multivariate Cox proportional hazard models were used to estimate the relations of protein expressions and clinical characteristics to overall survival. All reported P values are two-sided, and the level of significance was set at $P < 0.05$. Variables for multivariate analysis were selected by means of a forward stepwise approach, using a significance level of $P < 0.10$ for entering into or remaining in the model. All analyses were done with the use of the statistical software package StatView, version 5.0 (SAS Institute, Inc.).

Results

A total of 87 patients were eligible for the study. Chemotherapy began in July 1997 in the first patient and May 2004 in the last patient. The demographic characteristics of the patients at the start of first-line chemotherapy are shown in Table 1. There were 70 men (80%) and 17 women (20%), with a median age of 64 years. The associations of IGF-IR, EGFR, and HER2 expression on immunohistochemical assay to clinical outcome could be assessed in all patients. At the time of analysis, 79 patients (91%) had died and eight patients (9%) were alive.

The chemotherapy regimens received by the patients and the response rates are also listed in Table 1. The response rates to first-line chemotherapy in our study are comparable with those reported previously (2, 17–19).

Expression frequencies of IGF-IR, EGFR, and HER2 in primary tumors and associations with clinicopathologic features. All 87 of the samples showed positive immunohistochemical staining compared with the negative controls, i.e., without the primary antibodies. Semiquantitative data are summarized in Table 2, and typical examples of positive staining are shown in Fig. 1. Membranous expression was evaluated to be positive for IGF-IR in 67 tumors (77%), positive for EGFR in 55 (63%), and positive for HER2 in 16 (18%).

IGF-IR expression was significantly more common in intestinal type tumors than in diffuse type ($P = 0.002$, Mann-Whitney U test; Table 2). HER2-positive tumors were uncommon among diffuse type cancers.

We evaluated the association between protein expression levels and the anatomic extent of disease at the time of gastrectomy using the Japanese classification (20) to define pathologic stage. Pathologic stage did not correlate with the expression of IGF-IR, EGFR, or HER2 in primary tumors (Table 2).

Expressions of IGF-IR, EGFR, and HER2 according to response to first-line chemotherapy. In patients given S-1 monotherapy as first-line treatment, no significant associations were found between tumor response and the protein expressions of the primary tumors assessed according to a four-grade scale (IGF-IR, $P = 0.87$; EGFR, $P = 0.23$; HER2, $P = 0.50$; Mann-Whitney U test). In patients who received cisplatin + irinotecan as first-line chemotherapy, there were also no associations between tumor response and protein expressions (IGF-IR, $P = 0.91$; EGFR, $P = 0.39$; HER2, $P = 0.48$; Mann-Whitney U test). Other first-line regimens were not examined because the number of patients who responded to treatment was too small.

Expressions of IGF-IR, EGFR, and HER2, clinical characteristics, and overall survival since the start of first-line chemotherapy in all patients. The overall median survival time in our study was 14.1 months. Patients with advanced GC who had IGF-IR–positive tumors had slightly poorer survival (Fig. 2A). EGFR expression was unrelated to overall survival (Fig. 2B). HER2 expression was also unrelated to overall survival (Fig. 2C).

On univariate Cox regression analyses, no clinical characteristic significantly correlated with overall survival. A multivariate

Table 2. Results of immunohistochemical analysis and associations of protein expressions with histologic type and pathologic stage at gastrectomy

	Total no. of patients (%)	Histologic type			Pathologic stage* at gastrectomy					
		Intestinal ($n = 40$)	Diffuse ($n = 47$)	$P^\dagger$	Stage I ($n = 2$)	Stage II ($n = 11$)	Stage III ($n = 23$)	Stage IV ($n = 51$)	$r^\ddagger$	P
IGF-IR				**0.002**					0.128	0.24
Negative	20 (23)	5	15		1	3	9	7		
Positive	67 (77)	35	32		1	8	14	44		
Low	21 (24)	6	15		1	0	6	14		
Moderate	21 (24)	13	8		0	3	3	15		
High	25 (29)	16	9		0	5	5	15		
EGFR				0.33					0.133	0.22
Negative	32 (37)	12	20		1	6	9	16		
Positive	55 (63)	28	27		1	5	14	35		
Low	16 (18)	9	7		0	1	5	10		
Moderate	18 (21)	8	10		0	2	5	11		
High	21 (24)	11	10		1	2	4	14		
HER2				**0.001**					0.016	0.88
Negative	71 (82)	27	44		2	8	20	41		
Positive	16 (18)	13	3		0	3	3	10		
Low	5 (6)	3	2		0	1	1	3		
Moderate	2 (2)	2	0		0	0	0	2		
High	9 (10)	8	1		0	2	2	5		

NOTE: Significant P values are shown in bold.
*According to Japanese classification.
†Mann-Whitney U test.
‡Spearman's rank-correlation coefficient.

in 48 tumors (55%), that of IGF-IR and HER2 in 16 (18%), and that of EGFR and HER2 in 13 (15%; Table 4). Although a definite correlation was not found among the four-grade scores of these protein expressions, IGF-IR expression weakly correlated with EGFR and HER2 (Table 4).

No combination of protein expressions was significantly related to overall survival. Coexpression of IGF-IR and EGFR (n = 48) was associated with a median overall survival of 13.2 months, and patients with negative expression of either or both of these proteins (n = 39) had a median overall survival of 14.7 months (P = 0.50, log-rank test). Coexpression of IGF-IR and HER2 (median overall survival, 16.0 months n = 16 versus 13.6 months in other patients n = 71; log-rank P = 0.31), and that of EGFR and HER2 (median, 13.2 months n = 13 versus 14.1 months in other patients n = 74; log-rank P = 0.65) were also not significantly related to overall survival.

Discussion

In our study, 67 of the 87 cases (77%) of advanced GC were positive for IGF-IR expression on immunohistochemical assay, and such expression was a significant independent predictor of poor outcomes, as were well-recognized prognostic factors, such as performance status and the histologic type of tumor. In contrast, the expression of EGFR or HER2 was unrelated to overall survival. The frequency of EGFR expression (63%) in the present study was higher than those in previous studies (range, 31-47%) using immunohistochemical techniques to evaluate GC (21-23), whereas the frequency of HER2 expression (18%) was in agreement with previous findings (range, 10-23%; refs. 24-27). Although EGFR expression in GC remains poorly understood, a possible reason for our high frequency of EGFR expression was that our study was underpowered. Differences in EGFR expression among studies may also be ascribed to the lack of an established immunohistochemical scoring system commonly used to evaluate GC.

Because reports that the IGF system is involved in cancer progression, angiogenesis, metastasis, and resistance to apoptosis, IGF-IR has received considerable attention as a potential target for cancer therapy (28-31). During the past few years, intensive efforts have been directed toward the development of anti-IGF-IR drugs, such as receptor-specific blocking monoclonal antibodies and small molecule tyrosine kinase inhibitors. Two phase I/phase II clinical trials of new monoclonal antibodies are now under way (32, 33), as are many phase I and preclinical trials of other monoclonal antibodies and tyrosine kinase inhibitors. In GC, evidence supporting an association of IGF-IR expression with clinicopathologic characteristics and survival remains scant thus far. Our study showed a high rate of IGF-IR-positive expression in GC and provided evidence that such expression is related to poor outcomes. Our findings suggest that membranous IGF-IR

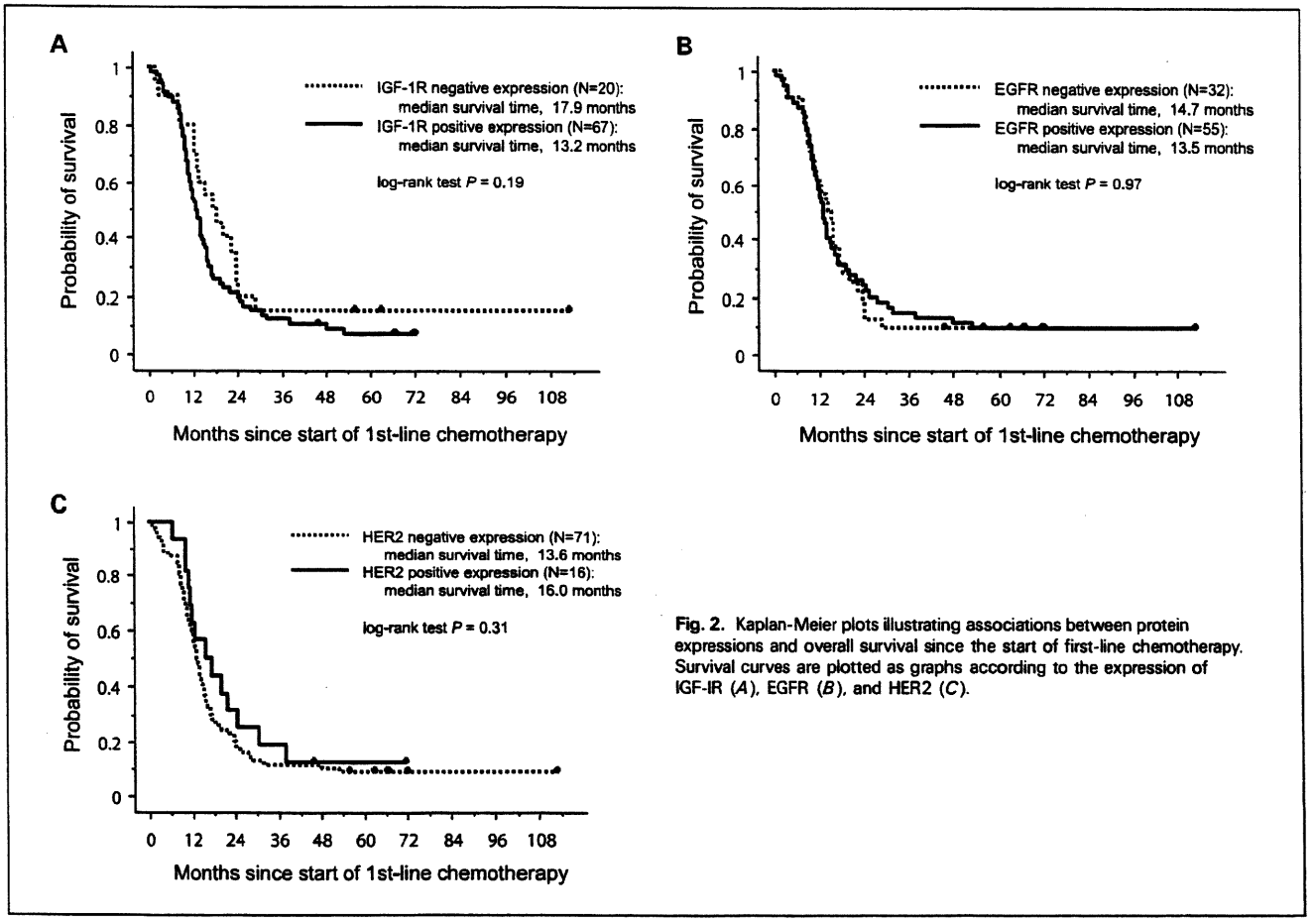

Fig. 2. Kaplan-Meier plots illustrating associations between protein expressions and overall survival since the start of first-line chemotherapy. Survival curves are plotted as graphs according to the expression of IGF-IR (*A*), EGFR (*B*), and HER2 (*C*).

our study. The trend toward poorer survival in patients with diffuse type GC is consistent with the findings of previous studies (45). Other possible reasons why the relation between IGF-IR expression and survival was insignificant on univariate analysis, but significant on multivariate analysis, were an underpowered study or the fact that only a single methodology of immunohistochemistry was used.

On the other hand, patients with HER2-positive tumors showed a slight but insignificant trend toward better survival (Fig. 2C). This finding might be related to the fact that HER2-positive expression was uncommon among diffuse type tumors (a well-known fact) which are significantly associated with poorer survival (Tables 2 and 3). Some studies have indicated that HER2 expression is an independent predictor of poor survival in GC (26, 46), whereas others have not (24, 25). Expression of EGFR in GC has also been linked to shorter overall survival, more advanced tumor stage, and lymph node metastases in some studies, but not in others (23, 47–49). EGFR expression was not related to any of these factors in our study. The results of immunohistochemical assays can be affected by many variables, including tissue fixation, choice of primary antibodies, and scoring systems, potentially leading to conflicting relations between the expressions of growth factor receptors and clinical outcomes. Immunohistochemical scoring systems of IGF-IR and EGFR differ among studies (16, 23, 49). Even for HER2, it remains unclear whether the scoring system used for breast cancer is valid for GC. The utilization of fluorescent *in situ* hybridization as an adjunct in GC or the automated quantification of immunohistochemical results may circumvent the subjective nature of immunohistochemical analyses. Nonetheless, a common scoring system for GC should be urgently established to accurately predict the clinical response to antagonists of these receptors, as well as to predict patient survival.

In conclusion, our study provides evidence that IGF-IR expression in GC specimens, poor performance status, and diffuse type cancer are significant predictors of poor survival in patients with advanced GC. We also showed that coexpression of IGF-IR and EGFR or IGF-IR and HER2 is relatively common in GC. Because the expression of IGF-IR has been associated with resistance to anti-EGFR and anti-HER2 therapies (35, 36), the potential therapeutic benefits of simultaneously targeting such receptors in patients with GC should be critically evaluated. Taken together, our findings suggest that anti-IGF-IR strategies may prove valuable in patients with GC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Hideko Morita, Hiromi Orita, and Mari Araake for help in collecting and organizing the clinical samples and Hideko Morita for providing excellent technical assistance.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74–108.
2. 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.
3. Ohtsu A, Yoshida S, Saijo N. Disparities in gastric cancer chemotherapy between the East and West. J Clin Oncol 2006;24:2188–96.
4. Furstenberger G, Senn HJ. Insulin-like growth factors and cancer. Lancet Oncol 2002;3:298–302.
5. Brodt P, Samani A, Navab R. Inhibition of the type I insulin-like growth factor receptor expression and signaling: novel strategies for antimetastatic therapy. Biochem Pharmacol 2000;60:1101–7.
6. Papa V, Gliozzo B, Clark GM, et al. Insulin-like growth factor-I receptors are overexpressed and predict a low risk in human breast cancer. Cancer Res 1993;53:3736–40.
7. Bonneterre J, Peyrat JP, Beuscart R, Demaille A. Prognostic significance of insulin-like growth factor 1 receptors in human breast cancer. Cancer Res 1990;50:6931–5.
8. Adams TE, McKern NM, Ward CW. Signalling by the type 1 insulin-like growth factor receptor: interplay with the epidermal growth factor receptor. Growth Factors 2004;22:89–95.
9. Gilmore AP, Valentijn AJ, Wang P, et al. Activation of BAD by therapeutic inhibition of epidermal growth factor receptor and transactivation by insulin-like growth factor receptor. J Biol Chem 2002;277:27643–50.
10. Huang SM, Harari PM. Epidermal growth factor receptor inhibition in cancer therapy: biology, rationale and preliminary clinical results. Invest New Drugs 1999;17:259–69.
11. Barnard JA, Beauchamp RD, Russell WE, Dubois RN, Coffey RJ. Epidermal growth factor-related peptides and their relevance to gastrointestinal pathophysiology. Gastroenterology 1995;108:564–80.
12. Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J 1997;16:1647–55.
13. Nicholson RI, Gee JM, Harper ME. EGFR and cancer prognosis. Eur J Cancer 2001;37 Suppl 4:S9–15.
14. Hayward JL, Rubens RD, Carbone PP, Heuson JC, Kumaoka S, Segaloff A. Assessment of response to therapy in advanced breast cancer. A project of the programme on clinical oncology of the International Union against Cancer, Geneva, Switzerland. Eur J Cancer 1978;14:1291–2.
15. Onn A, Correa AM, Gilcrease M, et al. Synchronous overexpression of epidermal growth factor receptor and HER2-neu protein is a predictor of poor outcome in patients with stage I non-small cell lung cancer. Clin Cancer Res 2004;10:136–43.
16. Cunningham MP, Essapen S, Thomas H, et al. Coexpression of the IGF-IR, EGFR and HER-2 is common in colorectal cancer patients. Int J Oncol 2006;28:329–35.
17. Koizumi W, Kurihara M, Nakano S, Hasegawa K. Phase II study of S-1, a novel oral derivative of 5-fluorouracil, in advanced gastric cancer. For the S-1 Cooperative Gastric Cancer Study Group. Oncology 2000;58:191–7.
18. Boku N, Ohtsu A, Shimada Y, et al. Phase II study of a combination of irinotecan and cisplatin against metastatic gastric cancer. J Clin Oncol 1999;17:319–23.
19. Boku N, Yamamoto S, Shirao K, et al. Randomized phase III study of 5-fluorouracil (5-FU) alone versus combination of irinotecan and cisplatin (CP) versus S-1 alone in advanced gastric cancer (JCOG9912) [suppl; abstract LBA4513]. J Clin Oncol 2007;25:18S.
20. Japanese Gastric Cancer A. Japanese classification of gastric carcinoma-2nd English Edition. Gastric Cancer 1998;1:10–24.
21. Yonemura Y, Sugiyama K, Fujimura T, et al. Epidermal growth factor receptor status and S-phase fractions in gastric carcinoma. Oncology 1989;46:158–61.
22. Lui VW, Grandis JR. EGFR-mediated cell cycle regulation. Anticancer Res 2002;22:1–11.
23. Gamboa-Dominguez A, Dominguez-Fonseca C, Quintanilla-Martinez L, et al. Epidermal growth factor receptor expression correlates with poor survival in gastric adenocarcinoma from Mexican patients: a multivariate analysis using a standardized immunohistochemical detection system. Mod Pathol 2004;17:579–87.
24. Jain S, Filipe MI, Gullick WJ, Linehan J, Morris RW. c-erbB-2 proto-oncogene expression and its relationship to survival in gastric carcinoma: an immunohistochemical study on archival material. Int J Cancer 1991;48:668–71.
25. Gurel S, Dolar E, Yerci O, et al. The relationship between c-erbB-2 oncogene expression and clinicopathological factors in gastric cancer. J Int Med Res 1999;27:74–8.
26. Nakajima M, Sawada H, Yamada Y, et al. The prognostic significance of amplification and overexpression of c-met and c-erb B-2 in human gastric carcinomas. Cancer 1999;85:1894–902.
27. Yano T, Doi T, Ohtsu A, et al. Comparison of HER2 gene amplification assessed by fluorescence *in situ* hybridization and HER2 protein expression assessed by immunohistochemistry in gastric cancer. Oncol Rep 2006;15:65–71.
28. Yu H, Rohan T. Role of the insulin-like growth factor family in cancer development and progression. J Natl Cancer Inst 2000;92:1472–89.
29. Baserga R. Targeting the IGF-1 receptor: from rags to riches. Eur J Cancer 2004;40:2013–5.
30. LeRoith D, Roberts CT, Jr. The insulin-like growth factor system and cancer. Cancer Lett 2003;195:127–37.
31. Adachi Y, Lee CT, Coffee K, et al. Effects of genetic

Jpn J Clin Oncol 2008;38(5)354–359
doi:10.1093/jjco/hyn035

Clinical Efficacy and Safety of Octreotide (SMS201-995) in Terminally Ill Japanese Cancer Patients with Malignant Bowel Obstruction

Yasuo Shima[1,5], Atshushi Ohtsu[2], Kuniaki Shirao[3,6] and Yasutsuna Sasaki[4,7]

[1]Palliative Care Unit, National Cancer Center Hospital East, Chiba, [2]Division of Gastrointestinal Oncology, National Cancer Center Hospital East, Chiba, [3]Division of Gastrointestinal Oncology, National Cancer Center Hospital, Tokyo and [4]Division of Oncology/Hematology, National Cancer Center Hospital East, Chiba, Japan

Received February 28, 2008; accepted April 10, 2008

Objective: In patients with advanced cancer, malignant bowel obstruction (MBO) causes gastrointestinal symptoms such as nausea and vomiting leading to severely impaired oral food intake. Thus, MBO markedly diminishes the quality of life (QOL) of these patients because placement of a nasogastric tube becomes necessary. Many studies have shown that octreotide (SMS201−995; SMS), a synthetic analog of somatostatin, is effective for controlling the symptoms of MBO. This study was conducted to assess the efficacy and safety of 300-μg/day initial dose of SMS in Japanese patients with MBO and to investigate the clinical benefit of patients achieved by the improvement of nausea/vomiting based on subjective assessment.

Methods: The subjects were patients with MBO that was refractory to other medical treatment and who had suffered at least two vomiting episodes per day for two consecutive days or had required a nasogastric tube. After enrollment, patients received SMS (300 μg/day) subcutaneously as a continuous injection for 6 days. Patients who responded to this 6-day course of treatment continued to receive the drug.

Results: Among 25 patients who were enrolled, 11 (44.0%) responded to treatment with resolution or improvement of nausea/vomiting. Their symptomatic improvement was assessed by quantitatively measuring the level of control of nausea/vomiting and by using a self-administered QOL questionnaire that evaluated the frequency and severity of nausea/vomiting, the proportion of patients enjoying recreational activities and the overall patient satisfaction with the therapy. SMS was well tolerated, and nausea and agitation were the only adverse events potentially related to this drug.

Conclusion: The results of the study confirmed that the 300-μg/day dose of SMS is safe and effective for patients with MBO uncontrolled by other therapies and suggested that the relief of symptoms with nausea/vomiting by SMS could contribute to improvement of the QOL of patients.

Key words: malignant bowel obstruction – octreotide – terminally ill cancer patients

INTRODUCTION

In patients with advanced cancer, malignant bowel obstruction (MBO) occurs primarily due to carcinomatosis because of the recurrence of gastrointestinal or ovarian cancer. Surveys performed at palliative care facilities of Japan have shown that the incidence of MBO is 10–16% (1,2), which is similar to that reported in the USA and Europe (3–5). Current treatments for MBO include (i)

For reprints and all correspondence: Yasuo Shima, Department of Palliative Medicine, Tsukuba Medical Center Hospital, 1-3-1 Amakubo, Tsukuba-city, Ibaraki, Japan. E-mail: shima@tmch.or.jp

[5]Present address: Department of Palliative Medicine, Tsukuba Medical Center Hospital, Ibaraki
[6]Present address: Department of Medical Oncology, Oita University, Faculty of Medicine, Oita
[7]Present address: Department of Clinical Oncology, Saitama Medical University International Medical Center, Hidaka, Saitama, Japan.

surgery to bypass/remove the obstruction; (ii) gastrointestinal drainage via a nasogastric tube or other means and (iii) medication such as antiemetics or other drugs. Surgical treatment is often contraindicated to the poor general condition of the patient, and placement of a nasogastric tube may be the only treatment available for inoperable cases. Some patients with MBO fail to respond to conventional drugs, such as antiemetics. A nasogastric tube can achieve symptomatic relief in such patients, but common complications include mucosal erosion and hemorrhage, esophagitis and aspiration pneumonia. Therefore, a tube is not recommended for terminally ill cancer patients in whom the quality of life (QOL) should hopefully be improved by palliative care.

As Mercadante et al. (6) first reported the use of SMS, a synthetic analog of somatostatin, for the management of MBO in 1993, several clinical studies have been conducted to evaluate its efficacy for nausea/vomiting due to MBO (5,7–10), primarily at palliative care facilities.

Accordingly, we conducted the first clinical study to assess the efficacy and safety of SMS for the control of nausea/vomiting in Japanese patients with MBO who were unlikely to respond to any other therapy. This study was also designed to evaluate the clinical benefit controlling nausea/vomiting for improvement of the QOL by patient self-assessment.

PATIENTS AND METHODS

Patients in this study were required to be hospitalized, to be between 20 and 74 years of age, to suffer from MBO that was refractory to medical treatment and to have a life expectancy of at least 3 weeks. Before being enrolled in this study, the patients also had at least two episodes of vomiting per day on two designated days or had marked drainage of bowel contents ($\geq$500 ml/day) from a nasogastric tube. Patients who retained hepatic function, as indicated by a total bilirubin $\leq$2.0 mg/dl, were eligible for the study. The study excluded patients with serious complications (e.g. active infection, pleunal effusion and gastrointestinal hemorrhage) and those with symptomatic brain metastasis.

After enrollment, patients received SMS (300 μg/day) subcutaneously as a continuous injection for 6 days. Patients who responded to this 6-day course of treatment continued to receive the drug. The dose of SMS was to be decreased to 150 μg/day in the event of Grade 2 or worse adverse events or if there was marked aggravation of nausea/vomiting.

Patients were assessed daily to determine the number of vomiting episodes, the severity of their nausea and (if relevant) the volume of fluid draining from the nasogastric tube. The volume of intravenous and oral fluid was also measured daily because of a probable influence on the volume of vomitus and tube drainage. The clinical benefit of treatment was also assessed daily during the 6-day treatment period using a self-administered QOL questionnaire (see Appendix 1). Patients were asked about the frequency and severity of nausea/vomiting, the intensity of pain, the amount and quality of sleep and the extent of their enjoyment of recreational activities (TV/radio, reading and conversation).

Table 1. Grading of vomiting by Japan Clinical Oncology Group Toxicity Criteria

Grade	Vomiting
0	No vomiting episodes
1	Nausea only
2	One to five vomiting episodes per 24 h
3	Six or more vomiting episodes per 24 h

Response criteria were based on the change from baseline (24 h before the start of treatment) to Day 6 in the severity of nausea/vomiting graded using the Toxicity Criteria of the Japan Clinical Oncology Group (JCOG) (11). Grading of vomiting by JCOG Toxicity Criteria is shown in Table 1. As shown in Table 2, the response to treatment was graded using three categories ['complete control' (CC), 'partial control' (PC) and 'no control' (NC)]. Patients with JCOG Grade 0 nausea/vomiting on Day 6 were assigned a rating of CC. The rating was PC if the JCOG grade for nausea/vomiting was decreased by one grade or more from baseline on Day 6. No change or an increase of JCOG grade was regarded as NC.

In patients with a nasogastric tube at baseline, extubation was allowed if drainage was reduced to >500 ml/day. After extubation, the response to the treatment was graded according to the following three categories defined by the JCOG grade of nausea/vomiting on Day 6: CC (JCOG Grade 0), PC (only one episode of vomiting per day or nausea only) and NC (nausea/vomiting $\geq$JCOG Grade 2).

The occurrence of adverse events and abnormal laboratory findings were considered for the evaluation of safety, and the severity of adverse drug reaction was grades in accordance with JCOG criteria.

With regard to the clinical laboratory testing, hematology, biochemistry and urine tests were performed just before the start of the treatment with study medication and after 7 days of treatment.

This study was approved by the institutional review board of the National Cancer Center and was conducted in compliance with the Japanese Good Clinical Practice Guidelines. In

Table 2. Criteria for the response to treatment on Day 6

Nasogastric tube (GT)	Complete control (CC)	Partial control (PC)	No control (NC)
Without GT	Grade 0	One grade or more decrease from baseline	No charge or increase grades
With GT*	Grade 0	Only one vomiting per 24 h or nausea only	

*Evaluated after removal of nasogastric tube.

Table 3. Demographic and baseline characteristics of the patients

	Variable	Patients (%)
Sex	Male	11 (44.0)
	Female	14 (56.0)
Age (years)	Range	41–67
	Median	53
Diagnosis of primary tumor	Gastric cancer	14 (56.0)
	Colon cancer	5 (20.0)
	Ovarian cancer	3 (12.0)
	Pancreatic cancer	2 (8.0)
	Cervical cancer	1 (4.0)
Complication(s)	No	16 (64.0)
	Yes	9 (36.0)
Nasogastric tube at baseline	No	17 (68.0)
	Yes	8 (32.0)
Previous gastrectomy	No	18 (72.0)
	Partial	2 (8.0)
	Total	5 (20.0)
Surgical treatment of bowel obstruction	No	21 (84.0)
	Yes	4 (16.0)
PS at baseline	PS 1	1 (4.0)
	PS 2	3 (12.0)
	PS 3	17 (68.0)
	PS 4	4 (16.0)

PS, performance status.

accordance with the Declaration of Helsinki, written informed consent was obtained from all patients prior to enrollment.

RESULTS

Twenty-five patients were enrolled and treated with SMS. Their demographic and baseline characteristics are summarized in Table 3. There were 11 men and 14 women with ages ranging from 41 to 67 years (median: 53 years). Gastric cancer was the most frequent type of malignancy ($n = 14$; 56.0%), followed by colon, ovarian and pancreatic cancer. At baseline, a nasogastric tube was already inserted in eight patients (32.0%) but not in 17 patients (68.0%). The baseline performance status (PS) was 3–4 in 21 patients (84.0%).

None of the subjects needed dose reduction to 150 μg/day according to the protocol criteria.

Response

The response to treatment is summarized in Table 4. Among the 25 patients treated, five (20%) had a response of CC and six (24%) had a response of PC with an overall response rate (CC plus PC) of 44% (95% confidence interval: 24.4–65.1%). Among the 17 patients without a nasogastric tube at baseline, three (17.6%) achieved CC and five (29.4%) achieved PC with an overall response rate of 47.1%. Among the eight patients with a nasogastric tube at baseline, two (25%) achieved CC and one (12.5%) achieved PC with an overall response rate of 37.5%.

In the entire study population, the median number of vomiting episodes per day was significantly reduced from 6.0 (range: 2.0–55) at baseline to 2.5 (range: 0–29) on Day 6 ($P = 0.0024$). Among the eight patients with a nasogastric tube at baseline, four showed a marked decrease in drainage on Day 2 (Fig. 1). All of the three responders (with a rating of CC or PC) in this subgroup showed a reduction in drainage that was sufficient for extubation and achieved symptomatic improvement.

After 6 days (144 h) of continuous therapy, 14 patients were judged to require further treatment with SMS. Treatment was continued for up to 46 days, with the median duration being 8 days.

Clinical Benefit (Subjective Assessment)

Subjective clinical assessment was done by an investigation of four categories (Appendix 1). Twenty-three of the 25 patients completed the self-administered questionnaire. The other two patients were unable to complete this questionnaire because of their poor general condition. In addition, the number of responders decreased to 16 patients on Day 6 because of disease progression. The efficacy of SMS was reflected by an improvement of QOL in terms of nausea/vomiting and enjoyment in activities.

Severity of Nausea/Vomiting

At baseline, 11 of 23 patients (47.8%) had moderate or severe nausea/vomiting. On Day 6, only six of 16 patients (37.5%) still had moderate nausea/vomiting and no patient

Table 4. Response

	Rating	CC	PC	NC	Not evaluated	Total	Response rate (CC + PC), % (95% CI)
	No. of patients (%)	5 (20.0%)	6 (24.0%)	13 (52.0%)	1 (4.0%)	25	44.0 (24.4–65.1)
At baseline	Nasogastric tube (−)	3 (17.6%)	5 (29.4%)	8 (47.1%)	1 (5.9%)	17	47.1 (23.0–72.2)
	Nasogastric tube (+)	2 (25.0%)	1 (12.5%)	5 (62.5%)	0 (0%)	8	37.5 (8.5–75.5)

CI, confidence interval.

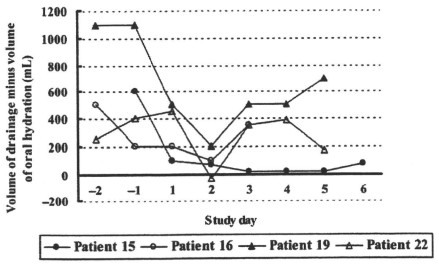

Figure 1. Changes in the net volume of nasogastric tube drainage in four patients who showed a substantial reduction.

rated nausea/vomiting as severe. Nausea/vomiting was absent or slight in nine of 16 patients (56.3%) on Day 6 compared with eight of 23 patients (34.8%) at baseline.

Change of Nausea/Vomiting Relative to Baseline

Nausea/vomiting was markedly or moderately alleviated in 11 of 23 patients (47.8%) on Day 1 and in 10 of 16 patients (62.5%) on Day 6.

Enjoyment of Recreational Activities

Recreational activities were never or hardly ever enjoyed by seven of 16 patients (43.8%) on Day 6 compared with 15 of 23 patients (65.2%) at baseline. The percentage of patients with fair or modest enjoyment of recreational activities increased from 17.4% (four of 23 patients) at baseline to 43.8% (seven of 16 patients) on Day 6. Figure 2 shows the changes in patients in the individual rating categories during SMS treatment (Days 1–6).

Safety

Adverse events occurred in nine patients. Among these, only two events in two patients (8.0%) (Grade 1 nausea and Grade 2 agitation) were judged to be potentially related to SMS. Potential treatment-related laboratory adverse events occurred in six patients (26.1%), including thrombocythemia, leukocytosis, increased ALP and increased γ-GTP. Thus, treatment with SMS was well tolerated and did not cause any serious or clinically significant adverse reactions.

DISCUSSION

Many recent studies have shown that SMS is useful for controlling gastrointestinal symptoms due to MBO in patients who have advanced cancer. In this study, we evaluated the efficacy and safety of 300-μg/day initial dose of SMS in Japanese patients with MBO. The primary efficacy endpoint was the change in vomiting episodes after treatment. To ensure objectivity of assessment, we used the JCOG Toxicity Criteria to grade the severity of emesis. In contrast, previous clinical studies have often used the World Health Organization (WHO) Toxicity Criteria (7) (Grade 1: nausea; Grade 2: transient vomiting; Grade 3: vomiting requiring therapy and Grade 4: intractable vomiting). Compared with the WHO criteria, the JCOG criteria (Grade 1: only nausea; Grade 2: 1–5 vomiting episodes per 24 h; Grade 3: ≥6 vomiting episodes per 24 h) seem to provide a more quantitative assessment of the severity of emesis. In this study, we also assessed the clinical benefit of treatment by using a self-administered QOL questionnaire. No previous clinical studies of SMS have included subjective assessment of efficacy by the patients. This is probably because of only a few eligible patients (e.g. terminally ill cancer patients) were in a satisfactory condition to answer a self-administered questionnaire. This was emphasized by the smaller number of respondents on Day 6 ($n = 16$) compared with baseline ($n = 23$) in our

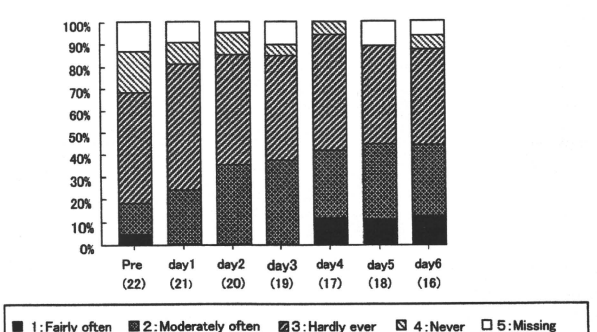

Figure 2. Changes of patients who could enjoy recreational activities rate (%).

study. As a result of previous findings and in consideration of the target disease (MBO refractory to other medical treatment), SMS was administered at 300 μg/day (the maximum effective dose) as a 24-h continuous subcutaneous injection using a pump. Dose escalation was not planned or performed because previous studies have shown that there is no significant additional benefit of higher doses.

Forty-four percent of the patients in this study responded to the treatment with SMS, and the overall response rate was slightly lower than that reported previously (5–10,12–14). Possible explanations for the less favorable response to SMS in the present study include differences in the general condition (poor PS), the underlying malignancies and the timing for assessment of the response to treatment.

Regarding the type of underlying malignancies, more than half of the patients enrolled in the present study had gastric cancer ($n = 14$; 56.0%). Analysis of response data obtained in this study revealed that five of the 14 patients with gastric cancer (35.7%) and six of the 11 patient with other cancer (54.5%) had a response of PC or better. Gastric cancer patients tended to have a lower response rate. Gastric cancer is a common malignancy in the Japanese population, but few earlier studies of SMS have involved patients with gastric cancer. Thus, the lower response rate of gastric cancer patients was partly responsible for the lower overall response rate in the present study.

The timing of assessment might also have affected the difference in the response rate. In this study, we assessed an estimate of response to SMS after 6 days of treatment by comparing the severity of nausea/vomiting as graded according to JCOG Toxicity Criteria between Days 0 (24 h before the starting treatment) and 6. The timing of assessment was based on the results of previous studies (6,7), which suggested no significant difference between the response on Day 6 (144 h) and the response observed over a longer treatment period. However, *post hoc* analysis of our data revealed that the comparison between Days 0 and 6 did not provide an accurate estimate of the response to SMS in some patients. In fact, the benefit of SMS could not be assessed correctly by Day 6 (the study endpoint) in some of the patients because of worsening of their symptoms due to disease progression. In overseas clinical studies (12,13), the response to SMS was assessed after only 3 days of treatment, so the longer treatment period before examination in this study might also have contributed to the lower response rate.

The patients' assessment of clinical benefit of treatment in terms of relief of gastrointestinal symptoms showed that the nausea/vomiting status tended to improve similarly to the assessments on JCOG Toxicity Scale, and the percentage of patients enjoying TV, radio, reading and conversation with others was particularly increased. The increase became progressively greater on each day of the 6-day treatment period (Fig. 2), suggesting that symptomatic improvement achieved by SMS may be associated with an improvement of QOL.

Initial treatment with 300 μg/day of SMS for 6 days was confirmed to be effective and safe for the controlling nausea/vomiting in Japanese patients with MBO. Further studies will be performed to evaluate the SMS therapy with respect to the duration of treatment, effect of higher doses and the usefulness of SMS treatment in relation to the location of obstruction in the upper or lower gastrointestinal tract, and investigation should be performed in more patients.

Further studies may also include assessment on Day 4 after 3 days of SMS treatment as done by Ripamonti coworkers (12–13) in overseas clinical study of SMS.

Acknowledgements

The authors are grateful to the patients and physicians who participated in this study, and are particularly indebted to Drs. E. Ariga, H. Minami, H. Fujii, Y. Matsumura and K. Muro from the National Cancer Center East/Central Hospital, Japan.

Funding

This study was sponsored by Novartis Pharma, Japan.

Conflict of interest statement

None declared.

References

1. Tsuneto S, Ikenaga M, Hosoi J, Kashiwagi T. Malignant intestinal obstruction. Current perspective and future directions palliative medicine. In: Eguchi K, editors. Tokyo: Springer 1998,25–32.
2. Maeno H. Octreotide for gastrointestinal symptoms of terminal cancer patients. *Terminal Care* 2001;11:181–5 (Japanese).
3. Ripamonti C. Management of bowel obstruction in advanced cancer. *Curr Opin Oncol* 1994;6:351–7.
4. Davis MP, Nouneh C. Modern management of cancer-related intestinal obstruction. *Curr Oncol Rep* 2000;2:343–50.
5. Mercadante S, Casuccio A, Mangione S. Medical treatment for inoperable malignant bowel obstruction: a qualitative systematic review. *J Pain Symptom Manage* 2007;33:217–23.
6. Mercadante S, Spoldi E, Caraceni A, Maddaloni S, Simonetti MT. Octreotide in relievjng gastrointestinal symptoms due to bowel obstruction. *Palliat Med* 1993;7:295–9.
7. Khoo D, Hall E, Motson R, Riley J, Denman K, Waxman J. Palliation of malignant intestinal obstruction using octreotide. *Eur J Cancer* 1994;30A:28–30.
8. Cascinu S, Del Ferro E, Catalano G. A randomized trial of octreotide vs best supportive care only in advanced gastrointestinal cancer patients refractory to chemotherapy. *Br J Cancer* 1995;71:97–101.
9. Mangili G, Franchi M, Mariani A, Zanaboni F, Rabaiotti E, Frigerio L, et al. Octreotide in the management of bowel obstruction in terminal ovarian cancer. *Gynecol Oncol* 1996;61:345–8.
10. Maeno H, Ikenaga M, Tsuneto S, Kashiwagi T. The effect of octreotide for bowel obstruction in terminally cancer patients. *Jpn J Clin Res Death Dying* 1996;19:49–52.
11. Tobinai K, Kohno A, Shimada Y, Watanabe T, Tamura T, Takeyama K, et al. Toxicity grading criteria of the Japan Clinical Oncology Group. *Jpn J Clin Oncol* 1993;23:250–7.
12. Ripamonti C, Mercadante S, Groff L, Zecca E, De Conno F, Casuccio A. A role of octreotide, scopolamine butylbromide, and hydration in symptom control of patients with inoperable bowel obstruction and nasogastric tubes: a prospective randomized trial. *J Pain Symptom Manage* 2000;19:23–34.
13. Mercadante S, Ripamonti C, Casuccio A, Zecca E, Groff L. Comparison of octreotide and hyoscine butylbromide in controlling

gastrointestinal symptoms due to malignant inoperable bowel obstruction. *Support Care Cancer* 2000;8:188–91.
14. Mystakidou K, Tsilika E, Kalaidopoulou O, Chondros K, Georgaki S, Papadimitriou L. Comparison of octreotide administration vs conservative treatment in the management of inoperable bowel obstruction in patients with far advanced cancer: a randomized, double-blind, controlled clinical trial. *Anticancer Res* 2002;22:1187–92.

APPENDIX

QOL QUESTIONNAIRE

Date:

- Question 1: How intense is your pain?
  (1) None
  (2) Slight
  (3) Moderate
  (4) Severe
- Question 2: How many vomiting episodes do you have per day?
- Question 3: How severe is your nausea and vomiting?
  (1) None
  (2) Slight
  (3) Moderate
  (4) Severe
- Question 4: Did the severity of your nausea and vomiting change after the start of the clinical trail?
  (1) Markedly improved
  (2) Moderately improved
  (3) Unchanged
  (4) Worse
- Question 5: How is your sleep quality?
  (1) Good
  (2) Fair
  (3) Poor
  (4) No sleep
- Question 6: Can you enjoy watching TV, listening to the radio, reading a book, or talking with others?
  (1) Fairly often
  (2) Moderately often
  (3) Hardly ever
  (4) Never

Jpn J Clin Oncol 2008;38(6)432–437
doi:10.1093/jjco/hyn043

A Phase II Study of Sequential Methotrexate and 5-fluorouracil Chemotherapy in Previously Treated Gastric Cancer: A Report from the Gastrointestinal Oncology Group of the Japan Clinical Oncology Group, JCOG 9207 Trial

Tetsuya Hamaguchi[1], Kuniaki Shirao[1,13], Noboru Yamamichi[2], Ichinosuke Hyodo[3], Wasaburo Koizumi[4], Shigeki Seki[5], Tetsuri Imamura[6], Hisanobu Honma[7], Atsushi Ohtsu[8], Narikazu Boku[8], Toshikazu Mukai[9], Seiichiro Yamamoto[10], Haruhiko Fukuda[11] and Shigeaki Yoshida[12], Gastrointestinal Oncology Study Group of Japan Clinical Oncology Group

[1]Division of Gastrointestinal Oncology, National Cancer Center Hospital, Tokyo, [2]Department of Surgery, Fukui Prefectural Hospital, Fukui, [3]Department of Internal Medicine, National Hospital Organization, Shikoku Cancer Center, Ehime, [4]Department of Internal Medicine, Kitasato University East Hospital, Kanagawa, [5]Department of Internal Medicine, Saku Central Hospital, Nagano, [6]Department of Gastroenterology, Sapporo-Kosei General Hospital, Hokkaido, [7]Department of Gastroenterology, National Hospital Organization, Hokkaido Cancer Center, Hokkaido, [8]Division of Gastrointestinal Oncology/Digestive Endoscopy, National Cancer Center Hospital East, Chiba, [9]Department of Internal Medicine, Kure Kyosai Hospital, Hiroshima, [10]Cancer Information Services and Surveillance Division, Center for Cancer Control and Information Services, National Cancer Center, Tokyo, [11]Clinical Trials and Practice Support Division, Center for Cancer Control and Information Services, National Cancer Center, Tokyo, Japan, [12]Director, National Cancer Center Hospital East, Chiba and [13]Department of Medical Oncology, Oita University, Faculty of Medicine, Oita, Japan

Received February 26, 2008; accepted May 7, 2008; published online May 30, 2008

Objective: As prognosis of advanced gastric cancer is still poor, a standard regimen after first-line fluorouracil (FU)-based chemotherapy has not yet been established. Therefore, we conducted a phase II study to evaluate the efficacy and toxicity of sequential treatment with methotrexate (MTX) and also 5-FU as second-line chemotherapy in patients with advanced gastric cancer.
Methods: Treatment consisted of weekly doses of MTX (100 mg/m^2, i.v. bolus), followed by 5-FU (600 mg/m^2, i.v. bolus) 3 h after MTX administration. Leucovorin rescue therapy (six doses of 10 mg/m^2, given at 6-h intervals) was commenced 24 h after a treatment with MTX. The primary endpoint was the response rate.
Results: Between December 1992 and June 1995, 56 patients were registered in this study and one was ineligible. All registered patients were included in all analyses. The median age of the patients was 60 years (20–75 years). Most patients (75%) had a performance status of 0 or 1, and 51 (90%) received 5-FU-based chemotherapy as first-line treatment. The major adverse events were myelosuppression and gastrointestinal toxicity. Grade 4 neutropenia occurred in 6.3% of the patients. The overall objective response rate was 9.0% [five partial responses among 56 patients, 95% confidence interval (CI): 3.0–20%]. The median overall survival time was 237 days, and the 1-year survival proportion was 21.4%.
Conclusions: Sequential MTX/5-FU therapy provides good survival outcomes with tolerable toxicity despite a limited response in patients with previously treated advanced gastric cancer. This regimen is now being evaluated in a randomized study in patients with pretreated advanced gastric cancer, by the Japan Clinical Oncology Group.

Key words: methotrexate – 5-fluorouracil – gastric cancer – second-line chemotherapy

For reprints and all correspondence: Kuniaki Shirao, Department of Medical Oncology, Oita University, Faculty of Medicine, 1-1 Idaigaoka, Hasama-machi, Yufu-city, Oita 879-5593, Japan. E-mail: kshirao@med.oita-u.ac.jp

INTRODUCTION

Gastric cancer is the most common cancer in Japan and many other Asian countries. Mortality statistics shows that around 50 000 patients die from gastric cancer every year in Japan (1). Primary tumor resection is the best strategy for the treatment of gastric cancer. In patients with curatively resected Stages I–III gastric cancer, the 5-year survival proportion is $\geq$50%, but remains $\leq$10% in Stage IV or recurrent disease. Some randomized trials have demonstrated that fluorouracil (FU)-based regimens improve survival proportions in patients with advanced gastric cancer, as compared with supportive care alone (2–4). Although this survival advantage is significant as first-line treatment, no randomized study has shown a survival benefit of any second-line regimen for patients with metastatic gastric cancer failed to first-line FU-based chemotherapy, as compared with best supportive care.

Methotrexate (MTX) enhances the cytotoxicity of 5-FU by inhibiting the synthesis of DNA, RNA or both when 5-FU is administered several hours after MTX (5,6). A meta-analysis of randomized trials of sequential MTX/5-FU therapy revealed a higher response rate and longer survival as compared with single-agent bolus 5-FU chemotherapy in patients with colorectal cancer. Toxicity with these sequential MTX/5-FU regimens was similar to that with 5-FU alone (i.e. vomiting, stomatitis, diarrhea and leukopenia) (7,8). The sequential MTX/5-FU therapy was also found to be effective against advanced gastric cancer as shown in phase II trials (9,10). One Japanese phase II trial of sequential MTX/5-FU therapy in advanced gastric cancer reported response rates of 23% [13 partial responses (PRs)/56 patients] and 41% (15 PRs/37 patients) with low- and intermediate dose MTX regimens, respectively (11). Several reports indicated that a 7- to 24-h interval between MTX and 5-FU may provide an advantage of efficacy. However, a longer than 7-h interval cannot be used on an outpatient basis. In Japan, a 3-h interval between MTX and 5-FU regimens has been verified to be safe and effective in patients with advanced gastric cancer (11). Therefore, we decided to adopt a 3-h interval in the present study.

In 1990s, the sequential MTX/5-FU therapy was widely used as an option for advanced gastric cancer in Japan. At that time, however, other active drugs, such as irinotecan (CPT-11) and taxanes, were unavailable. The results of *in vitro* studies showed that bolus and continuous administrations of FU had different mechanisms of cytotoxicity and resistance, thereby resulting in incomplete cross-resistance between pulse and prolonged exposure to FU (18,19). Therefore, the sequential MTX/5-FU therapy, in which 5-FU was given by bolus infusion, was used empirically without clinical evidence in patients who failed to respond to infusional FU-based regimens, such as 5-FU alone or 5-FU/cisplatin. This background led to the present phase II clinical trial assessing the efficacy and toxicity of sequential MTX/5-FU chemotherapy in patients with pretreated advanced gastric cancer, conducted by the Japan Clinical Oncology Group (JCOG 9207 study).

PATIENTS AND METHODS

Eligibility

All patients enrolled in this trial fulfilled the following eligibility criteria: (i) histologically confirmed gastric cancer, (ii) unresectable or recurrent disease, (iii) treated with one prior regimen until disease progression or unacceptable toxicity after discontinuing palliative or adjuvant chemotherapy, (iv) a wash-out period of at least 4 weeks since the last chemotherapy treatment, (v) measurable or assessable disease, (vi) age $\leq$75 years, (vii) performance status (PS) $\leq$3 on the Eastern Cooperative Group (ECOG) scale, (viii) adequate bone marrow function (WBC $\geq$ 4000/mm^3, platelets $\geq$ 100 000/mm^3), (ix) adequate liver function (serum total bilirubin level $\leq$2.0 mg/dl, transaminase level $\leq$2.5-fold the upper limit of normal), (x) adequate renal function [serum creatinine $\leq$ 1.5 mg/dl, blood urea nitrogen (BUN) $\leq$ 25 mg/dl, creatinine clearance $\geq$ 50 ml/min], (xi) a normal electrocardiogram, (xii) a life expectancy of at least 8 weeks and (xiii) provision of written informed consent. Patients with active gastrointestinal bleeding, synchronous carcinomas, large amounts of pleural effusion or ascites, central nervous system metastasis, concurrent uncontrolled disease, or severe psychiatric disease were excluded. Pregnant or nursing women were also excluded. The study protocol was approved by the JCOG Clinical Trial Review Committee and by the institutional review board of each participating center.

Treatment Plan

The treatment schedule consisted of a weekly dose of MTX (100 mg/m^2, i.v. bolus) followed by 5-FU (600 mg/m^2, i.v. bolus) after a 3-h interval. Leucovorin rescue therapy (10 mg/m^2 orally or i.v. every 6 h, for a total of six doses) was commenced 24 h after MTX administration. To prevent MTX-induced nephrotoxicity, acetazolamide (250 mg) was given intravenously immediately after MTX infusion, and sodium bicarbonate (33.3 mEq) dissolved in 500 ml of normal saline was administered by drip infusion for urinary alkalinization during the 3-h interval between the doses of MTX and 5-FU. Before each cycle, the patients had to meet the following criteria: WBC $\geq$ 3000/mm^3, platelet count $\geq$75 000/mm^3, adequate liver and renal function as defined in the eligibility criteria, a PS of 0–3 and no grade $\geq$2 toxicity. The treatment was terminated if the disease progressed within 4 weeks or if a complete response (CR), PR or minor response (MR) was not achieved within 8 weeks. Otherwise, the treatment was repeated until disease progression or severe toxicity was confirmed.

Evaluation of Response and Toxicity

Baseline evaluations included a complete medical history, physical examination, complete blood cell count, serum chemistry, creatinine clearance, urinary analysis, electrocardiography, gastroscopy, gastrography, abdominal computed

tomography, abdominal ultrasonography and chest radiography. Hematologic, serum chemical and urinary analyses and symptoms were monitored on a weekly basis during the treatment. The objective response was evaluated every 4 weeks. CR, PR, no change (NC), progressive disease (PD) or not evaluated (NE) were defined according to the response assessment criteria proposed by the Japanese Research Society of Gastric Cancer (12). The tumor response was confirmed by central review. Toxicity was evaluated according to the JCOG common toxicity criteria (13) that were established on the basis of the National Cancer Institute Common Toxicity Criteria, ver.1.

Statistical Methods

The primary endpoint of this study was the tumor response rate. The secondary endpoints were overall survival and toxicity. Sample size was determined by feasibility reasons. Within a reasonable length of time (1.5 year of accrual), 15 participating institutions can recruit 50 subjects. This produces the width of 25% of its 95% CI for a point estimate around 30%.

An interim analysis was planned to test for the treatment inefficacy by examining whether the 90% upper confidence limit of the response rate would exceed 25% for the first evaluable 20–25 patients. Overall survival was calculated from the date of registration until the date of death, using the Kaplan–Meier method, and the CIs were calculated using the Greenwood's formula.

All the analyses were conducted using SAS software (ver. 6.12; SAS Institute, Inc., Cary, NC, USA).

RESULTS

Patient Population and Study Treatment

Between December 1992 and June 1995, 56 patients were enrolled in the study at 15 hospitals. Although one patient was ineligible because of no previous chemotherapy, all analyses were conducted for all registered patients. Table 1 lists the demographic data, baseline disease and pretreatment characteristics. Thirty-eight men and 18 women were registered. The median age of the patients was 60 years (range, 25–75 years), and 42 of 56 patients (75%) had a good PS of 0 or 1. Fifty-one patients (91%) had received 5-FU-based chemotherapy as first-line treatment. A total of 419 doses of sequential MTX/5-FU therapy were administered to 56 patients. The median number of doses was 5 (range, 1–31). Forty-four of the 56 enrolled patients (78%) received four or more doses of sequential MTX/5-FU therapy. Treatment was terminated because of the disease progression in 38 patients, toxicity in seven, patient refusal in four, death in four and others in three.

Table 1. Patient characteristics

Characteristic	Total ($n = 56$)
Age (years)	
Median	60
Range	20–75
Gender	
Male	38
Female	18
ECOG performance status	
0	12
1	30
2	12
3	2
Macroscopic type of primary cancer	
1	2
2	14
3	22
4	11
Others	6
Histological type	
Intestinal type	27
Diffuse type	29
Metastatic sites	
Lymph nodes	39
Liver	20
Peritoneum	8
Lung	3
Krukenberg's tumor	3
Douglas' metastasis	2
Bone	1
Malignant ascites	8
Pleural effusion	1
Skin	1
Prior chemotherapy	
5-FU-based regimen	51
Non-FU regimen	4*

*One patient was ineligible due to no prior chemotherapy. ECOG, Eastern Cooperative Group.

Toxicity

Toxic effects occurring during the study are summarized in Table 2. The major toxicities were myelosuppression and gastrointestinal toxicity. Grade 3 and 4 neutropenia occurred in 10 and 6% of the patients, respectively. Severe thrombocytopenia was infrequent. The incidence of Grade 3/4 diarrhea was 3.6%. Mild nausea and vomiting (Grade 1 and 2) were frequent (63.6%). A Grade 4 elevation of total bilirubin was observed in one patient (2%) who was later found to have obstructive jaundice caused by disease progression. Early death, defined as death within 30 days from the last dose of chemotherapy, occurred in nine patients. The causal

Table 2. Toxicity profiles

Toxicity	JCOG grade					Total	Grade 3/4 (%)
	0	1	2	3	4		
Hematological toxicity							
Leucopenia	22	14	16	3	0	55	5.5
Neutropenia	16	15	9	5	3	48	16.7
Anemia	6	20	24	5	—	55	9.1
Thrombocytopenia	48	4	2	1	0	55	1.8
Non-hematological toxicity							
Nausea/vomiting	18	22	13	2	—	55	3.6
Diarrhea	35	12	6	2	0	55	3.6
Stomatitis	36	17	0	1	1	55	1.8
Alopecia	46	8	1	—	—	55	—
Allergic reaction	54	1	0	0	0	55	0
Fever	41	8		1	0	55	1.8
Peripheral neuropathy	53	2	0	0	0	55	0
Total bilirubin	39	—	7	4	1	51	9.8
AST	33	16	2	0	0	51	0
ALT	41	10	0	0	0	51	0
Creatinine	43	6	1	0	0	50	0
Hyponatremia	26	20	5	1	0	52	1.9
Hypokalemia	39	11	2	0	0	52	0
Body weight loss	11	12	6	0	0	29	0
ECOG PS	0	1	2	3	4	Total	
	6	19	18	9	2	54	

PS, performance status; JCOG, Japan Clinical Oncology Group; AST, aspartate aminotransferase; AL, alanine aminotransferase.

relationship between the early deaths and the study treatment was evaluated by the JCOG Data and Safety Monitoring Committee. Seven of the nine deaths were judged as 'death due to PD'. The other two deaths were evaluated to be treatment related. One of the treatment-related deaths was caused by cardiogenic shock, probably because of a 5-FU-related ischemic cardiac event.

Efficacy

The tumor responses of all registered patients were assessed and confirmed by central review (Table 3). The response of the patient who had not previously received chemotherapy was classified as NE. Only five of the 56 patients had an objective PR (response rate; 9.0%, 95% CI, 3–20%). The survival curve is shown in Fig. 1. The median overall survival time was 237 days (95% CI, 145–281 days). The 1-year survival proportion was 21.4% (95% CI, 10.7–32.2%), and the 2-year survival proportion was 3.6% (95% CI, 0–8.4%).

Table 3. Treatment response

CR	0
PR	5
NC	31
PD	14
NE	6
RR	9.0%; 95% CI, 3–20%

CR, complete response; PR, partial response; NC, no change; PD, progressive disease; NE, not able to be evaluated; RR, response rate; CI, confidence interval.

DISCUSSION

5-FU-based chemotherapy is considered a standard therapy for advanced gastric cancer. However, 5-FU-based combination regimens of chemotherapy have not been shown to prolong overall survival as compared with 5-FU alone (14–17). Furthermore, the potential benefits of second-line chemotherapy for patients with pretreated gastric cancer remain unclear, and few prospective studies have been conducted.

Although this study showed that the sequential MTX/5-FU therapy possessed limited antitumor activity as second-line chemotherapy, despite an MST of 237 days (95% CI, 145–281), and 1- and 2-year survival proportions of 21.4% (95% CI, 10.7–32.2%) and 3.6% (95% CI, 0–8.4%), respectively. These survival data were similar to those obtained for first-line chemotherapy with several regimens at that time. Possible reasons for the good survival may include good patients' clinical characteristics. At the baseline evaluation, the median age of the patients was 60 years (range, 20–75 years), and most patients had a good PS of 0 or 1. Another possible reason is a tumor stabilization effect of this combination regimen. Probably because nearly all patients had received 5-FU-based chemotherapy as first-line treatment, 56% of patients had NC, for a disease control rate (PR + NC) of 65%. The toxicity of the regimen can be considered tolerable. The proportion of patients with toxicity in our study was similar to that with the MTX/5-FU therapy used as first-line treatment (11).

Although the response rate of the present study was only 9%, the study regimen had good survival outcomes with tolerable toxicity. Given that survival with the best supportive care is ~3–4 months (3,4), this sequential MTX/5-FU therapy can be considered to be an option for standard second-line treatment.

Recently, second-line chemotherapy with paclitaxel or bi-weekly irinotecan has produced response rates of 27 and 18%, respectively (19,20), although these data were derived by subset analysis. Peritoneal dissemination of gastric cancer may cause serious complications, such as intestinal obstruction, ascites and hydronephrosis with renal dysfunction. In patients with these conditions, cisplatin or irinotecan, drugs active against gastric cancer, are difficult to use because of an

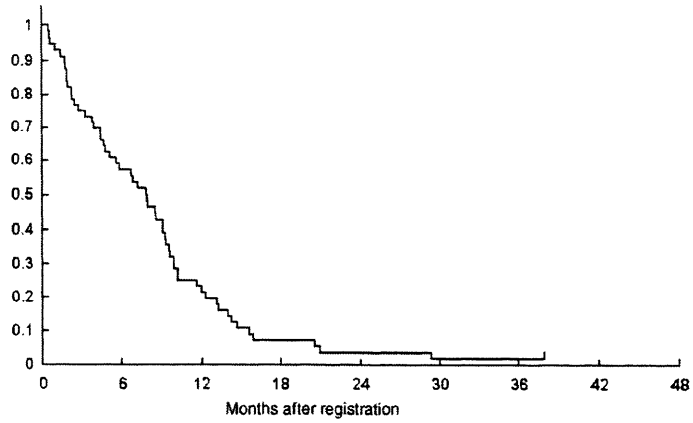

Figure 1. Overall survival.

increased risk of toxicity. The MTX/5-FU therapy is considered to be effective and safe as first-line treatment in patients who have the advanced gastric cancer with peritoneal dissemination, especially malignant ascites (21). On the basis of these results, a randomized phase II trial comparing the MTX/5-FU therapy with paclitaxel in patients with pretreated advanced gastric cancer who have mainly peritoneal disease is now being conducted by the JCOG (protocol JCOG 0407).

Acknowledgements

We would like to thank Dr Kenichi Nakamura for his helpful comments, Dr Naoki Ishizuka for his help in statistical analysis and Ms Aya Kimura for data management.

Funding

This work was supported by Grants-in-aid for Cancer Research (8S-1, 11S-3 and 11S-4) and by the Second-term Comprehensive 10-year Strategy for Cancer Control (H10-Gan-027, H12-Gan-012) from the Ministry of Health, Labor and Welfare, Japan.

Conflict of interest statement

None declared.

References

1. Statistics of Cancer. Center for Information Services. National Cancer Center, Japan, http://ganjoho.ncc.go.jp/public/statistics/pub/update.html.
2. Murad AM, Santiago FF, Petroianu A, Rocha PR, Rodrigues MA, Rausch M. Modified therapy with 5-fluorouracil, doxorubicin, and methotrexate in advanced gastric cancer. *Cancer* 1993;72:37–41.
3. Glimelius B, Hoffman K, Haglund U, Nyren O, Sjoden PO. Initial or delayed chemotherapy with best supportive care in advanced gastric cancer. *Ann Oncol* 1994;5:189–90.
4. Pyrhonen S, Kuitunen T, Nyandoto P, Kouri M. Randomized comparison of fluorouracil, epidoxorubicin and methotrexate (FEMTX) plus best supportive care alone in patients with non-resectable gastric cancer. *Br J Cancer* 1995;71:587–91.
5. Fernandes DJ, Bertino JR. 5-Fluorouracil–methotrexate synergy: enhancement of 5-fluorodeoxyuridylate binding to thymidylate synthase by dihydropteroylpolyglutamates. *Proc Natl Acad Sci USA* 1980;77:5663–7.
6. Cadman E, Heimer R, Davis L. Enhanced 5-fluorouracil nucleotide formation after methotrexate administration: explanation for drug synergism. *Science* 1979;205:1135–7.
7. Nordic Gastrointestinal Tumor Adjuvant Therapy Group. Superiority of sequential methotrexate, fluorouracil and leucovorin to fluorouracil alone in advanced symptomatic colorectal carcinoma: a randomized trial. *J Clin Oncol* 1989;7:1437–46.
8. Advanced Colorectal Cancer Meta-Analysis Project. Meta-analysis of randomized trials testing the biochemical modulation of fluorouracil by methotrexate in metastatic colorectal cancer. *J Clin Oncol* 1994; 12:960–9.
9. Perez JE, Lacava JA, Dominguez ME, Rodriguez R, Barbieri MR, Ortiz EH et al. Biochemical modulation of 5-fluorouracil by methotrexate in patients with advanced gastric carcinoma. *Am J Clin Oncol* 1998;21:452–7.
10. Dickinson R, Preagrave P, Levi J, Milliken S, Woods R. Sequential moderate-dose methotrexate and 5-fluorouracil in advanced gastric adenocarcinoma. *Cancer Chemother Pharmacol* 1989;24:67–8.
11. Sasaki T, Ota K, Ibayashi J, Sakata Y, Matsuoka T, Ishikawa M et al. Randomized multicenter trial of sequential methotrexate and 5-fluorouracil versus 5-fluorouracil alone in advanced gastric cancer. *Jpn J Cancer Chemother* 1989;16:2545–55.
12. Japanese Research Society for Gastric Cancer. Response assessment of chemotherapy for gastric carcinoma. Japanese Classification of Gastric Carcinoma. 1st edn Tokyo, Kanehara, 1995,90–104.
13. Tobinai K, Kohno A, Shimada Y, Watanabe T, Tamura T, Takeyama K et al. Toxicity grading criteria of the Japan Clinical Oncology Group. The Clinical Trial Review Committee of the Japan Clinical Oncology Group. *Jpn J Clin Oncol* 1993;23:250–7.
14. Cocconi G, DeLisi V, Di Blasio B. Randomized comparison of 5-FU alone or combined with mitomycin and cytarabine (MFC) in the treatment of advanced gastric cancer. *Cancer Treatment Rep* 1982;66:1263–6.
15. Cullinan SA, Moertel CG, Fleming TR, Rubin JR, Krook JE, Everson LK et al. A comparison of three chemotherapeutic regimens in the treatment of advanced pancreatic and gastric carcinoma. Fluorouracil vs fluorouracil and doxorubicin vs fluorouracil, doxorubicin, and mitomycin. *JAMA* 1985;253:2061–7.
16. Kim NK, Park YS, Heo DS, Suh C, Kim SY, Park KC et al. A phase III randomized study of 5-fluorouracil and cisplatin versus 5-fluorouracil, doxorubicin, and mitomycin C versus 5-fluorouracil alone in the treatment of advanced gastric cancer. *Cancer* 1993;71:3813–8.
17. Ohtsu A, Shimada Y, Shirao K, Boku N, Hyodo I, Saito H 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.
18. Sobrero AF, Aschele C, Bertino JR. Fluorouracil in colorectal cancer-a tale of two drugs: implications for biochemical modulation. *J Clin Oncol* 1997;15:368–81.
19. Yamada Y, Shirao K, Ohtsu A, Boku N, Hyodo I, Saitoh H et al. Phase II trial of paclitaxel by three-hour infusion for advanced gastric cancer with short premedication for prophylaxis against paclitaxel-associated hypersensitivity reactions. *Ann Oncol* 2001;12:1133–7.
20. Futatsuki K, Wakui A, Nakao I, Sakata Y, Kambe M, Shimada Y et al. Late phase II study of irinotecan hydrochloride (CPT-11) in advanced gastric cancer. CPT-11 Gastrointestinal Cancer Study Group. *Jpn J Cancer Chemother* 1994;21:1033–8.
21. Yamao T, Shimada Y, Shirao K, Ohtsu A, Ikeda N, Hyodo I 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.

Drug Metab. Pharmacokinet. 23 (2): *SNP1* (139)-*SNP9* (147) (2008).

SNP Communication

Genetic Variations and Haplotypes of *ABCC2* Encoding MRP2 in a Japanese Population

Kimie SAI[1,2,*], Yoshiro SAITO[1,2], Masaya ITODA[1], Hiromi FUKUSHIMA-UESAKA[1], Tomoko NISHIMAKI-MOGAMI[2], Shogo OZAWA[1,3,a], Keiko MAEKAWA[1,2], Kouichi KUROSE[1,4], Nahoko KANIWA[1,4], Manabu KAWAMOTO[5], Naoyuki KAMATANI[5], Kuniaki SHIRAO[6,b], Tetsuya HAMAGUCHI[6], Noboru YAMAMOTO[6], Hideo KUNITOH[6], Yuichiro OHE[6], Yasuhide YAMADA[6], Tomohide TAMURA[6], Teruhiko YOSHIDA[7], Hironobu MINAMI[8,c], Yasuhiro MATSUMURA[9], Atsushi OHTSU[10], Nagahiro SAIJO[11] and Jun-ichi SAWADA[1,2]

[1]*Project Team for Pharmacogenetics,* [2]*Division of Functional Biochemistry and Genomics,*
[3]*Division of Pharmacology,* [4]*Division of Medicinal Safety Science,*
National Institute of Health Sciences, Tokyo, Japan
[5]*Division of Genomic Medicine, Department of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan*
[6]*Division of Internal Medicine, National Cancer Center Hospital*
[7]*Genomics Division, National Cancer Center Research Institute, National Cancer Center, Tokyo, Japan*
[8]*Division of Oncology/Hematology,*
[9]*Investigative Treatment Division, Research Center for Innovative Oncology,*
[10]*Division of GI Oncology/Digestive Endoscopy,*
[11]*Deputy Director, National Cancer Center Hospital East, Chiba, Japan*

Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: The multidrug resistance-associated protein 2 (MRP2) encoded by the *ABCC2* gene is expressed in the liver, intestine and kidneys and preferentially exports organic anions or conjugates with glucuronide or glutathione. In this study, all 32 exons and the 5′-flanking region of *ABCC2* in 236 Japanese were resequenced, and 61 genetic variations including 5 novel nonsynonymous ones were detected. A total of 64 haplotypes were determined/inferred and classified into five *1 haplotype groups (*1A, *1B, *1C, *1G, and *1H) without nonsynonymous substitutions and *2 to *9 groups with nonsynonymous variations. Frequencies of the major 4 haplotype groups *1A (−1774delG), *1B (no common SNP), *1C (−24C>T and 3972C>T), and *2 [1249G>A (Val417Ile)] were 0.331, 0.292, 0.172, and 0.093, respectively. This study revealed that haplotype *1A, which has lowered activity, is quite common in Japanese, and that the frequency of *1C, another functional haplotype, was comparable to frequencies in Asians and Caucasians. In contrast, the haplotypes harboring 3972C>T but not −24C>T (*1G group), which are reportedly common in Caucasians, were minor in Japanese. Moreover, the allele 1446C>T (Thr482Thr), which has increased activity, was not detected in our Japanese population. These findings imply possible differences in MRP2-mediated drug responses between Asians and Caucasians.

Keywords: ABCC2; MRP2; genetic variation; haplotype; amino acid change

Received; October 15, 2007, Accepted; December 5, 2007

**To whom correspondence should be addressed:* Kimie SAI, Ph.D., *Division of Functional Biochemistry and Genomics, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel: +81-3-3700-9478, Fax: +81-3-3707-6950, E-mail: sai@nihs.go.jp*
[a]Present address: Department of Pharmacodynamics and Molecular Genetics, Faculty of Pharmaceutical Sciences, Iwate Medical University, Iwate, Japan.
[b]Present address: Department of Medical Oncology, OITA University Faculty of Medicine, Yufu, Japan
[c]Present address: Medical Oncology, Department of Medicine, Kobe University Hospital and Graduate School of Medicine, Kobe, Japan.

As of October 7, 2007, the novel variations reported here are not found in the database of Japanese Single Nucleotide Polymorphisms (http://snp.ims.u-tokyo.ac.jp/), dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), or PharmGKB Database (http://www.pharmgkb.org/).

This study was supported in part by the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, and Health and Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare.

Introduction

The multidrug resistance-associated protein 2 (MRP2) or canalicular multispecific organic anion transporter (cMOAT) is a 190–200 kDa transmembrane glycoprotein comprised of 1545 amino acids and belongs to the superfamily C of ATP-binding cassette (ABC) transporters. This transporter is expressed on hepatic canalicular membranes, intestinal apical membranes, luminal membranes of renal proximal tubules, placental epithelial cells, and the blood brain barrier.[1] MRP2 exports endogenous and exogenous substances, preferentially organic anions or conjugates with glucuronide, glutathione and sulfate.[1-3] This protein originally identified in cisplatin-resistant tumor cells[4] is shown to confer drug resistance to other anti-cancer drugs, such as vincristine and doxorubicin.[5,6]

MRP2 is encoded by the *ABCC2* gene located on chromosome 10q24 and consists of 32 exons (31 coding exons) and spans 69 kb. Several *ABCC2* genetic variations have been detected in patients with Dubin-Johnson syndrome (DJS), an autosomal recessive disease characterized by hyperbilirubinemia with conjugated bilirubin or increased coproporphyrin excretion in urine.[2,7] Recent studies on *ABCC2* have identified common single nucleotide polymorphisms (SNPs) such as −24C>T and −3972C>T (Ile 1324Ile) among several ethnic populations, and several studies have suggested their association with altered MRP2 expression or function.[8-17] In more recent studies on *ABCC2* haplotypes covering an extended 5′-flanking region, close linkages were found among −1549A>G in the 5′-flanking region and two common SNPs −24C>T and −3972C>T (Ile1324Ile).[8] In addition, as possible functional SNPs, −1774delG in Koreans[8] and −1019A>G in Caucasians[10] were reported. However, there is little information on detailed haplotype structures throughout the gene, and comprehensive haplotype analysis in Japanese has not yet been conducted.

We previously analyzed *ABCC2* genetic variations within all 32 exons and the proximal 5′-flanking region (approximately 800 bp upstream of the translation initiation site) using established cell lines derived from Japanese cancer patients to obtain preliminary information on *ABCC2* SNPs in Japanese.[18] In this study, to reveal *ABCC2* haplotype structures in Japanese, we resequenced the *ABCC2* gene including the distal 5′-upstream region (approximately 1.9 kb upstream from the translation initiation site) as well as all 32 exons in 236 Japanese subjects and conducted haplotype analysis using the detected genetic polymorphisms.

Materials and Methods

Human DNA samples: Genomic DNA samples were obtained from blood leukocytes of 177 Japanese cancer patients at two National Cancer Center Hospitals (Tokyo and Chiba, Japan) and Epstein-Barr virus-transformed lymphoblastoid cells prepared from 59 healthy Japanese volunteers at the Tokyo Women's Medical University under the auspices of the Pharma SNP consortium (Tokyo, Japan). Written informed consent was obtained from all subjects. Ethical review boards of all participating organizations approved this study.

PCR conditions for DNA sequencing: We sequenced all 32 exons of the *ABCC2* gene and approximately 800 bp upstream of the translation initiation codon (proximal 5′-flanking region) as described previously and also extended the sequenced region to 1.9 kb upstream of the translation initiation site (distal 5′-flanking region). Briefly, for amplification of the proximal 5′-flanking region and 32 exons, 5 sets of multiplex PCR were performed from 200 ng of genomic DNA using 1.25 units of Z-taq (Takara Bio. Inc., Shiga, Japan) with 0.3 uM each of the mixed primers as shown in **Table 1** [1st PCR]. The first PCR conditions consisted of 30 cycles of 98°C for 5 sec, 55°C for 5 sec, and 72°C for 190 sec. Next, each exon was amplified separately using the 1st PCR product by Ex-Taq (0.625 units, Takara Bio. Inc.) with appropriate primers (0.3 uM) (**Table 1**) [2nd PCR]. The conditions for the second round PCR were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min. For amplification of the distal 5′-flanking region, multiplex PCR was performed from 25 ng of genomic DNA using 1 unit of Ex-Taq (Takara Bio. Inc.) with 0.4 uM each of the 2 sets of primers as shown in **Table 1** [PCR]. The PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min.

Following the PCR, products were treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the sequencing primers listed in **Table 1** (Sequencing). Excess dye was removed by a DyeEx-96 kit (Qiagen, Hilden, Germany), and the eluates were analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). All variations were confirmed by sequencing PCR products generated from new amplifications from genomic DNA. Genbank NT_030059.12 was used as the reference sequence.

Linkage disequilibrium (LD) and haplotype analyses: Hardy-Weinberg equilibrium and LD analyses were performed using SNPAlyze 3.1 software (Dynacom Co., Yokohama, Japan). Pairwise LDs were shown as rho square (r^2) and $|D'|$ values in **Figure 1**. Diplotype configurations (haplotype combinations) were inferred by LDSUPPORT software, which determined the posterior probability distribution of diplotype configurations for each subject based on estimated haplotype frequencies[19].

Results and Discussion

In this study, sixty-one *ABCC2* genetic variations including 36 novel ones were detected in 236 Japanese subjects

Table 1. Primer sequences used in this study

Amplified or sequenced region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified region[a]
PCR (Ex-taq)			
5′-Flanking (for −1.9 k to −1.7 k)	CCACCAGTGCCAAGAGAAGTAT	CACAAGTCATCTGGAAAACACA	20289134–20289443
5′-Flanking (for −1.7 k to −950)	ATGAGGTGGTATCTAACTGTGG	AAATGTTTTCTGTAGGGACGGG	20289392–20290182
1st PCR (Z-taq)			
5′-Flanking (for −1.2 k) to exon 6	ATACTGCATGGGTGGTTATG	AACCTGCCTCCAAATTTTTC	20289942–20303347
Exons 7 to 11	GGAGAATCACTTTGAAGCCG	CTAGCAAGTGTGAGGGGTGT	20304874–20314079
Exons 12 to 19	TCTGTGAATGTGGCAAAACT	GGATCTACCAAGAATTTAGC	20315189–20328004
Exons 20 to 25	GATGAGCATTTTCAATTTAC	TCAGTTCACCCAGCACTTAT	20338211–20344941
Exons 26 to 32	GAGCAAGACCTTGTCTCATA	CCATGGATGAATCTCAGATA	20349821–20360334
2nd PCR (Ex-taq)			
5′-Flanking (for −880 to −130)	GGAAGATCGCTTGAACCCAT	TCATCCCAACCATTTAATCG	20290245–20290994
Exon 1	TTGTTGGCCAGCTCTGTTG	TTCTGGTTCTTGTTGGTGAC	20290810–20291254
Exon 2	GGGTAAGGCTGGATATGGAT	CTGGCTCTACCTGAGACAAT	20292767–20293194
Exon 3	CACCGGAAACCATTCTGTTC	TTTGCCTCACTATGGATCCC	20300442–20300773
Exon 4	GCCAGATTAGTCACGACAGT	CCAAAGGAAGTCTACATGGCC	20301708–20302134
Exon 5	CAGGTAAGGAAAAAAAAGAGTGG	CCTTGTCATAAAATGGTCTG	20301966–20302418
Exon 6	TATGCCAGAAAATCTGATTA	AGGTGGAACATGAGCTTGAGT	20302499–20303070
Exon 7	GGTGGAGATAGCCTCTGACC	TGCACTGAGAAGTATGAAGTGC	20305320–20305728
Exon 8	CCTGTACAGAGAAGGCCACG	TGCGGTCTTCATGAACACAA	20307385–20307816
Exon 9	GGCTTTGGACAATTCTGGTC	TCCACCCATTGTCTGTGAAC	20308539–20309038
Exon 10	AGGCAAGAAGTCACAGTGCC	TTGCCCAAACTCCCATTAAG	20312158–20312650
Exon 11	ACAGTCAGGCAAGGGCTATG	GACAGGAGGACATGAAACAA	20313420–20313873
Exon 12	GATTTCTATTCCCCACATTT	GAGCTGGGGGTATGGTACAA	20315554–20315983
Exon 13	GTGACCTTGGAGAAGATATT	CTCTTGAAAGTTTACCAGCA	20316189–20316623
Exon 14	TTGCTCAAGGACTGAAATAG	CCTGCTTATCCTCAGAAGAG	20318223–20318732
Exon 15	GGTCTCATGGTCTCATTCTA	GGGTTTATCCTGCACTAGTA	20319650–20320025
Exon 16	AGAAGCACTTTGGGGTCTTGTA	GCTGAAATGGGAAGGAGAATC	20321144–20321581
Exon 17	GCTGAAAAACGATAGTCCAA	TCAACTAGATTACCCCTGTGT	20325354–20325863
Exons 18 and 19	TCACAGGGTGACAAGCAAC	TTGAATCTCTGGGTAGTTTG	20326820–20327678
Exon 20	GAAACCAGCAAGATCAGAGGA	TCACTCAGCTGGCATCAAAG	20338493–20338929
Exon 21	TGACTGTGACATCTGCTTGC	GGACAGAGGACATATTGCTCC	20338927–20339248
Exons 22 and 23	GCATTGTATTTCAGCATTGT	ACAGTGTTGTCTAGGGGGAC	20339701–20340506
Exon 24	GAACACACAGAATCCAACAGA	TCACTTCAGCTTCAGACAGT	20342562–20343001
Exon 25	TCTCATTGGTCTCCTCCTCG	AATTTCACACCACTAGCCAT	20344186–20344672
Exon 26	GAGGCATTGCCTAAGAGTGC	AAAGATGGAGCCAGGGTTTG	20350122–20350523
Exons 27 and 28	GGCAAGGATTGTCTTTCTTA	CGACAGCTGCGGTAAGTCTG	20351928–20352954
Exon 29	AGAGATGGAGTAGCCAGTCAC	CAGCCACAAATGCATATTACC	20353790–20354262
Exon 30	GAAGCTCAACCACAAACCAG	GCTCGACCAGTTTTCAAGAG	20355106–20355610
Exon 31	GCAAGGTACAGCTAGTTGAA	GCGTGATGTAAAATTTTGGC	20358730–20359248
Exon 32	GCTGTGGCTCATTGATTTTC	AAGGTGATAAAACAGAAATG	20359651–20360213
Sequencing			
5′-Flanking (for −1.7 k)	CCACCAGTGCCAAGAGAAGTAT	CACAAGTCATCTGGAAAACACA[b]	
(for −1.7 k to −1.3 k)	GGTATCTAACTGTGGTTTTG	GAAGGAAAGGAGTCAAAGGAAC	
(for −1.5 k to −950)	TCCCACACTGAATGCTGCCTTT	TAGGGACGGGGTCTCACTAT	
(for −880 to −400)	GGAAGATCGCTTGAACCCAT[b]	ATGTGCAGTTTCGCTTCTG	
(for −570 to −130)	CATATAGGCTCACACTGGAT	TCATCCCAACCATTTAATCG[b]	
Exon 1	TGGTTCCTTTTATGTATGGC	GTTCTTGTTGGTGACCACCC	
Exon 2	AAAGCAGTGGGATGTGCTG	TGTCTCTACTGTGCACCAAGG	
Exon 3	CACCGGAAACCATTCTGTTC[b]	TTTGCCTCACTATGGATCCC[b]	
Exon 4	CCTCCTTTCTTCCCATGTTC	CTCAACTTGATGCCATTTAC	
Exon 5	TGGGGCAACCTCTAACTCATA	TGAGACCCAGACATCTTAAA	
Exon 6	TTAGGGTCTCCAAATAAACA	ACTTTCAGAGGAGTGAGAGAGT	
Exon 7	GGTGGAGATAGCCTCTGACC[b]	TGCACTGAGAAGTATGAAGTGC[b]	
Exon 8	CCTGTACAGAGAAGGCCACG[b]	CACAATGCTGTAAGGTTAAG	
Exon 9	GGCTTTGGACAATTCTGGTC[b]	TCCACCCATTGTCTGTGAAC[b]	
Exon 10	GTGCCTTGGAGAAGCTGTGT	TTGCCCAAACTCCCATTAAG[b]	
Exon 11	TCACTGGGCACCTCAAGTTC	GGAATCCATCACCTCTACCA	
Exon 12	ACATTTTGGGGACTATATCT	ATGCCAGCTAGTCTATCAAA	
Exon 13	GGAGGCTGGATGATCCTTAAG	CTCTTGAAAGTTTACCAGCA[b]	
Exon 14	CATCTGTCTATGGTGGGATA	ATAGGCTCAAGACAAATCTC	
Exon 15	GATTTCATTCACCTCCTGTT	CATTTCCCCATGCATTCTAT	
Exon 16	CCAATCTTGAGGGGAAATCT	TCCAAGACCTCACCTACTAGC	

Table 1. continued

Amplified or sequenced region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified region[a]
Exon 17	GTGGAATAACTACAAGCACG	TCAACTAGATTACCCCTGTGT[b]	
Exon 18	GGTGACAAGCAACAAAACTA	CCACCATCTTCCCTGTCTTA	
Exon 19	GATGCTCATGTAGGAAAACA	TTTACCATTCCACCCATGGC	
Exon 20	GGCTTCTCTCTCCTTGTTCA	CAAAGAAACAAAGGAAGAGC	
Exon 21	TGACTGTGACATCTGCTTGC[b]	GGACAGAGGACATATTGCTCC[b]	
Exon 22	GCATTGTATTTCAGCATTGT[b]	GATATTTGATGCATGGACGA	
Exon 23	GAATCTGTCTGGACCCTGTA	GTCTAGGGGGACATAATAAT	
Exon 24	ACACACAGAATCCAACAGAT	TCAACATATGACTAAATGGC	
Exon 25	GGAGCCTCTCATCATTCTGC	TTTCACACCACTAGCCATGC	
Exon 26	CCGATCAAGTCAAACCCTCT	TTTGAACCTCAGTCTTCTTT	
Exon 27	TTTCCTTACTCCCTTGTAGA	AAACTTTAGGGACCCATTAT	
Exon 28	CTGCTACCCTTCTCCTGTTC	CCTTCCCTCTGATACTGTGT	
Exon 29	TACCTCCTGTGACTGTGAAT	CAGCCACAAATGCATATTACC[b]	
Exon 30	GCCAGTCCTATCCACCATCT	AACACGAGGAACACGAGGAG	
Exon 31	GATCTGGAACATGAAAATGG	TTTTGGCCAGATTACTTGAC	
Exon 32	GCTCATTGATTTTCACTGCT	AAGGCAAAGGAATAATTATCG	

[a]The reference sequence is NT_030059.12.
[b]The same primer that was used for the 2nd PCR.

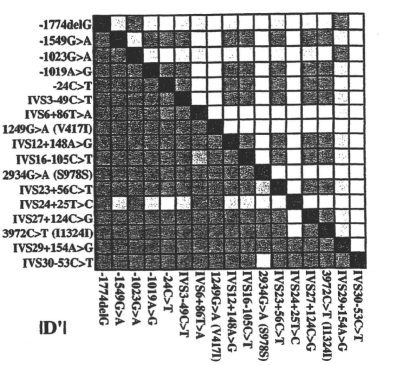

Fig. 1. Linkage disequilibrium (LD) analysis of *ABCC2*
Pairwise LD (r^2 values and |D′|) of polymorphisms detected in no less than 3% of allele frequencies is shown as a 10-graded blue color.

(**Table 2**). All detected variations were in Hardy-Weinberg equilibrium ($p > 0.05$). Novel variations consisted of 5 non-synonymous and 4 synonymous variations in the coding region, 22 in the intronic regions, 3 in the 5′-flanking region, 1 in the 3′- flanking region, and 1 in the 3′-UTR. The novel non-synonymous variations were 1177C>T (Arg393Trp), 1202A>G (Tyr401Cys), 2358C>A (Asp786Glu), 2801G>A (Arg934Gln), and 3320T>G (Leu1107Arg), and their frequencies were 0.002. No statistically significant differences were found in the allele frequencies of all variations between 177 cancer patients and 59 healthy subjects ($P > 0.05$, Fisher's exact test), although a larger number of subjects would be needed to conclude.

The frequency of the known common SNP −24C>T (0.173) was comparable to those reported in Asians (0.17–0.25)[8,12,20] and Caucasians (0.15–0.23)[9,10,14,15,21]. The allele frequency of another common SNP, 3972C>T (Ile1324Ile) (0.216), was also comparable to those in Asians (0.22–0.30)[8,12,20] but lower than those in Caucasians (0.32–0.37)[9,10,14,15,21]. The other major variations in the 5′-flanking region, −1774delG and −1549G>A, were found at frequencies of 0.343 and 0.203, respectively, and these values were similar to those obtained in Koreans (0.34 and 0.21, respectively).[8] However, the relatively frequent SNPs 1446C>G (Thr482Thr) (allele frequency = 0.125), IVS15-28C>A (0.333) and IVS28+16G>A (0.167) in Caucasians[17] were not detected in our study.

The LD profile of the *ABCC* variations (no less than 3% allele frequency) is shown in **Figure 1**. As assessed by r^2 values, close linkages were observed among −1774delG, −1023G>A and IVS29+154A>G, and among −1549G>A, −1019A>G, −24C>T, IVS3-49C>T, IVS12+148A>G, IVS15+169T>C, IVS16-105C>T, IVA23+56C>T, IVS27+124C>G, and 3972C>T (Ile1324Ile). It must be noted that complete linkage was observed between −1549G>A and −1019A>G in our population. In |D′| values, strong LD was also observed almost throughout the region analyzed. Overall, since close associations between the variations were observed throughout the entire *ABCC2* gene, the region sequenced was analyzed as a single LD block for the haplotype inference.

The *ABCC2* haplotype structures were analyzed using 61 detected genetic variations and a total of 64 haplotypes were identified/inferred. **Figure 2** summarizes the haplotypes and their grouping. Our nomenclature system is based on the recommendation of Nebert.[22] Haplotypes without

Table 2. Summary of *ABCC2* variations detected in this study

SNP ID			Reference	Location	Position		Nucleotide change	Amino acid change	Frequency (total = 472)
This Study	dbSNP (NCBI)	JSNP			NT_030059.12	From the translational initiation site or from the end of the nearest exon			
MPJ6_AC 2082			8	5′-Flanking	**20289354**	−1774	acttatcttgttG/_ttttttttttt		0.343
MPJ6_AC 2078[a]				5′-Flanking	**20289538**	−1590	tttaatttgttaG/Atgtatgtttgct		0.002
MPJ6_AC 2079			8, 10, 17	5′-Flanking	**20289579**	−1549	tccttatagtatG/Attgtggatatta		0.203
MPJ6_AC 2080			9, 17	5′-Flanking	**20290105**	−1023	tgggaggccaagG/Acagaaggattgt		0.343
MPJ6_AC 2081			10, 17	5′-Flanking	**20290109**	−1019	aggccaaggcagA/Gaggattgttgaa		0.203
MPJ6_AC 2028[a]				5′-Flanking	**20290395**	−733	acagtttctagcG/Tactgatgccacc		0.004
MPJ6_AC 2029				5′-Flanking	**20290395**	−733	acagtttctagcG/Aactgatgccacc		0.002
MPJ6_AC 2030[a]				5′-Flanking	**20290715**	−413	ttgcagcagaagC/Tgaaactgcacat		0.002
MPJ6_AC 2003		**ssj0000371**	9, 12, 15–18, 20, 26	Exon 1	**20291104**	−24	tagaagagtcttC/Tgttccagacgca		0.174
MPJ6_AC 2004			18	Exon 1	**20291105**	−23	agaagagtcttcG/Attccagacgcag		0.006
MPJ6_AC 2031		**ssj0000386**	17, 26	Intron 3	**20301785**	IVS3 − 49	ctccccctcagtcC/Ttcggtagtggc		0.203
MPJ6_AC 2032[a]				Intron 6	**20302837**	IVS6 + 86	tattttattattT/Attttttgagat		0.076
MPJ6_AC 2033[a]				Exon 7	**20305479**	732	caagtttgaaacG/Acacatgaagaga	Thr244Thr	0.002
MPJ6_AC 2066[a]				Intron 7	**20307421**	IVS7 − 69	tcacaggctgacC/Gaccctggagctg		0.002
MPJ6_AC 2067[a]				Intron 7	**20307423**	IVS7 − 67	acaggctgaccaC/Acctggagctgct		0.002
MPJ6_AC 2035[a]				Exon 9	**20308814**	1177	ggtgtaaaagtaC/Tggacagctatca	Arg393Trp	0.002
MPJ6_AC 2068[a]				Exon 9	**20308839**	1202	tggctttctgtatA/Gtaagaaggtaag	Tyr401Cys	0.002
MPJ6_AC 2036[a]				Intron 9	**20308859**	IVS9 + 13	gtaagcagaataC/Tggcaggtatcac		0.002
MPJ6_AC 2037[a]				Exon 10	**20312319**	1227	gaccctatccaaC/Tttggccaggaag	Asn409Asn	0.002
MPJ6_AC 2009		**ssj0000388**	17, 18, 20, 23–26	Exon 10	**20312341**	1249	aaggagtacaccG/Attggagaaacag	Val417Ile	0.097
MPJ6_AC 2010			18	Exon 10	**20312549**	1457	ccaagagtaagaC/Tcattcaggtaag	Thr486Ile	0.019
MPJ6_AC 2069[a]				Intron 11	**20315600**	IVS11 − 67	taaaacatgggtG/Agatcagatacac		0.002
MPJ6_AC 2038		**ssj0000390**	26	Intron 12	**20315952**	IVS12 + 148	ccgccccatgccA/Gcttttcctcctt		0.210
MPJ6_AC 2039[a]				Intron 13	**20318344**	IVS13 − 73	tcatggactaacG/Aacaaagtcaaaa		0.002
MPJ6_AC 2070[a]				Intron 14	**20318515**	IVS14 + 14	taaataaatttgG/Taagttgcttccc		0.002
MPJ6_AC 2040[a]				Intron 14	**20318521**	IVS14 + 20	aatttggaagtt(del/ins)[b]cagcaaactga		0.002
MPJ6_AC 2071[a]				Intron 14	**20318594**	IVS14 + 93	agcaaactgagaG/Tagagtgtgggaga		0.002
MPJ6_AC 2041[a]				Intron 14	**20319757**	IVS14 − 62	cggagagagacaC/Tgtgagggcagac		0.002
MPJ6_AC 2042[a]				Intron 14	**20319758**	IVS14 − 61	ggagagagacacG/Atgagggcagaca		0.006
MPJ6_AC 2043		**ssj0000393**	26	Intron 15	**20320054**	IVS15 + 169	aaagcaaaggttT/Ctcagccccttcc		0.210
MPJ6_AC 2044[a]				Intron 15	**20321170**	IVS15 − 131	gtcttgtatatcC/Gaaggcaaatttt		0.004
MPJ6_AC 2045[a]				Intron 16	**20325422**	IVS16 − 169	ttgagtcctgagA/Tgtggaataacta		0.004
MPJ6_AC 2046		**ssj0000396**	17	Intron 16	**20325486**	IVS16 − 105	tgcacagttattC/Taaatttaagctc		0.214
MPJ6_AC 2072[a]				Exon 18	**20327159**	2358	tctttctagatgaC/Acccctgtctgca	Asp786Glu	0.002
MPJ6_AC 2012			18, 20, 23	Exon 18	**20327167**	2366	atgacccccctgtC/Tgcagtggatgc	Ser789Phe	0.008
MPJ6_AC 2073[a]				Intron 19	**20327555**	IVS19 + 3	gaagccacaggtA/Gtgtaagaaggat		0.002
MPJ6_AC 2047[a]				Intron 19	**20327645**	IVS19 + 93	agtatccagtgaA/Tctagatttggaa		0.002
MPJ6_AC 2048				Intron 20	**20338745**	IVS20 + 29	gctggcagccctcC/Agtcagctctata		0.002
MPJ6_AC 2049[a]				Exon 21	**20339052**	2801	cctgaaaactcG/Agaatgtgaatag	Arg934Gln	0.002
MPJ6_AC 2015		**ssj0000398**	8, 18, 26	Exon 22	**20339944**	2934	aggattgttttcG/Aatattcttcatc	Ser978Ser	0.040
MPJ6_AC 2050[a]				Exon 22	**20340061**	3051	cgactatccagcA/Gtctcagagggac	Ala1017Ala	0.002
MPJ6_AC 2051[a]				Exon 23	**20340337**	3181	cacaagcaactgC/Tgaacaatatcc	Leu1061Leu	0.002
MPJ6_AC 2052		**ssj0000399**	17, 26	Intron 23	**20340470**	IVS23 + 56	ggatctttctgaC/Tagggaggaatta		0.222
MPJ6_AC 2074[a]				Exon 24	**20342724**	3320	ttacatgcttccT/Gggggataatcag	Leu1107Arg	0.002
MPJ6_AC 2053				Intron 24	**20342843**	IVS24 + 25	atggctaagtcaT/Ccctttccttcctc		0.030
MPJ6_AC 2075[a]				Intron 24	**20342880**	IVS24 + 62	agcccagcctctT/Ctcctgagaatct		0.002
MPJ6_AC 2054				Intron 24	**20342926**	IVS24 + 108	cactcactcctcC/Tcctcagcagctt		0.023
MPJ6_AC 2055[a]				Intron 24	**20344318**	IVS24 − 56	agaaaggaggaaG/Aatggtggatgcc		0.002
MPJ6_AC 2056[a]				Intron 26	**20352061**	IVS26 − 21	atgatgatttttcA/Ggtctttctggttt		0.002
MPJ6_AC 2057[a]				Intron 27	**20352227**	IVS27 + 44	ggcaaaaacaacA/Gtgcaactcctttc		0.008
MPJ6_AC 2058		**ssj0000404**	17, 26	Intron 27	**20352307**	IVS27 + 124	aaagttttctttC/Gctctaactcaaa		0.222
MPJ6_AC 2076			26	Exon 28	**20352688**	3927	ccaagtgcggtaC/Tcgacctgagctg	Tyr1309Tyr	0.002
MPJ6_AC 2022		**ssj0000407**	8, 12, 13, 17, 18, 20, 26	Exon 28	**20352733**	3972	cacttgtgacatC/Tggtagcatggag	Ile1324Ile	0.216
MPJ6_AC 2059[a]				Intron 28	**20352920**	IVS28 + 172	agggaaggatagC/Tagccaggggatca		0.004
MPJ6_AC 2060[a]				Intron 29	**20354201**	IVS29 + 136	cttgagctagttC/Tcctagggatggac		0.002
MPJ6_AC 2061		**ssj0000408**	26	Intron 29	**20354219**	IVS29 + 154	gatggacacgtcA/Gttttccagaactt		0.367
MPJ6_AC 2062		**IMS-JST090926**	17	Intron 29	**20355209**	IVS29 − 35	cttttctggcatG/Aagccccaacagc		0.015
MPJ6_AC 2063[a]				Intron 30	**20358793**	IVS30 − 92	gggggttttgaA/Gagtctgatctgg		0.008
MPJ6_AC 2064		**IMS-JST185750**		Intron 30	**20358832**	IVS30 − 53	ccccctgccctgC/Tgtctttccttgg		0.051
MPJ6_AC 2077[a]				3′-UTR	**20359975**	*61[c]	taatttttattttT/Gtataaaatacag		0.002
MPJ6_AC 2065[a]				3′-Flanking	**20360190**	*193+83[c]	ttattccttttgcC/Gtttcatttctgt		0.002a8

[a]Novel genetic variation

[b]delGCTTCCCAAACTTATTCGCAGTACTGGTGCCAGAATTTTGATAATACAAGAGCTTAGTAG/insTATTTACCT

[c]Numbered from the termination codon.