend that every GIST should be considered as potentially malignant and therefore be resected.[2,3,8,9] Hence, it is very important to apply tools that help in differentiating GISTs from other benign SMTs, such as the implementation of IHC staining panels.[4,6,7] In our study, spindle cell tumors comprise the vast majority of our EUS-FNA diagnoses (93 of 141 lesions).[4,6,7] We used a directed IHC staining panel with c-kit, CD34, actin, and S-100 antibodies for differentiating spindle cell tumors into leiomyoma, schwan-

TABLE 5. Summary of univariate and multivariate analyses of factors associated with EUS-FNA sampling adequacy

Variable	Univariate analysis* *P* value	Multivariate analysis† *P* value (adjusted OR; 95% CI)
SMT location within the stomach	.572 NS	–
Tumor size on EUS (<20 mm vs ≥20 mm)	.022	.14 (0.46; 95% CI, 0.16-1.3)
EUS layer of origin	.489 NS	–
EUS echo pattern (homogenous vs heterogeneous)	.001	.002 (0.1; 95% CI, 0.02-0.4)
No. needle passes (≤2 vs >2)	.427 NS	–

EUS-FNA, EUS-guided FNA; *OR*, odds ratio; *CI*, confidence interval; *SMT*, submucosal tumor; *NS*, not significant.
*Chi-square test.
†Multivariate logistic regression analysis (insufficient vs sufficient sample).

noma, and GIST. Sixty-four spindle cell lesions were adequately stained for these antibodies, and of them, only 48 cases were counted for accuracy calculations because they had a definitive final diagnosis. Other IHC stains may be needed in selected cases, such as chromogranin, synaptophysin, and keratin in carcinoid tumors and calponin in glomus lesions.[6,7]

On evaluating the predictors for sampling adequacy, only a heterogeneous echo pattern was found significant in a multivariate analysis. It may be possible that the higher cellularity and proliferation rate are related to a more heterogeneous echo pattern. In contrast, Hoda et al[16] have previously reported that there were no identifiable factors that affected the yield of EUS-FNA.

We categorized our results into *diagnostic*, *suggestive*, and *nondiagnostic*, because previous reports have variably interpreted positive IHC staining results, especially for GISTs. Some authors are conservative, considering them as suggestive tools only, and their rationale is the presence of staining heterogeneity.[17,20,21] Others trust IHC results and rely upon them for treatment decision making.[5-7] We followed the former conservative approach and used a well-characterized criterion standard for final diagnosis to allow for robust conclusions. Because the cases used to calculate the performance characteristics of EUS-FNA in the present series were definitively diagnosed, our results may serve as a benchmark for future interventions. The main shortcoming of the present study is its retrospective nature and the potential for bias in selecting patients who were referred for surgery or chemotherapy. The strength of this study is the large number of gastric SMTs with EUS-FNA sampling and a well-defined criterion standard.

Based our results, we recommend a short algorithmic approach for the diagnosis of gastric SMTs. An initial EUS can rule out extraluminal, hyperechoic, and third-layer (submucosal) lesions. For hypoechoic lesions that originate from the fourth (muscle) layers, EUS-FNA should be performed even for small lesions, and IHC stains with a panel of CD34, c-kit, actin, and S-100 should be done if spindle cells are found.

In conclusion, EUS-FNA with 22-gauge needles is an accurate and safe method for diagnosing gastric SMTs and for delineating malignant lesions with the adjunctive and selective use of a limited panel of IHC stains.

REFERENCES

1. Hedenbro JL, Ekelund M, Wetterberg P. Endoscopic diagnosis of submucosal gastric lesions: the results after routine endoscopy. Surg Endosc 1991;5:20-3.
2. Polkowski M. Endoscopic ultrasound and endoscopic ultrasound guided fine-needle biopsy for the diagnosis of malignant submucosal tumors. Endoscopy 2005;37:635-45.
3. Miettinen M, Lasota J. Gastrointestinal stromal tumors: definition, clinical, histological, immunohistochemical, and molecular genetic features and differential diagnosis. Virchows Arch 2001;438:1-12.
4. Ando N, Goto H, Niwa Y, et al. The diagnosis of GI stromal tumors with EUS-guided fine needle aspiration with immunohistochemical analysis. Gastrointest Endosc 2002;55:37-43.
5. Matsui M, Goto H, Niwa Y, et al. Preliminary results of fine needle aspiration biopsy histology in upper gastrointestinal submucosal tumors. Endoscopy 1998;30:750-5.
6. Yamaguchi U, Hasegawa T, Masuda T, et al. Differential diagnosis of gastrointestinal stromal tumor and other spindle cell tumors in the gastrointestinal tract based on immunohistochemical analysis. Virchows Arch 2004;445:142-50.
7. Stelow EB, Murad FM, Debol SM, et al. A limited immunocytochemical panel for the distinction of subepithelial gastrointestinal mesenchymal neoplasms sampled by endoscopic ultrasound–guided fine-needle aspiration. Am J Clin Pathol 2008;129:219-25.
8. Fletcher CD, Berman JJ, Corless C, et al. Diagnosis of gastrointestinal stromal tumors: a consensus approach. Hum Pathol 2002;33:459-65.
9. Joensuu H. Risk stratification of patients diagnosed with gastrointestinal stromal tumor. Hum Pathol 2008;39:1411-9.
10. Chak A. EUS in submucosal tumors. Gastrointest Endosc 2002;56:S43-S48.
11. Hunt GC, Smith PP, Faigel DO. Yield of tissue sampling for submucosal lesions evaluated by EUS. Gastrointest Endosc 2003;57:68-72.
12. Polkowski M, Gerke W, Jarosz D, et al. Diagnostic yield and safety of endoscopic ultrasound guided trucut biopsy in patients with gastric submucosal tumors: a prospective study. Endoscopy 2009;41:329-34.
13. Okubo K, Yamao K, Nakamura T, et al. Endoscopic ultrasound guided fine-needle aspiration biopsy for the diagnosis of gastrointestinal stromal tumors in the stomach. J Gastroenterol 2004;39:747-53.

14. Chen VK, Eloubeidi MA. Endoscopic ultrasound-guided fine-needle aspiration of intramural and extraintestinal mass lesions: diagnostic accuracy, complication assessment, and impact on management. Endoscopy 2005;37:984-9.
15. Akahoshi K, Sumida Y, Matsui N, et al. Preoperative diagnosis of gastrointestinal stromal tumor by endoscopic ultrasound-guided fine needle aspiration. World J Gastroenterol 2007;13:2077-82.
16. Hoda KM, Rodriguez SA, Faigel DO. EUS-guided sampling of suspected GI stromal tumors. Gastrointest Endosc 2009;69:1218-23.
17. Arantes V, Logrono R, Faruqi S, et al. Endoscopic sonographically guided fine-needle aspiration yield in submucosal tumors of the gastrointestinal tract. J Ultrasound Med 2004;23:1141-50.
18. Vander Noot MR III, Eloubeidi MA, Chen VK, et al. Diagnosis of gastrointestinal tract lesions by endoscopic ultrasound-guided fine-needle aspiration biopsy. Cancer 2004;102:157-63.
19. Chatzipantelis P, Salla C, Karoumpalis I, et al. Endoscopic ultrasound-guided fine needle aspiration biopsy in the diagnosis of gastrointestinal stromal tumors of the stomach: a study of 17 cases. J Gastrointest Liver Dis 2008;17:15-20.
20. Liegl B, Kepten I, Le C, et al. Heterogeneity of kinase inhibitor resistance mechanisms in GIST. J Pathol 2008;216:64-74.
21. Medeiros F, Corless CL, Duensing A, et al. KIT-negative gastrointestinal stromal tumors: proof of concept and therapeutic implications. Am J Surg Pathol 2004;28:889-94.

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Characteristic methylation profile in CpG island methylator phenotype-negative distal colorectal cancers

Byonggu An[1,2], Yutaka Kondo[1], Yasuyuki Okamoto[1], Keiko Shinjo[1,3], Yukihide Kanemitsu[4], Koji Komori[4], Takashi Hirai[4], Akira Sawaki[5], Masahiro Tajika[5], Tsuneya Nakamura[5], Kenji Yamao[5], Yasushi Yatabe[6], Makiko Fujii[1], Hideki Murakami[1], Hirotaka Osada[1,3], Tohru Tani[2], Keitaro Matsuo[7], Lanlan Shen[8], Jean-Pierre J. Issa[8] and Yoshitaka Sekido[1,3]

[1] Division of Molecular Oncology, Aichi Cancer Center Research Institute, Chikusa-Ku, Nagoya 464-8681, Japan
[2] Department of Surgery, Shiga University of Medical Science, Seta Tsukinowa-cho, Otsu City, Shiga 520-2192, Japan
[3] Department of Cancer Genetics, Program in Function Construction Medicine, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya, 466-8550, Japan
[4] Department of Gastroenterological Surgery, Aichi Cancer Center Central Hospital, Chikusa-Ku, Nagoya 464-8681, Japan
[5] Department of Gastroenterology, Aichi Cancer Center Central Hospital, Chikusa-Ku, Nagoya 464-8681, Japan
[6] Department of Pathology and Molecular Diagnostics, Aichi Cancer Center Hospital, Chikusa-Ku, Nagoya 464-8681, Japan
[7] Division of Epidemiology and Prevention, Aichi Cancer Center Research Institute, Chikusa-Ku, Nagoya 464-8681, Japan
[8] Department of Leukemia, The University of Texas at M.D. Anderson Cancer Center, Houston, TX 77030, USA

Aberrant DNA methylation is involved in colon carcinogenesis. Although the CpG island methylator phenotype (CIMP) is defined as a subset of colorectal cancers (CRCs) with remarkably high levels of DNA methylation, it is not known whether epigenetic processes are also involved in CIMP-negative tumors. We analyzed the DNA methylation profiles of 94 CRCs and their corresponding normal-appearing colonic mucosa with 11 different markers, including the five classical CIMP markers. The CIMP markers were frequently methylated in proximal CRCs ($p < 0.01$); however, *RASSF1A* methylation levels were significantly higher in distal CRCs, the majority of which are CIMP-negative ($p < 0.05$). Similarly, methylation levels of *RASSF1A* and *SFRP1* in the normal-appearing mucosae of distal CRC cases were significantly higher than those in the proximal CRC cases ($p < 0.05$). They were also positively correlated with age (*RASSF1A*, $p < 0.01$; *SFRP1*, $p < 0.01$). Microarray-based genome-wide DNA methylation analysis of 18 CRCs revealed that 168 genes and 720 genes were preferentially methylated in CIMP-negative distal CRCs and CIMP-positive CRCs, respectively. Interestingly, more than half of the hypermethylated genes in CIMP-negative distal CRCs were also methylated in the normal-appearing mucosae, indicating that hypermethylation in CIMP-negative distal CRCs is more closely associated with age-related methylation. By contrast, more than 60% of the hypermethylated genes in CIMP-positive proximal CRCs were cancer specific ($p < 0.01$). These data altogether suggest that CpG island promoters appear to be methylated in different ways depending on location, a finding which may imply the presence of different mechanisms for the acquisition of epigenetic changes during colon tumorigenesis.

Colorectal cancer (CRC) is one of the most common human malignancies worldwide. CRC cells accumulate several genetic and epigenetic alterations in cancer-related genes to achieve malignant status.[1] Mutations in genes controlling the *KRAS/BRAF*, *APC/β-catenin* and *TP53* pathways are well known for their contribution to tumorigenesis.[2] Epigenetic alterations, including DNA hypermethylation of CpG island promoters and global DNA hypomethylation, have been reported to occur early in colorectal carcinogenesis.[3] Hypermethylation of CpG island promoters is closely associated with the transcriptional silencing of tumor suppressor genes, whereas global hypomethylation can lead to chromosomal instability.[4]

Recent cumulative studies in CRCs have suggested the existence of an accumulation of high rates of aberrant promoter hypermethylation in a subset of CRCs known as the CpG island methylator phenotype (CIMP).[5,6] CIMP-positive CRCs exhibit distinct genetic and clinical features, including high rates of *BRAF* and *KRAS* mutations, low rates of *TP53* mutations, a specific histology (mucinous, poorly differentiated), proximal location and characteristic clinical outcomes.[7] Given these clinicopathological features, CIMP-related carcinogenesis may proceed through a specific pathway in which the

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Correspondence to: Yutaka Kondo, Division of Molecular Oncology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan, Tel.: +81-52-764-2993, Fax: +81-52-764-2993, E-mail: ykondo@aichi-cc.jp

epigenetic changes that occur in premalignant cells determine subsequent genetic changes, thereby fostering the progression of these clones.[8] However, it is not known whether epigenetic processes are also involved in CIMP-negative CRCs that frequently emerge in the distal colon.

It has been suggested that proximal and distal CRCs show differences in epidemiological incidence, morphology and molecular biological characteristics.[9–12] Indeed, CIMP-positive CRCs are more frequently found in the proximal than the distal colon, suggesting that intensive accumulation of aberrant DNA methylation is more closely associated with proximal colon carcinogenesis. In contrast, the significance of aberrant DNA methylation is not well understood in distal colon carcinogenesis, where CIMP-negative CRCs are more common. If distinct mechanisms of colon carcinogenesis exist based on their site of origin, it is possible that the DNA methylation behavior and set of hypermethylated genes in distal CRCs are different from those in proximal CIMP-positive CRCs.

In this study, we analyzed the methylation status of CRCs both quantitatively and genome wide, in addition to other clinical and molecular characteristics. We elicited the significance of DNA methylation in CIMP-negative distal CRC compared with CIMP-positive proximal CRC. We also assessed the methylation status of normal-appearing mucosae by location, since DNA methylation, a factor of the field defect (also known as field cancerization) related to epimutagen exposure that leads to cancer formation, may differ by location and CIMP status.[13]

Material and Methods

Tissue samples

Samples of primary CRCs and their corresponding normal-appearing colonic mucosae were collected in accordance with institutional policy from 95 individuals who underwent surgical resection at the Aichi Cancer Center Central Hospital, Nagoya, Japan. All patients provided written informed consent. The specimens examined showed a high cellularity of cancer cells without definite evidence of necrosis. The corresponding normal-appearing colonic mucosae of CRC patients were sampled from two distinct sites: 2 cm and 10 cm from the cancer. We also obtained colonic biopsy samples of normal-appearing mucosae from the cecum and/or rectum of 38 colon polyp patients and the corresponding colon polyps from 22 patients. Pathological finding of all colon polyps is compatible with adenoma. The proximal colon consists of the cecum, and the ascending and transverse colon, and the distal colon consists of the descending and sigmoid colon, and rectum. Genomic DNA was extracted using a standard phenol-chloroform method.

Bisulfite pyrosequencing methylation analysis and bisulfite sequencing analysis

We performed a bisulfite treatment as previously reported.[14] Briefly, 2 μg of genomic DNA was converted and resuspended in 30 μl of water. DNA methylation levels were measured by a highly quantitative method using pyrosequencing technology with 11 methylation markers (Pyrosequencing AB, Uppsala, Sweden). Each assay included positive controls (samples after SssI treatment; New England Biolabs, Ipswich, MA) and negative controls (samples after whole genomic amplification using GenomiPhi V2; GE Healthcare, Piscataway, NJ), mixing experiments to rule out bias and repeat experiments to assess reproducibility. A detailed protocol of pyrosequencing was described previously.[15,16] The methylation levels at different C sites measured by pyrosequencing were averaged to represent the degree of methylation in each sample for each gene. Bisulfite-PCR products of the *MGMT* and *RASSF1A* promoters and *SFRP1* promoters were cloned and sequenced (Invitrogen, Carlsbad, CA). At least 10 clones were sequenced for each sample. The PCR conditions, primer sequences and sequencing primer sequences of the 11 markers are listed in Supporting Information Table 1.

Methylated CpG island amplification microarray

We analyzed 18 CRCs with methylated CpG island amplification microarray (MCAM; average patient age was 65.5 years, ranging from 44 to 79 years): 7 CIMP-positive proximal CRCs and 11 CIMP-negative distal CRCs. All were randomly selected from CRCs as classified *via* the five CIMP markers. Eight corresponding normal-appearing colonic mucosae from CRC patients (average age was 62.5 years, ranging from 52 to 75 years) were also analyzed with MCAM. Seventeen of the 18 CRCs were derived from the 94 CRC samples examined by pyrosequencing analysis, while one was newly added. As normal controls, we used normal-appearing colonic mucosae from two males and two females who, according to our pyrosequencing analysis, showed no aberrant methylation in any of the 11 markers. The background of the analyzed samples is listed in Supporting Information Table 2. A detailed protocol of MCAM was described previously.[16–18] We used a human custom-promoter array from Agilent Technologies (G4497A; Agilent Technologies, Santa Clara, CA) containing 15,134 probes corresponding to 6,157 unique genes, which we had initially validated by the MCAM method in a previous study.[16] Arrays were scanned on an Agilent scanner and analyzed using Feature Extraction software. Normalization was achieved with a linear per-array algorithm according to the manufacturer's protocol (Agilent Technologies).

Hierarchical clustering analysis

Cluster analysis was performed using an agglomerative hierarchical clustering algorithm (http://rana.lbl.gov/EisenSoftware.htm).[19] For specimen clustering, pairwise similarity measures among specimens were calculated using Cluster 3.0 software or Minitab 15 statistical software (http://www.minitab.com) based on DNA methylation intensity measurements across all genes. Dendrograms and heat maps were