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Combination of O^6-methylguanine-DNA methyltransferase and thymidylate synthase for the prediction of fluoropyrimidine efficacy

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ABSTRACT

We investigated the correlation between the response to fluoropyrimidines as first-line therapy and the expressions of genes in patients with primary colorectal cancer (CRC). The study group comprised 92 patients with metastatic CRC. Total RNA was isolated from laser-captured tumour cells in surgically resected primary lesions, and gene expression was quantitatively evaluated by real-time RT-PCR assay. Low thymidylate synthase (TS), low γ-glutamyl hydrolase, high reduced folate carrier 1, high O^6-methylguanine-DNA methyltransferase (MGMT) and low cyclin E expressions were associated with a good response ($P = 0.0030$, 0.0250, 0.0120, 0.0030 and 0.0020, respectively) on univariate analysis. On multivariate logistic regression analysis, TS and MGMT remained independent predictors of the response. The clinical response rates were 63.2% in the low TS or high MGMT group and 14.3% in high TS and low MGMT group ($P < 0.0001$). The combination of high TS and low MGMT expression is a significant predictor of a poor response to fluoropyrimidine treatment.

1. Introduction

The median survival time of patients with colorectal cancer (CRC) has improved in the past 10 years because of the development of new agents with advantages over 5-fluorouracil (5-FU), including irinotecan hydrochloride (CPT-11) and oxaliplatin. CPT-11 or oxaliplatin monotherapy, however, was not shown to be more effective than bolus 5-FU/leucovorin (LV) in terms of response and median survival time. CPT-11 or oxaliplatin plus bolus or infusional 5-FU/LV regimens were found to be clearly more effective than 5-FU/LV, resulting in a doubling of the tumour response rate and prolongation of median survival time by 2–3 months. Regimens combining CPT-11 or oxaliplatin with fluoropyrimidines are now key first- and second-line chemotherapies for CRC. Response rates with these regimens, however, remain around 40–50%, prompting investigations of molecular predictors of the response to specific chemotherapeutic regimens. In this study, we evaluated molecular markers that could be used to predict the clinical outcomes of treatment with fluoropyrimidine-based

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regimens, now widely used to treat CRC. Because data on CPT-11-based regimens not including fluoropyrimidines will be difficult to obtain in the future, we also investigated such regimens used for second-line treatment in this study.

Evaluations of regimens including fluoropyrimidines alone as well as those including CPT-11 without fluoropyrimidines are required to produce benchmarks for predicting the efficacy of combined treatment with fluoropyrimidines and CPT-11.

Many potential predictors of the response to fluoropyrimidines have been reported. Several enzymes involved in the targeting, metabolism and catabolism of fluoropyrimidines have been extensively studied, including thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD).[1–3] The enzymes concerning folinic acid metabolism and transport are also the important factors involved in the efficacy of biochemical modulation of 5-FU by LV; among these being folylpolyglutamate synthetase (FPGS), γ-glutamyl hydrolase (GGH) and reduced folate carrier 1 (RFC1).[4,5]

The role of molecular markers in predicting the response to CPT-11-based chemotherapy remains largely unclear, as compared with oxaliplatin-based chemotherapy for CRC, for which several promising markers have been identified.[6] Recently, comprehensive analysis based on the microarray gene expression also have been performed to clarify the predictive markers for CPT-11/5-FU/LV treatment.[7] DNA topoisomerase I (TOPO I) may be a useful predictor of the response to CPT-11-based treatments in colon cancer cell lines as well as in patients with metastatic CRC.[8] Factors involved in DNA-repair systems, such as excision repair cross-complementing 1 (ERCC1) and O^6-methylguanine-DNA methyltransferase (MGMT), have also been investigated recently with respect to their role in resistance to CPT-11.[9,10] The relations between response and factors involved in drug detoxification, such as glutathione S-transferase pi (GSTpi), have been studied for many chemotherapeutic agents, including CPT-11.[9,10] On the other hand, epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and cell-cycle-regulation genes, such as cyclin E, have been reported to be related to the outcomes of patients with CRC.[11]

To gain further insight into potentially useful markers, we investigated the correlation between clinical response and the expressions of genes involved in the targeting, metabolism or catabolism of fluoropyrimidines, drug detoxification, cell cycles and DNA-repair systems in patients with metastatic or recurrent CRC who received first-line fluoropyrimidine-based regimens with or without LV or second-line CPT-11-based regimens.

2. Patients and methods

2.1. Patient selection and chemotherapy

This clinical–biological correlative study was performed retrospectively in a consecutive series of patients who underwent surgery for primary CRC at our hospital between 1996 and 2003 and received first-line fluoropyrimidine-based regimens for metastatic or recurrent CRC. Their responses to treatment and outcomes were confirmed. Patients who received second-line CPT-11-based chemotherapy were analysed as a subgroup. Approval for this study was obtained from the institutional review board of the National Cancer Center Hospital, Tokyo.

Fluoropyrimidines included 5-FU/l-LV (5-FU 600 mg/m^2 bolus and l-LV 250 mg/m^2 div, weekly × 6, q 8 weeks), continuous infusion of 5-FU (5-FU 250 mg/m^2/day), uracil-tegafur (UFT)/LV (UFT 300 mg/day and LV 75 mg/day per os, 4 weeks on and 1 week off), UFT alone (UFT 300 mg/day per os, 4 weeks on and 1 week off) and TS-1 (TS-1 80, 100 or 120 mg/day per os, 4 weeks on and 2 weeks off). CPT-11-based chemotherapy included CPT-11 alone (CPT-11 150 mg/m^2 div, biweekly) and CPT-11/mitomycin C (CPT-11 150 mg/m^2 div and mitomycin C 5 mg/m^2 bolus, biweekly).

2.2. Clinical evaluation and response criteria

Clinical response was evaluated every 6–8 weeks by CT imaging. Responders to treatment were classified as those patients whose tumours shrank by 50% or more, as estimated on two observations not less than 6 weeks apart. More precisely, a complete response (CR) was defined as the complete disappearance of all evidence of tumour, while a partial response (PR) was defined as a greater than 50% decrease in the sum of the products of the largest perpendicular diameters of all measurable lesions, without the occurrence of new lesions. Amongst those classified as non-responders, stable disease (SD) was defined as a change of less than 25% in tumour size, and progressive disease (PD) was defined as an increase of greater than 25% in the area of the measurable tumour deposits or the appearance of new lesions. Time to progression (TTP) during first-line or second-line chemotherapy was defined as the period from the starting date of chemotherapy to the date on which progression was confirmed.

2.3. Laboratory methods

Ten-micrometre-thick sections of resected primary CRC tumours were obtained from identified areas with the highest tumour concentration and were then mounted on uncoated glass slides. For histologic diagnosis, representative sections were stained with haematoxylin and eosin by standard methods. Before microdissection, sections were stained with nuclear fast red (NFR, American MasterTech Scientific, Lodi, CA). The sections of interest were selectively isolated by laser capture microdissection (P.A.L.M. Microsystem, Leica, Wetzlar, Germany), according to standard procedures.[12] The dissected particles of tissue were transferred to a reaction tube containing 400 μl of RNA lysis buffer.

The samples were homogenised and heated at 92 °C for 30 min. Fifty microlitres of 2 M sodium acetate was added at pH 4.0, followed by 600 μl of freshly prepared phenol/chloroform/isoamyl alcohol (250:50:1). The tubes were vortexed for 15 s, placed on ice for 15 min, and then centrifuged at 13,000 rpm for 8 min in a chilled (8 °C) centrifuge. The upper aqueous phase was carefully removed and placed in a 1.5-mL centrifuge tube. Glycogen (10 μl) and 300–400 μl of isopropanol were added and the samples were vortexed for 10–15 s. The tubes were chilled at −20 °C for 30–45 min to precipitate the RNA. The samples were then washed in 500 μl of 75% v/v ethanol and air-dried for 15 min. The pellet was

resuspended in 50 μl of 5 mM Tris. Finally, cDNA was prepared as described by Lord and colleagues.[13]

Quantification of the 12 genes of interest and an internal reference gene (β-actin) was done using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System, TaqMan®, Perkin–Elmer [PE] Applied Biosystems, Foster City, CA). The PCR reaction mixture consisted of 1200 nM of each primer, 200 nM of probe, 0.4 U of AmpliTaq gold polymerase, 200 nM each of dATP, dCTP, dGTP and dTTP, 3.5 mM of $MgCl_2$ and 1 × Taqman buffer A containing a reference dye. The final volume of the reaction mixture was 20 μl (all reagents from PE Applied Biosystems, Foster City, CA). Cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 46 cycles of 95 °C for 15 s and 60 °C for 1 min. The primers and probes used are listed in Table 1. Gene expression values (relative mRNA levels) are expressed as ratios (differences between Ct values) between the gene of interest and the internal reference gene (β-actin).

2.4. *Statistical analysis*

To evaluate the association of gene expressions with response and TTP, gene expression levels were categorised into low and high values. To determine cutoff values, the maximally selected χ^2 method was employed.[14–16] For each observed value, patients were classified as falling below or equal to that value, or above that value. The maximally selected χ^2-test statistic was used to compare the response rates of the two resulting groups of patients (below or equal to the value versus above the value). The value that yielded the largest χ^2-test statistic (the maximal χ^2 statistic) was selected as the optimal cutoff point. To determine the P-value associated with the maximal χ^2 statistic, we performed 2000 bootstrap-like simulations. For each simulation, a randomly selected value was drawn (with replacement) from the set of observed values and assigned to each of the observed responses; the maximal χ^2 statistic was calculated based on this set of randomly matched values and responses. The corrected P-value was calculated as the proportion of the 2000 simulated maximal statistics that was larger than the original maximal χ^2 statistic. This analysis was repeated using the log-rank test to compare TTP. If promising significant predictive variables were found on this analysis, multivariate logistic regression analysis was preformed for the response to fluoropyrimidines. Stepwise variable selection was done using a significance level of 0.01 for entering into or remaining in the model.

All reported P-values are two-sided, and the level of significance was set at $P < 0.05$, except for stepwise variable selection. All analyses were performed using the statistical software package R, version 2.4.1 and the SAS statistical package, version 9.1.3 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. *Demographics and patients assessed for response and TTP*

A total of 92 Japanese patients (54 men and 38 women; median age, 61 years; range 27–77 years) were evaluated (Table 2). Seventy of these patients (43 men and 27 women; median age, 62 years; range 27–77 years) had received 5-FU/LV regimens, and 63 had received CPT-11 as second-line chemotherapy; 43 patients with CPT-11 and 20 patients with CPT-11/ mitomycin C. Gene expression levels of TS, DPD, FPGS, GGH, RFC1, TOPO I, ERCC1, MGMT, GSTpi, EGFR, VEGF and cyclin E were assessed in all patients, and the relations of these levels to response and TTP were examined.

The response to first-line fluoropyrimidine-based chemotherapy was CR in 2 patients (2%), PR in 39 (42%), NC in 30 (33%) and PD in 21 (23%). The median TTP was 5.1 months. The response to first-line 5-FU/LV treatment was CR in 2 patients (3%), PR in 31 (44%), NC in 21 (30%) and PD in 16 (23%). The median TTP was 5.0 months. For second-line CPT-11-based chemotherapy, the response was PR in 9 patients (14%), NC in 32 (50%) and PD in 22 (36%). The median TTP was 3.5 months.

Table 1 – Primers and probes

Gene	GenBank Accession	Forward primer (5′–3′)	Reverse primer (5′–3′)	Taqman probe (5′–3′)
β-Actin	NM_001101.2	GAGCGCGGCTACAGCTT	TCCTTAATGTCACGCACGATTT	ACCACCACGGCCGAGCGG
TS	NM_001071.1	GCCTCGGTGTGCCTTTCA	CCCGTGATGTGCGCAAT	TCGCCAGCTACGCCCTGCTCA
DPD	NM_000110.2	AGGACGCAAGGAGGGTTTG	GTCCGCCGAGTCCTTACTGA	CAGTGGCTACAGTCTCGAGTCTGCCAGT
FPGS	M98045	GGCTGGAGGAGACCAAGGAT	CATGAGTGTCAGGAAGCGGA	CAGCTGTGTCTCCATGCCCCCCTAC
GGH	NM_003878	GCGAGCCTCGAGCTGTCTA	AATATTCCGATGATGGGCTTCTT	ACCCCACGGCGACACCGC
RFC1	NM_194255.1	CATCGCCACCTTTCAGATT	TGGCAAAGAACGTGTTGAC	CCCGAAGACCAGGGCACAGA
TOPO I	NM_003286	TGTAGCAAAGATGCCAAGGT	TGTTATCATGCCGGACTTCT	CCTTCTCCTCCTCCAGGACATAAGTGGA
ERCC1	NM_001983.2	GGGAATTTGGCGACGTAATTC	GCGGAGGCTGAGGAACAG	CACAGGTGCTCTGGCCCAGCACATA
MGMT	NM_002412	CGTTTTCCAGCAAGAGTCGTT	GAAATCACTTCTCCGAATTTCACA	TCAGCAGCTTCCATAACACCTGTCTGG
GSTpi	X06547	CCTGTACCAGTCCAATACCATCCT	TCCTGCTGGTCCTTCCCATA	TCACCTGGGCCGCACCCTTG
EGFR	X00588	TGCGTCTCTTGCCGGAAT	GGCTCACCCTCCAGAAGGTT	ACGCATTCCCTGCCTCGGCTG
VEGF	NM_003376.4	AGTGGTCCCAGGCTGCAC	TCCATGAACTTCACCACTTCGT	TGATTCTGCCCTCCTCCTTCTGCCAT
Cyclin E	NM_001238	CAGCTTATTGGGATTTCATCTTT	ATACGCAAACTGGTGCAACT	TGCAGCCAAACTTGAGGAAATCTATCC

TS: thymidylate synthase, DPD: dihydropyrimidine dehydrogenase, FPGS: folylpolyglutamate synthetase, GGH: γ-glutamyl hydrolase, RFC1: reduced folate carrier 1, TOPO I: DNA topoisomerase I, ERCC1: excision repair cross-complementing 1, MGMT: O^6-methylguanine-DNA methyltransferase, GSTpi: glutathione S-transferase pi, EGFR: epidermal growth factor receptor, VEGF: vascular endothelial growth factor.

Table 2 – Characteristics of 92 patients treated with first-line fluoropyrimidine

Characteristic	Frequency
Median age, years (range)	61 (27–77)
Gender	
Male	54
Female	38
PS	
0	65
1	26
2	1
Metastatic site	
Liver	64
Lung	44
Lymph node	27
Peritoneum	19
Ovary	2
Bone	1
Regimens	
5-FU/l- LV	70
5-FU continuous infusion	10
UFT/LV	9
UFT	1
TS-1	2
Clinical response	
Complete response	2
Partial response	39
Stable disease	30
Progressive disease	21

PS: Performance status of Eastern Cooperative Oncology Group; 5-FU: 5-fluorouracil; it l-LV: l-leucovorin; UFT, uracil-tegafur; LV: leucovorin.

3.2. *Gene expression levels and clinical outcome of patients receiving first-line fluoropyrimidine-based treatment*

Median gene expression levels relative to the level of the house-keeping gene β-actin, used as an internal reference, are shown in Table 3. For descriptive purposes, we call gene expressions below the designated cut-point 'low' while those above the designated cut-point are called 'high'. The results of univariate analysis for response and TTP are shown in Table 3. Low TS, low GGH, high RFC1, high MGMT and low cyclin E expression levels were significantly associated with a good response to fluoropyrimidines on univariate analysis ($P = 0.0030$, 0.0250, 0.0120, 0.0030 and 0.0020, respectively). Low TS, low GGH, high RFC1, low TOPO I, high MGMT, low GSTpi and low cyclin E expression levels significantly correlated with a long TTP in patients given fluoropyrimidine on univariate analysis ($P = 0.027$, 0.023, 0.045, 0.025, 0.039, 0.002 and 0.009, respectively).

Seventy of the 92 patients given fluoropyrimidines had received 5-FU/LV regimens. Low TS, high FPGS, low GGH, high RFC1, high MGMT and low cyclin E expression levels were significantly associated with a good response to 5-FU/LV on univariate analysis ($P = 0.0060$, 0.0350, 0.0355, 0.0415, 0.0030 and 0.0015, respectively). Low GGH, low GSTpi, high VEGF and low cyclin E expression levels significantly correlated with a long TTP in patients given 5-FU/LV on univariate analysis ($P = 0.016$, 0.045, 0.032 and 0.003, respectively).

3.3. *Multiple logistic regression analysis of clinical response in patients receiving first-line fluoropyrimidines*

Among the expressions of TS, GGH, RFC1, MGMT and cyclin E, which were significantly associated with response as well as TTP in patients given fluoropyrimidines, TS and MGMT expressions continued to be independent predictors of the response to fluoropyrimidines on multiple logistic regression analysis. The clinical responses of patient groups divided according to the cutoff values of TS and MGMT expressions are shown in Table 4. The sensitivity of low TS or high MGMT for the response to fluoropyrimidines (responding patients in the low TS and high MGMT groups/responding patients in all groups) was 0.88, and the specificity of high TS and low MGMT for the response to fluoropyrimidines (non-responding patients in the high TS and low MGMT groups/non-responding patients in all groups) was 0.59. The positive predictive value of low TS or high MGMT for the response to fluoropyrimidines was 0.63, and the negative predictive value of high TS and low MGMT was 0.86. The shortest TTP was observed in the group of patients with TS above and MGMT below the respective cutoff values ($P = 0.083$) (Fig. 1). The median TTP was 5.7 months in patients with low TS or high MGMT and 3.3 months in those with both high TS and low MGMT.

3.4. *Gene expression levels and clinical outcome of patients receiving second-line CPT-11-based treatment*

The results of univariate analyses for response and TTP are shown in Table 5. High TS, high FPGS, low ERCC1, high MGMT, high GSTpi and low VEGF expressions significantly correlated with a good clinical response to second-line CPT-11 treatment ($P = 0.0085$, 0.0145, 0.0015, 0.0215, 0.0155 and 0.0165, respectively). No significant correlation was demonstrated between any gene expression and TTP for second-line chemotherapy.

4. Discussion

Our primary end-point was to clarify the gene expression levels of enzymes involved in the targeting, metabolism or catabolism of fluoropyrimidines, the metabolism or transport of folinic acid, DNA-repair systems and drug detoxification systems and thereby identify predictors of clinical outcomes in patients with CRC who receive fluoropyrimidines. The presence of both high TS and low MGMT expression levels was found to be a significant predictor of a poor response to fluoropyrimidine therapy. Clinically, this combination would have an important role in the selection of first-line treatment for CRC with regimens such as FORFIRI (CPT-11/5-FU/LV) or CPT-11 alone.

MGMT is a DNA-repair enzyme that removes alkyl adducts from O^6-methylguanine. Since the MGMT gene is usually not mutated or deleted in human cancers, loss of MGMT function is probably due mostly to epigenetic changes.[17] Abnormal MGMT activity causes O^6-methylguanine to accumulate in cellular DNA, potentially resulting in the activation of oncogenes or inactivation of tumour suppressor genes, followed

Table 3 – Univariate analysis of gene expression levels and clinical outcome (A: response, B: time to progression) in 92 patients treated with first-line fluoropyrimidine-based regimens

Gene	Number of patients	mRNA expression levels relative to β-actin × 10^{-3}, Median (range)	Cut-point	Bootstrap P-value	RR (%) in low group	RR (%) in high group
A: Correlation between response and gene expression						
TS	92	1.4 (0–10.9)	1.37	0.0030	60.0	29.8
DPD	87	0.22 (0–1.16)	0.48	0.0995	41.3	66.7
FPGS	92	0.49 (0.04–1.69)	0.49	0.1035	36.0	54.8
GGH	91	2.17 (0–9.94)	1.21	0.0250	68.4	38.9
RFC1	92	1.67 (0–8.79)	0.87	0.0120	18.8	50.0
TOPO I	92	1.71 (0–4.93)	1.59	0.1845	53.5	36.7
ERCC1	92	0.41 (0–2.95)	0.38	0.1680	36.4	52.1
MGMT	92	1.75 (0–64.73)	2.59	0.0030	34.3	72.0
GSTpi	92	2.19 (0.48–7.6)	3.71	0.0815	48.2	18.2
EGFR	92	0.86 (0–5.31)	0.48	0.2140	57.9	41.1
VEGF	92	3.86 (0.88–24.3)	4.83	0.2890	40.0	55.6
Cyclin E	92	0.51 (0–2.28)	0.99	0.0020	50.7	13.3

Gene	Number of patients	Cut-point	Bootstrap P-value	Median TTP (day) in low group	Median TTP (day) in high group
B: Correlation between time to progression (TTP) and gene expression					
TS	92	1	0.027	230	132
DPD	87	0.31	0.128	141	232
FPGS	92	0.75	0.293	141	165
GGH	91	4.87	0.023	151	64
RFC1	92	0.87	0.045	68	155
TOPO I	92	2.68	0.025	155	105
ERCC1	92	0.6	0.296	137	165
MGMT	92	3.22	0.039	134	253
GSTpi	92	2.47	0.002	169	86
EGFR	92	0.6	0.161	148	141
VEGF	92	7.06	0.108	141	232
Cyclin E	92	1.09	0.009	155	57

Table 4 – Predictive value of TS, MGMT and their combination for the response to fluoropyimidne (TS and MGMT were selected as independent variables in multiple logistic regression analysis)

Gene expression status	Number of non-responding patients	Number of responding patients	RR (%)	P-value (χ^2-test)
All	51	41	44.6	–
Low MGMT	44	23	34.3	0.0012
High MGMT	7	18	72.0	
Low TS	18	27	60.0	0.0036
High TS	33	14	29.8	
High MGMT or low TS	21	36	63.2	<.0001
Low MGMT and high TS	30	5	14.3	

TS: thymidylate synthase, MGMT: O^6-methylguanine-DNA methyltransferase.

by carcinogenesis.[17–20] In previous studies, the significance of the correlation between MGMT promoter hypermethylation or loss of MGMT expression and patients' prognosis was controversial,[21–25] and its prognostic value for patients treated with specific regimens of anticancer agents remains a matter of debate.

As for CRC, abnormal MGMT expression has been examined in many studies in connection with microsatellite instability (MSI) or CpG island methylator phenotype (CIMP).[25–27] Kohonen-Corish and colleagues reported that low-MSI characterised a distinct subgroup of patients with stage C colon cancer who had poor outcomes.[25] They also found that loss or reduced MGMT protein expression was associated with the low-MSI phenotype, but was not a prognostic factor for overall survival in colon cancer.[25] Recent studies have shown that 5-FU-based adjuvant chemotherapy improves overall

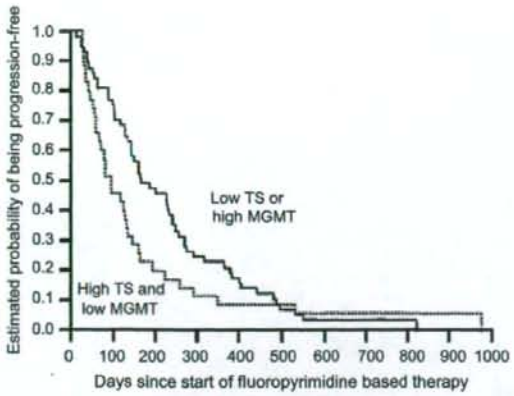

Fig. 1 – Time to progression in patients given fluoropyrimidines according to the cutoff levels of TS and MGMT expression ($p = 0.083$). TS: thymidylate synthase, MGMT: O^6-methylguanine-DNA methyltransferase.

survival in patients who have non-high-MSI CRC as compared with those who have high-MSI CRC.[28,29] On the other hand, Nagasaka and colleagues reported that the later the tumour stage at diagnosis, the less likely MGMT promoter will be methylated; in addition, the recurrence rate associated with oral fluoropyrimidine-based adjuvant chemotherapy was significantly higher in patients with unmethylated MGMT than in those with methylated MGMT (not adjusted for MSI status).[27] To date, the value of MGMT as a prognostic or predictive marker for fluoropyrimidines remains controversial. In this study, MGMT expression was found to be the most significant biomarker for the response to fluoropyrimidines. Furthermore, MGMT combined with TS, one of the most promising enzymes for predicting clinical outcomes of fluoropyrimidine treatment, was shown to be a powerful predictor of the response to fluoropyrimidines.[1,30]

In the subset analysis of 70 patients who received 5-FU/LV as first-line chemotherapy, three enzymes involved in the formation, degradation and transfer into cells of folates, i.e. FPGS, GGH and RFC1, were significantly related to response and TTP. It suggests that high FPGS, low GGH and high RFC1 activity promotes optimal modulation of 5-FU by LV, probably by augmenting the retention of high levels of reduced polyglutamated folates in tumours. We also investigated the predictive value of gene expression levels in patients who received second-line CPT-11-based chemotherapy. Ideally, predictive markers should be evaluated by large prospective randomised trials in patients receiving first-line chemotherapy, and our results suggested that TS, FPGS, ERCC1, MGMT,

Table 5 – Univariate analysis of gene expression levels and clinical outcome (A: response, B: time to progression) in 63 patients treated with second-line CPT-11-based regimens

Gene	Number of patients	Cut-point	Bootstrap P-value	RR (%) in low group	RR (%) in high group
A: Correlation between response and gene expression					
TS	63	1.43	0.0085	5.7	25.0
DPD	60	0.42	0.4735	11.5	25.0
FPGS	63	0.83	0.0145	10.9	37.5
GGH	62	1.1	0.2390	0.0	15.4
RFC1	63	0.77	0.3420	28.6	12.5
TOPO I	63	2.13	0.1630	10.6	25.0
ERCC1	63	0.11	0.0015	44.4	9.3
MGMT	63	1.74	0.0215	6.3	22.6
GSTpi	63	2.43	0.0155	5.3	28.0
EGFR	63	0.39	0.0810	33.3	11.1
VEGF	63	1.97	0.0165	37.5	10.9
Cyclin E	63	1.02	0.056	11.1	33.3
Gene	Number of patients	Cut-point	Bootstrap P- value	Median TTP (day) in low group	Median TTP (day) in high group
B: Correlation between time to progression (TTP) and gene expression					
TS	63	1.38	0.089	97	103
DPD	60	0.14	0.231	99	102
FPGS	63	0.35	0.096	151	98
GGH	62	1.04	0.058	57	102
RFC1	63	1.32	0.221	103	98
TOPO I	63	2.71	0.100	98	113
ERCC1	63	0.11	0.465	119	99
MGMT	63	1.64	0.086	87	110
GSTpi	63	2.89	0.060	92	155
EGFR	63	0.39	0.119	118	98
VEGF	63	2.78	0.243	151	92
Cyclin E	63	0.78	0.321	87	113

TS: thymidylate synthase, DPD: dihydropyrimidine dehydrogenase, FPGS: folylpolyglutamate synthetase, GGH: γ-glutamyl hydrolase, RFC1: reduced folate carrier 1, TOPO I: DNA topoisomerase I, ERCC1: excision repair cross-complementing 1, MGMT: O^6-methylguanine-DNA methyltransferase, GSTpi: glutathione S-transferase pi, EGFR: epidermal growth factor receptor, VEGF: vascular endothelial growth factor.

GSTpi and VEGF are candidate genes for the prediction of the response to CPT-11.

On the other hand, the methodologies in this study to evaluate mRNA levels in the primary tumours with real time RT-PCR offered several advantages. It can easily overcome problems associated with sample volume and tumour heterogeneity by obtaining specimens from primary tumours by laser-captured microdissection. Furthermore, formalin-fixed, paraffin-embedded specimens of the primary tumours are obtained from nearly all patients with CRC. However, only a few studies have examined the relation between levels of molecular markers in primary colorectal tumours and associated metastases.[10,31,32] Since we analysed samples from primary tumours to predict the response of metastatic lesions to chemotherapy, the clinical value of our technique must be validated in larger prospective studies. In addition, we should bear in mind that all patients in our study were Japanese. The potential importance of ethnicity in studies of gene expressions should be taken into account in prospective clinical trials in the future.

Our findings suggest that the presence of both high TS and low MGMT expression is a significant predictor of a poor response to fluoropyrimidine treatment, and the diagnostic value of these predictive markers should be validated in larger cohorts of patients. Furthermore, future studies should also evaluate predictive markers for chemotherapy in patients who receive oxaliplatin-based or CPT-11-based regimens as first-line treatment. A combined analysis of these results might provide new insights into the optimal design for randomised clinical trials.

Conflicts of interest statement

Yoshihiro Okayama and Toshinori Oka are employees of Optimal Medication Research Laboratory, Taiho Pharmaceutical Co., Ltd., Tokushima, Japan.

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Synergistic antitumor activity of the novel SN-38-incorporating polymeric micelles, NK012, combined with 5-fluorouracil in a mouse model of colorectal cancer, as compared with that of irinotecan plus 5-fluorouracil

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The authors reported in a previous study that NK012, a 7-ethyl-10-hydroxy-camptothecin (SN-38)-releasing nano-system, exhibited high antitumor activity against human colorectal cancer xenografts. This study was conducted to investigate the advantages of NK012 over irinotecan hydrochloride (CPT-11) administered in combination with 5-fluorouracil (5FU). The cytotoxic effects of NK012 or SN-38 (an active metabolite of CPT-11) administered in combination with 5FU was evaluated *in vitro* in the human colorectal cancer cell line HT-29 by the combination index method. The effects of the same drug combinations was also evaluated *in vivo* using mice bearing HT-29 and HCT-116 cells. All the drugs were administered i.v. 3 times a week; NK012 (10 mg/kg) or CPT11 (50 mg/kg) was given 24 hr before 5FU (50 mg/kg). Cell cycle analysis in the HT-29 tumors administered NK012 or CPT-11 *in vivo* was performed by flow cytometry. NK012 exerted more synergistic activity with 5FU compared to SN-38. The therapeutic effect of NK012/5FU was significantly superior to that of CPT-11/5FU against HT-29 tumors ($p = 0.0004$), whereas no significant difference in the antitumor effect against HCT-116 tumors was observed between the 2-drug combinations ($p = 0.2230$). Cell-cycle analysis showed that both NK012 and CPT-11 tend to cause accumulation of cells in the S phase, although this effect was more pronounced and maintained for a more prolonged period with NK012 than with CPT-11. Optimal therapeutic synergy was observed between NK012 and 5FU, therefore, this regimen is considered to hold promise of clinical benefit, especially for patients with colorectal cancer.

Key words: NK012; SN-38; 5-fluorouracil; drug delivery system; colorectal cancer

The 5-year survival rates of colorectal cancer (CRC) have improved remarkably over the last 10 years, accounted for in large part by the extensively investigated agents after 5-fluorouracil (5FU). Irinoetcan hydrochloride (CPT-11), a water-soluble, semi-synthetic derivative of camptothecin, is one such agent that has been shown to be highly effective, and currently represents a key-drug in first- and second-line treatment regimens for CRC. CPT-11 monotherapy, however, has not been shown to yield superior efficacy, including in terms of the median survival time, to bolus 5FU/leucovorin (LV) alone.[1] In 2 Phase III trials, the addition of CPT-11 to bolus or infusional 5FU/LV regimens clearly yielded greater efficacy than administration of 5FU/LV alone, with a doubling of the tumor response rate and prolongation of the median survival time by 2–3 months.[1,2]

CPT-11 is converted to 7-ethyl-10-hydroxy-camptothecin (SN-38), a biologically active and water-insoluble metabolite of CPT-11, by carboxylesterases in the liver and the tumor. SN-38 has been demonstrated to exhibit up to a 1,000-fold more potent cytotoxic activity than CPT-11 against various cancer cells *in vitro*.[3] The metabolic conversion rate is, however, very low, with only <10% of the original volume of CPT-11 being metabolized to SN-38[4,5]; conversion of CPT-11 to SN-38 also depends on genetic interindividual variability of the activity of carboxylesterases.[6] Direct use of SN-38 itself for clinical cancer treatment must be shown to be identical in terms of both efficacy and toxicity.

Some drugs incorporated in drug delivery systems (DDS), such as Abraxane and Doxil, are already in clinical use.[7,8] The clinical benefits of DDS are based on their EPR effect.[9] The EPR effect is based on the pathophysiological characteristics of solid tumor tissues: hypervascularity, incomplete vascular architecture, secretion of vascular permeability factors stimulating extravasation within cancer tissue, and absence of effective lymphatic drainage from the tumors that impedes the efficient clearance of macromolecules accumulated in solid tumor tissues. Several types of DDS can be used for incorporation of a drug. A liposome-based formulation of SN-38 (LE-SN38) has been developed, and a clinical trial to assess its efficacy is now under way.[10,11]

Recently, we demonstrated that NK012, novel SN-38-incorporating polymeric micelles, exerted superior antitumor activity and less toxicity than CPT-11.[12] NK012 is characterized by a smaller size of the particles than LE-SN38; the mean particle diameter of NK012 is 20 nm. NK012 can release SN-38 under neutral conditions even in the absence of a hydrolytic enzyme, because the bond between SN-38 and the block copolymer is a phenol ester bond, which is stable under acidic conditions and labile under mild alkaline conditions. The release rate of SN-38 from NK012 under physiological conditions is quite high; more than 70% of SN-38 is released within 48 hr. We speculated that the use of NK012, in place of CPT-11, in combination with 5FU may yield superior results in the treatment of CRC. In the present study, we evaluated the antitumor activity of NK012 administered in combination with 5FU as compared to that of CPT-11 administered in combination with 5FU against CRC in an experimental model.

Material and methods

Cells and animals

The human colorectal cancer cell lines used, namely, HT-29 and HCT-116, were purchased from the American Type Culture Collection (Rockville, MD). The HT-29 cells and HCT-116 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Cell Culture Technologies, Gaggenau-Hoerden, Germany), penicillin, streptomycin, and amphotericin B (100 units/mL, 100 μg/mL, and 25 μg/mL, respectively; Sigma, St. Louis, MO) in a humidified atmosphere containing 5% CO_2 at 37°C.

BALB/c *nu/nu* mice were purchased from SLC Japan (Shizuoka, Japan). Six-week-old mice were subcutaneously (s.c.)

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inoculated with 1×10^6 cells of HT-29 or HCT-116 cell line in the flank region. The length (a) and width (b) of the tumor masses were measured twice a week, and the tumor volume (TV) was calculated as follows: $TV = (a \times b^2)/2$. All animal procedures were performed in compliance with the Guidelines for the Care and Use of Experimental Animals established by the Committee for Animal Experimentation of the National Cancer Center; these guidelines meet the ethical standards required by law and also comply with the guidelines for the use of experimental animals in Japan.

Drugs

The SN-38-incorporating polymeric micelles, NK012, and SN-38 were prepared by Nippon Kayaku (Tokyo, Japan).[12] CPT-11 was purchased from Yakult Honsha (Tokyo, Japan). 5FU was purchased from Kyowa Hakko (Tokyo, Japan).

Cell growth inhibition assay

HT-29 cells were seeded in 96-well plates at a density of 2,000 cells/well in a final volume of 90 μL. Twenty-four hours after seeding, a graded concentration of NK012 or SN-38 was added concurrently with 5FU to the culture medium of the HT-29 cells in a final volume of 100 μL for drug interaction studies. The culture was maintained in the CO_2 incubator for an additional 72 hr. Then, cell growth inhibition was measured by the tetrazolium salt-based proliferation assay (WST assay; Wako Chemicals, Osaka, Japan). WST-1 labeling solution (10 μL) was added to each well and the plates were incubated at 37°C for 3 hr. The absorbance of the formazan product formed was detected at 450 nm in a 96-well spectrophotometric plate reader. Cell viability was measured and compared to that of the control cells. Each experiment was carried out in triplicate and was repeated at least 3 times. Data were averaged and normalized against the nontreated controls to generate dose-response curves.

Drug interaction analysis

The nature of interaction between NK012 or SN-38 and 5FU against HT-29 cells was evaluated by median-effect plot analyses and the combination index (CI) method of Chou and Talalay.[13] Data analysis was performed using the Calcusyn software (Biosoft, NY, USA). NK012 or SN-38 was combined with 5FU at a fixed ratio that spanned the individual IC_{50} values of each drug. The IC_{50} values were determined on the basis of the dose-response curves using the WST assay. For any given drug combination, the CI is known to represent the degree of synergy, additivity or antagonism. It is expressed in terms of fraction-affected (Fa) values, which represents the percentage of cells killed or inhibited by the drug. Isobologram equations and Fa/CI plots were constructed by computer analysis of the data generated from the median effect analysis. Each experiment was performed in triplicate with 6 gradations and was repeated at least 3 times. The resultant dose-response curves were averaged, to create a single composite dose-response curve for each combination.

In vivo analysis of the effects of NK012 combined with 5FU as compared to those of CPT-11 combined with 5FU

When the mean tumor volumes reached ~93 mm^3, the mice were randomly divided into test groups consisting of 5 mice per group (Day 0). The drugs were administered i.v. *via* the tail vein of the mice. In the groups administered NK012 or 5FU as single agents, the drug was administered on Days 0, 7 and 14. In the combined treatment groups, NK012 or CPT-11 was administered 24 hr before 5FU on Days 0, 7 and 14, according to the previously reported combination schedule for CPT-11 and 5FU.[14] Complete response (CR) was defined as tumor not detectable by palpation at 90 days after the start of treatment, at which time-point the mice were sacrificed. Tumor volume and body weight were measured twice a week. As a general rule, animals in which the tumor volume exceeded 2,000 mm^3 were also sacrificed.

Experiment 1. Evaluation of the effects of NK012 combined with 5FU and determination of the maximum tolerated dose (MTD) of NK012/5FU. By comparing the data between NK012 administered as a single agent and NK012/5FU, we evaluated the effects of the combined regimen against the s.c HT-29 tumors. A preliminary experiment showed that combined administration of NK012 15 mg/kg + 5FU 50 mg/kg every 6 days caused drug-related lethality (data not shown). To determine the MTD, therefore, we set the dosing schedule of the combined regimen at 5 or 10 mg/kg of NK012 + 50 mg/kg of 5FU three times a week.

Experiment 2. Comparison of the antitumor effect of NK012/5FU and CPT-11/5FU. Based on a comparison of the data between NK012/5FU and CPT-11/5FU against the s.c. HT-29 and HCT-116 tumors, we investigated the feasibility of the clinical application of NK012/5FU for the treatment of CRC. CPT-11/5FU was administered three times a week at the respective MTDs of the 2 drugs as previously reported, that is, CPT11 at 50 mg/kg and 5FU at 50 mg/kg, respectively.[14] NK012/5FU was administered once three times a week at the respective MTDs of the 2 drugs determined from Experiment 1.

Cell cycle analysis

Samples from the HT-29 tumors that had grown to 80–100 mm^3 were removed from the mice at 6, 24, 48, 72 and 96 hr after the administration of NK012 alone at 10 mg/kg or CPT-11 alone at 50 mg/kg. The samples were excised, minced in PBS and fixed in 70% ethanol at −20°C for 48 hr. They were then digested with 0.04% pepsin (Sigma chemical Co., St Lous, MO) in 0.1 N HCL for 60 min at 37°C in a shaking bath to prepare single-nuclei suspensions. The nuclei were then centrifuged, washed twice with PBS and stained with 40 μg/mL of propidium iodide (Molecular Probes, OR) in the presence of 100 μg/mL RNase in 1 mL PBS for 30 min at 37°C. The stained nuclei were analyzed with B-D FACSCalibur (BD Biosciences, San Jose, CA), and the cell cycle distribution was analyzed using the Modfit program (Verity Software House Topsham, ME).

Statistical analyses

Data were expressed as mean ± SD. Data were analysed with Student's t test when the groups showed equal variances (F test), or Welch's test when they showed unequal variances (F test). $p < 0.05$ was regarded as statistically significant. All statistical tests were 2-sided.

Results

Antiproliferative effects of NK012 or SN-38 administered in combination with 5FU

Figure 1*a* shows the dose-response curves for NK012 alone, 5FU alone and a combination of the two. The IC_{50} levels of NK012 and 5FU against the HT-29 cells were 39 nM and 1 μM, respectively, and the IC_{50} level of SN-38 was 14 nM (data not shown). Based on these data, the molar ratio of NK012 or SN-38:5FU of 1:1,000 was used for the drug combination studies.

Figures 1*b* and 1*c* show the median-effect and the combination index plots. Combination indices (CIs) of <1.0 are indicative of synergistic interactions between 2 agents; additive interactions are indicated by CIs of 1.0, and antagonism by CIs of >1.0. Figure 1*c* shows the combination index for NK012 and 5FU, when 2 drugs are supposed to be mutually exclusive. Marked synergism was observed between Fa 0.2 and 0.6. Theoretically, the CI method is the most reliable around an Fa of 0.5, suggesting synergistic effects of the combination of NK012 and 5FU. This synergistic effect was more evident than that of SN-38/5FU (Fig. 1*d*).

In vivo *effect of combined NK012 and 5FU*

Experiment 1. Dose optimization and effect of combined NK012 and 5FU against HT-29 tumors. Comparison of the relative tumor volumes on Day 40 revealed significant differences between

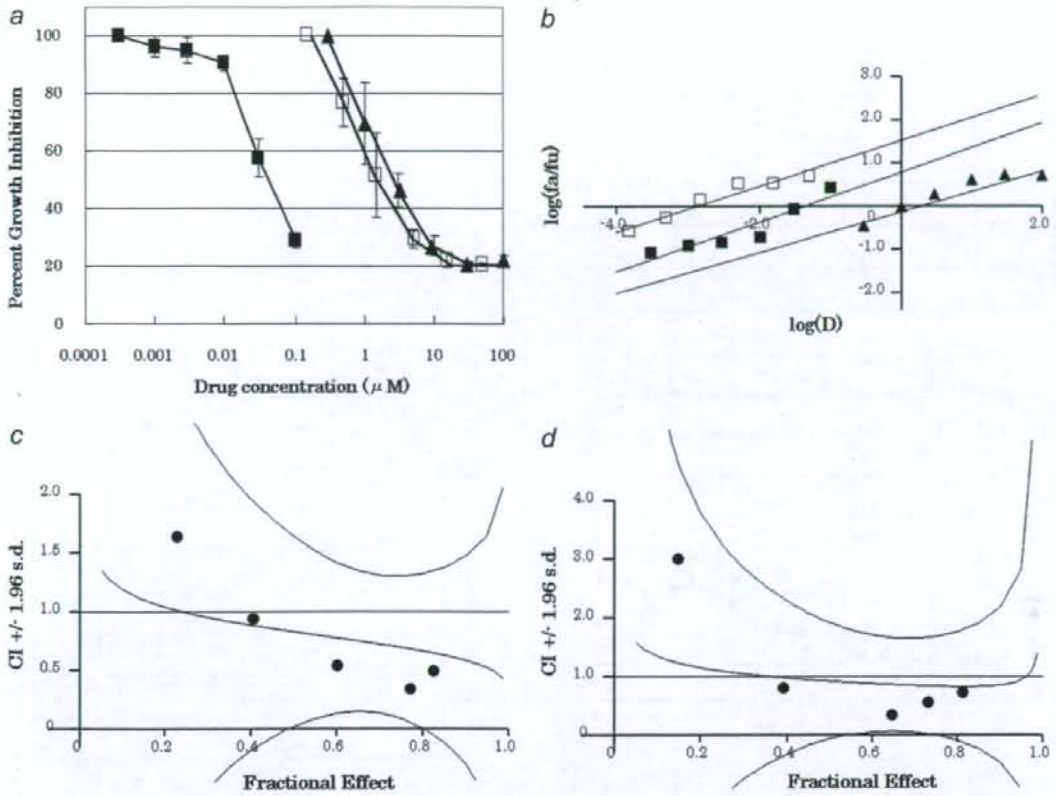

FIGURE 1 – Interaction of NK012 and 5FU *in vitro*. (*a*) Dose-response curves for NK012 alone (■), 5FU alone (▲) and their combination (□) against HT-29 cells. HT-29 cells were seeded at 2,000 cells/well. Twenty-four hours after seeding, a graded concentration of NK012 or 5FU was added to the culture medium of the HT-29 cells. Cell growth inhibition was measured by WST assay after 72 hr of treatment. Cell viability was measured and compared with that of the control cells. Each experiment was carried out independently and repeated at least 3 times. Points, mean of tripricates; bars, SD. (*b*) Median effect plot for the interaction of NK012 and 5FU. (*c*, *d*) Combination index for the interaction as a function of the level of effect (fraction effect = 0.5 is the IC_{50}). The straight line across the CI value of 1.0 indicates additive effect and CIs above and below indicate antagonism and synergism, respectively. The molar ratio of NK012/5FU (*c*) or SN-38/5FU (*d*) at 1:1,000 was tested by CI analysis. Black circles represent the CIs of the actual data points, solid lines represent the computer-derived CIs at effect levels ranging from 10 to 100% inhibition of cell growth, and the dotted lines represent the 95% confidence intervals.

those in the mice administered NK012 alone and those administered NK012/5FU at 5 mg/kg of NK012 ($p = 0.018$) (Fig. 2*a*). Although there was no statistically significant difference in the relative tumor volume measured on Day 54 between the mice administered NK012 alone and NK012/5FU at 10 mg/kg of NK012 ($p = 0.3050$), a trend of superior antitumor effect was demonstrated in the group treated with NK012/5FU at 10 mg/kg of NK012 (Fig. 2*a*). The CR rates were 20, 40 and 60% for 5 mg/kg NK012 + 50 mg/kg 5FU, 10 mg/kg NK012 alone and 10 mg/kg NK012 + 50 mg/kg 5FU, respectively. The schedule of 10 mg/kg NK012 + 50 mg/kg 5FU resulted in no remarkable toxicity in terms of body weight changes, and these doses were determined as representing the MTDs (Fig. 2*b*).

Experiment 2. Comparison of the antitumor effect of combined NK012/5FU and CPT-11/5FU against HT-29 and HCT-116 tumors. The therapeutic effect of NK012/5FU on Day 60 was significantly superior to that of CPT-11/5FU against the HT-29 tumors ($p = 0.0004$) (Fig. 3*a*). A more potent antitumor effect, namely, a 100% CR rate, was obtained in the NK012/5FU group as compared to the 0% CR rate in the CPT-11/5FU group. Although no statistically significant difference in the relative tumor volume on Day 61 was demonstrated between the NK012/5FU and CPT-11/5FU in the case of the HCT-116 tumors ($p = 0.2230$), a trend of superior antitumor effect against these tumors was observed in the NK012/5FU treatment group (Fig. 3*b*). The CR rates for the case of the HCT-116 tumors were 0% in both NK012/5FU and CPT-11/5FU groups.

Specificity of cell cycle perturbation

We studied the differences in the effects between NK012 10 mg/kg and CPT-11 50 mg/kg on the cell cycle (Fig. 4*a*). The data indicated that both NK012 and CPT-11 tended to cause accumulation of cells in the S phase, although the effect of NK012 was stronger and maintained for a more prolonged period than that of CPT-11; the maximal percentage of S-phase cells in the total cell population in the tumors was 34% at 24 hr after the administration of CPT-11, whereas it was 39% at 48 hr after the administration of NK012 (Figs. 4*b*, and 4*c*).

Discussion

Our primary endpoint was to clarify the advantages of NK012 over CPT-11 administered in combination with 5FU. We demonstrated that combined NK012 and 5FU chemotherapy exerts more

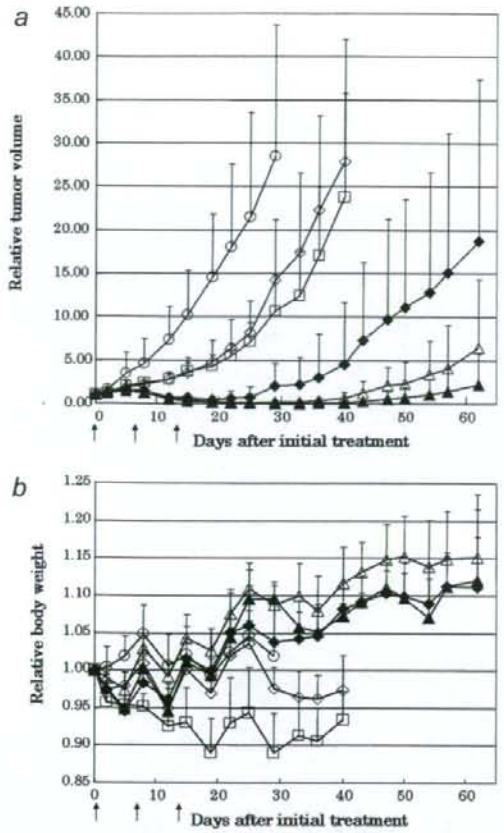

FIGURE 2 – Effect of NK012 alone or NK012 in combination with 5FU against HT-29 tumor-bearing mice. Points, mean; bars, SD. (*a*) Antitumor effect of each regimen on Days 0, 7 and 14. (○) control, (□) 5FU 50 mg/kg alone, (◇) NK012 5 mg/kg alone, (◆) NK012 5 mg/kg 24 hr before 5FU 50 mg/kg, (△) NK012 10 mg/kg alone, (▲) NK012 10 mg/kg 24 hr before 5FU 50 mg/kg. (*b*) Changes in the relative body weight. Data were derived from the same mice as those used in the present study.

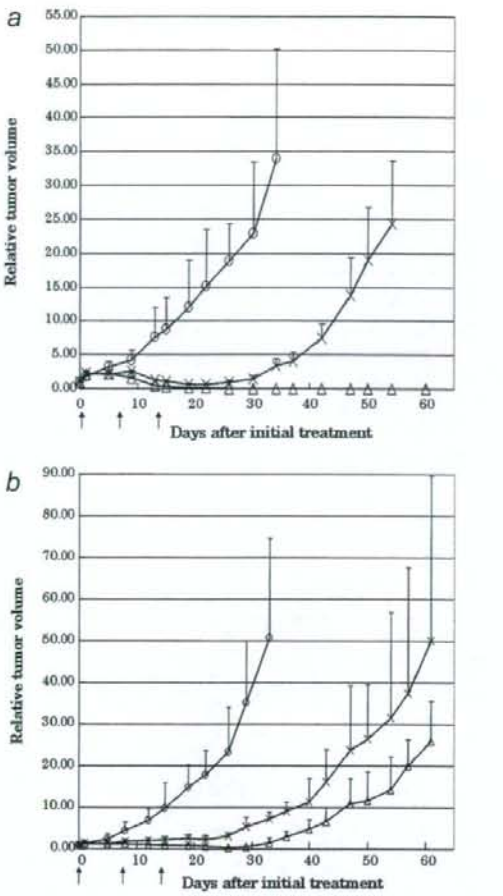

FIGURE 3 – Effect of NK012/5FU as compared with that of CPT11/5FU against HT-29 (*a*) or HCT-116 (*b*) tumor-bearing mice. Antitumor effect of each schedule on Days 0, 7 and 14. (○) control, (×) CPT-11 50 mg/kg 24 hr before 5FU 50 mg/kg, (△) NK012 10 mg/kg 24 hr before 5FU 50 mg/kg. Points, mean; bars, SD.

synergistic activity *in vitro* and significantly greater antitumor activity against human CRC xenografts as compared to CPT-11/5FU. The combination of NK012 and 5FU is considered to hold promise of clinical benefit for patients with CRC.

CPT-11, a topoisomerase-I inhibitor, and 5FU, a thymidilate synthase inhibitor, have been demonstrated to be effective agents for the treatment of CRC. A combination of these 2 drugs has also been demonstrated to be clearly more effective than either CPT-11 or 5FU/LV administered alone *in vivo* and in clinical settings.[1,2,14] Administration of 5FU by infusion with CPT-11 was shown to be associated with reduced toxicity and an apparent improvement in survival as compared to that of administration of the drug by bolus injection with CPT-11.[1,2] This synergistic enhancement may result from the mechanism of action of the 2 drugs; CPT-11 has been reported to cause accumulation of cells in the S phase, and 5FU infusion is known to cause DNA damage specifically in cells of the S phase.[14] On the basis of this background, our results suggesting the more pronounced and more prolonged accumulation of the tumor cells in the S phase caused by NK012 as compared with that by CPT-11 may explain the more effective synergy of the former administered with 5FU infusion. This may be attributable to accumulation of NK012 due to the enhanced permeability and retention (EPR) effect.[9] It is also speculated that NK012 allows sustained release of free SN-38, which may move more freely in the tumor interstitium.[15] Otherwise NK012 itself could internalize into cells to localize in several cytoplasmic organelles as reported by Savic *et al.*[16] These characteristics of NK012 may be responsible for its more potent antitumor activity observed in this study, because CPT-11 has been reported to show time-dependent growth-inhibitory activity against the tumor cells.[17]

The major dose-limiting toxicities of CPT-11 are diarrhea and neutropenia. SN-38, the active metabolite of CPT-11, may cause CPT-11-related diarrhea as a result of mitotic -inhibitory activity.[18] Because it undergoes significant biliary excretion, SN-38 may have a potentially long residence time in the gastrointestinal tract that may be associated with prolonged diarrhea.[19,20] In our previous report, we evaluated the tissue distribution of SN-38 after administration of an equimolar amount of NK012 (20 mg/kg) and CPT-11 (30 mg/kg), and found no difference in the level of SN-38 accumulation in the small intestine.[12] A significant antitumor effect of NK012 with a lower incidence of diarrhea was also dem-

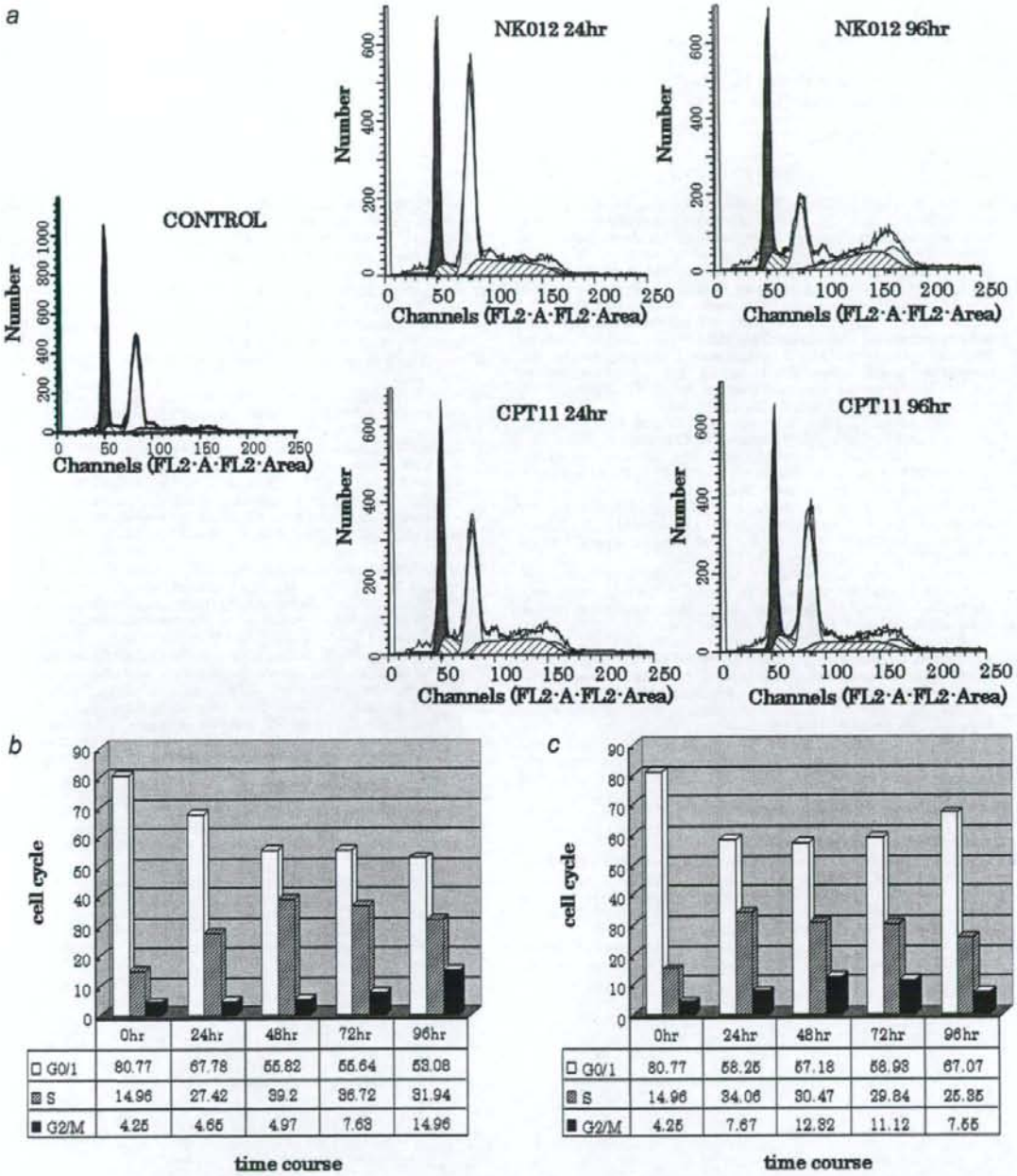

FIGURE 4 – Cell cycle analysis of HT-29 tumor cells collected 24, 48, 72 and 96 hr after administration of NK012 at 10 mg/kg alone or CPT-11 at 50 mg/kg alone using the Modfit program (Verity Software House Topsham, ME). (*a*) Cell cycle analysis of HT-29 tumor cells 24 and 96 hr after administration of NK012 at 10 mg/kg or CPT-11 at 50 mg/kg, respectively. (*b*) Cell cycle distribution of tumor cells 0, 24, 48, 72 and 96 hr after treatment with NK012 at 10 mg/kg. (*c*) Cell cycle distribution of tumor cells 0, 24, 48, 72 and 96 hr after treatment with CPT-11 at 50 mg/kg.

onstrated as compared to that observed with CPT-11 in a rat mammary tumor model.[21] Combined administration of CPT-11 with 5FU/LV infusion appears to be associated with acceptable toxicity in patients with CRC. In addition, no significant difference in the frequency of Grade 3/4 diarrhea was noted between patients treated with FOLFIRI (CPT-11 regimen with bolus and infusional 5FU/LV) and those treated with FOLFOX6 (oxaliplatin regimen with bolus and infusional 5FU/LV).[22,23] Our *in vivo* data actually revealed no severe body weight loss in the NK012/5FU group. Consequently, we expect that the NK012/5FU regimen, especially

with infusional 5FU, may be an attractive arm for a Phase III trial in CRC, with CPT-11/5FU as the control arm. We have already initiated a Phase I trial of NK012 in patients with advanced solid tumors based on the data suggesting higher efficacy and lower toxicity of this preparation than CPT-11 *in vivo*.[12]

In conclusion, we demonstrated that combined NK012 and 5FU chemotherapy exerts significantly greater antitumor activity against human CRC xenografts as compared to CPT-11/5FU, indicating the necessity of clinical evaluation of this combined regimen.

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ORIGINAL ARTICLE

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Efficacy and safety profile of imatinib mesylate (ST1571) in Japanese patients with advanced gastrointestinal stromal tumors: a phase II study (STI571B1202)

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Abstract

Background. Imatinib mesylate, an inhibitor of KIT, ABL protein, and platelet-derived growth factor receptor α (PDGFRα) tyrosine kinase, has recently been found to have a dramatic antitumor effect on gastrointestinal stromal tumor (GIST). The aim of this study was to assess the efficacy and safety of imatinib mesylate in Japanese patients with advanced GIST.

Methods. Patients with measurable lesions were enrolled between April 1, 2002, and September 20, 2002, using a design based on previous phase II studies in the United States and the European Union. The diagnosis of GIST was proven histologically with positive immunostaining for KIT (CD117). Imatinib mesylate was administered at a dose of either 400 mg or 600 mg once a day. Pharmacokinetic parameters and mutation analysis of c-*kit* were also assessed in a subgroup of patients.

Results. A total of 74 patients (28 receiving imatinib mesylate at 400 mg/day; 46 receiving 600 mg/day); median age, 56.0 years, were enrolled. No patient had a complete response, 51 patients (69%) had a partial response, and 19 patients (26%) had stable disease. The median progression-free survival time was 96 weeks. The estimated 3-year overall survival (Kaplan-Meier) rate for all patients was 73.6%. The most frequent adverse effects related to the drug were nausea (78%), diarrhea (70%), dermatitis (62%), facial edema (61%), edema of the lower limbs (58%), vomiting (54%), and eyelid edema (51%). Most of the adverse effects were mild and manageable.

Conclusion. Imatinib mesylate is generally safe and has significant activity in the treatment of advanced GIST in Japanese patients.

Key words Gastrointestinal stromal tumor · Imatinib · Phase II

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Introduction

Gastrointestinal stromal tumor (GIST), the most common mesenchymal tumor in the gut, is generally considered to be malignant, with different degrees of clinicopathological aggressiveness.[1] Although surgery is the mainstay of therapy for GIST, there are still cases of primary unresectable advanced GIST, and GIST frequently relapses even after apparently complete resection. After recurrence, conventional chemotherapy or radiotherapy is ineffective, and the median survival of patients with a relapse of GIST has been estimated to be almost 1 year.[2] Until recently, the prognosis of patients with advanced or metastatic GIST was dismal.

Gain-of-function mutations of the c-*kit* gene or the platelet-derived growth factor receptor α (*PDGFRα*) gene are found in sporadic as well as in familial GISTs and are thought to be causative factors for GIST.[3,4] GIST usually expresses the KIT protein, with fewer than 5% of GISTs being immunohistochemically negative for this protein. In the latter cases, patients usually have mutations in the *PDGFRα* gene. Constitutively activated KIT or PDGFRα plays a central role in the pathogenesis as well as progression of GIST tumor cells.[5-7] Imatinib mesylate, a selective tyrosine kinase inhibitor of BCR-ABL, KIT, and PDGFR proteins, has had a significant impact in the treatment of chronic myeloid leukemia (CML).[8] Early reports of clinical trials in the United States and the European Union have also shown that the drug is active against advanced and/or metastatic GIST.[9-11] Based on this evidence, we conducted a phase II study to evaluate the efficacy, safety, and pharmacokinetics of imatinib mesylate in Japanese patients with unresectable and/or metastatic GIST.

Patients and methods

Patients

Eligibility criteria required patients aged between 20 and 75 years of age with histologically proven KIT (CD117)-positive unresectable or metastatic GIST. Before study entry, the pathological specimens were reviewed centrally by a single pathologist.

Inclusion criteria also included the following: at least one measurable and previously untreated lesion detected by computed tomographic (CT) scan or magnetic resonance imaging (MRI), an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2, adequate organ function, as follows: neutrophils 1500/μl or more, platelets 100000/μl or more, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) within 2.5 times the upper limit of normal (ULN; 5 × ULN with liver metastasis), bilirubin less than 1.5 × ULN, creatinine less than 1.5 × ULN, a life expectancy of at least 2 months, and written informed consent. Patients were not allowed to receive any chemotherapy, surgery, or radiotherapy for at least 4 weeks before study entry. This study was performed at seven medical centers in Japan after approval by each institutional review board.

Treatment schedule

The initial 28 patients, who were enrolled between April 1, 2002, and June 7, 2002, were assigned to take 400 mg imatinib mesylate. However, after a United States Food and Drug Administration (FDA) review, the recommended dose in the United States was changed from 400 mg to 400 mg or 600 mg. Accordingly, a 600-mg cohort was added to the protocol, and 46 patients, who were enrolled between June 28, 2002, and September 20, 2002, were assigned to take 600 mg imatinib mesylate once daily. The drug was taken within 2 h after a meal.

Dose escalation to 800 mg was allowed if the tumor progressed and the patients tolerated the initial dose. Patients whose tumors progressed despite the dose increment were withdrawn from the study.

Evaluation of response and toxicity

The response of the tumor to imatinib was evaluated after 1 month, after 3 months, and every 3 months thereafter, or whenever medically indicated. Assessments were performed according to the standard Southwest Oncology Group (SWOG) criteria and were based solely on CT or MRI findings. Responses were classified as follows: complete response (CR; disappearance of all disease that could be measured and evaluated); partial response (PR; >50% decrease in the sum of the products of the perpendicular diameters of all measurable lesions, absence of progression, and absence of new lesions); stable disease (SD; a response that did not qualify as a CR, a PR, or progressive disease); or progressive disease (PD; a >50% increase, or an increase of >10 cm^2 [whichever was smaller] in the sum of the products of the perpendicular diameters of all measurable lesions, worsening of a lesion that could be evaluated, the reappearance of any lesion, the presence of a new lesion, or failure of the patient to return for evaluation because of a deteriorating condition).

Toxic effects were recorded in accordance with the National Cancer Institute Common Toxicity Criteria version 2.

Pharmacokinetics

For pharmacokinetic analysis, blood samples were obtained from 21 patients hospitalized in the two hospitals (National Cancer Center Hospital and National Cancer Center Hospital East). Plasma samples were collected before, and 1, 2, 3, 8, 24, 48, and 72 h after administration of the agent on day 1 and before, 1, 2, 3, 8, and 24 h after administration of the agent on day 29. Plasma imatinib and CGP74588 (a major metabolite of imatinib) concentrations were determined by a liquid chromatography/tandem mass spectrometry assay.[12] Pharmacokinetic parameters were calculated by means of noncompartmental analysis.

Immunohistochemical and genetic analyses

Immunohistochemical and genetic analyses were performed on paraffin-embedded sections of formalin-fixed tissues. Immunohistochemical analysis for the detection of KIT (CD117) was performed using a polyclonal rabbit antibody (A4502; DAKO, Kyoto, Japan) and the Envision method with antigen retrieval.[13] Tumor specimens from patients were analyzed for c-*kit* mutations of exon 9, 11, 13, or 17, using polymerase chain reaction (PCR) amplification of genomic DNA and direct DNA sequencing.[14] This approach

was validated by comparison with reverse transcriptase (RT)-PCR sequencing of the entire c-*kit* coding sequence in a subset of patients. These laboratory studies were performed under the guidelines for genetic analysis of each institution, and written informed consent for genetic analysis was obtained from all participants.

Statistics

The primary endpoint of this study was the response rate, and secondary endpoints were safety and pharmacokinetics. Originally, a sample size in the 400-mg-group of at least 15 evaluable patients per arm was determined based on a Fleming single-stage procedure under conditions of P0 = 5%, P1 = 32%, alpha = 2.5% (one sided) and power = 90%. With these sample sizes, the widths of the 95% confidence interval were no more than ±20% and ±15%, respectively. Progression-free survival (PFS) was analyzed as time-to-event data for all patients. The starting date for PFS was the date of the first dose. The earliest occurrence of any progression, or death due to any cause, was used as the PFS. Patients, who had neither progressed, died, nor discontinued from the trial for any reason other than "condition no longer requires therapy", were censored for analysis at the time of their last tumor assessment. Overall survival (OS) was analyzed as time-to-event for all patients. The start date was the first date of study medication. The date of death was included in follow-up data. Patients who did not die were censored at the last date they were known to be alive.

All analyses were performed using Statistical Analysis Software (version 8.0; SAS Institute, Cary, NC, USA), and a value of $P < 0.05$ was considered significant.

Results

Demographics

Between April 1, 2002, and September 20, 2002, 81 patients were recruited for this study, and of these, 74 patients with positive immunoreactivity for KIT protein by central pathological review were registered. There were 48 men and 26 women, with a median age of 56 years (range, 24 to 74 years; Table 1). Twenty-eight patients received initial daily doses of 400 mg of imatinib and 46 patients received 600 mg daily. Sixty-seven (91%) and 20 (27%) patients had a prior history of surgery and chemotherapy, respectively, while no patient had had radiotherapy. Patients who had previously undergone chemotherapy had received between one and five courses.

The primary site of the GIST was the esophagus ($n = 2$), stomach ($n = 33$), small intestine ($n = 34$), colon ($n = 2$), or elsewhere ($n = 3$). Metastatic/relapse sites at registration (some patients have multiple metastatic/relapse sites) were the liver ($n = 52$), peritoneum ($n = 38$), local ($n = 12$), lung ($n = 6$), skin ($n = 3$), bone ($n = 2$), and adrenal gland ($n = 2$).

Pharmacokinetics

The pharmacokinetics of imatinib was evaluated in 21 patients on day 1 and on day 29 (Table 2). The plasma concentration of imatinib reached a peak approximately 3 to 6 h after oral administration, and then declined in a monophasic manner with a half-life of 16 to 18 h. On day 1, maximum concentration (Cmax) values were 2.51 and

Table 1. Patient demographics

Demographics	400 mg ($n = 28$)	600 mg ($n = 46$)	Either dose ($n = 74$)
Age (years)			
Median	54.0	56.5	56.0
Range	24–70	33–74	24–74
Sex, *n* (%)			
Male	19 (67.9)	29 (63.0)	48 (64.9)
Female	9 (32.1)	17 (37.0)	26 (35.1)
ECOG performance status, *n* (%)*			
0	22 (78.6)	35 (76.1)	57 (77.0)
1	6 (21.4)	8 (17.4)	14 (18.9)
2	0	3 (6.5)	3 (4.1)
Primary site of tumor, *n* (%)			
Esophagus	1 (3.6)	1 (2.2)	2 (2.7)
Stomach	11 (39.3)	22 (47.8)	33 (44.6)
Intestine	14 (50.0)	20 (43.5)	34 (45.9)
Colon	2 (7.1)	0	2 (2.7)
Others	0	3 (6.5)	3 (4.1)
Previous treatment, *n* (%)			
Surgery	25 (89.3)	42 (91.3)	67 (90.5)
Chemotherapy	8 (28.6)	12 (26.0)	20 (27.0)
Radiotherapy	0	0	0

*ECOG, Eastern Cooperative Oncology Group

Table 2. Pharmacokinetic (PK) parameters of imatinib in GIST patients on day 1 and day 29 following once-daily oral administration of imatinib at doses of 400 mg and 600 mg

PK parameter	Mean ± SD (Range)	
	400 mg (n = 9)	600 mg (n = 12)
Day 1		
T_{max} (h)	3.23 ± 1.91 (1.10–8.00)	6.36 ± 2.48 (2.97–8.17)
C_{max} (μg/ml)	2.51 ± 1.00 (1.29–3.78)	3.45 ± 2.20 (1.38–7.65)
AUC_{0-24} (μg*h/ml)	34.7 ± 13.6 (13.7–53.0)	56.1 ± 40.8 (21.5–141)
$t_{1/2}$ (h)	15.5 ± 1.9 (12.7–18.2)	18.2 ± 4.6 (11.8–2.81)
Day 29[a]		
T_{max} (h)	3.24 ± 2.05[b] (1.00–8.00)	3.61 ± 2.02[c] (1.92–8.08)
C_{max} (μg/ml)	2.86 ± 0.87[b] (1.77–4.27)	3.11 ± 0.65[c] (2.30–4.37)
AUC_{0-24} (μg*h/ml)	47.6 ± 17.0[b] (16.1–73.8)	58.9 ± 11.7[d] (45.9–75.5)
$t_{1/2}$ (h)	20.0 ± 4.9[b] (9.10–25.6)	25.5 ± 8.4[d] (17.8–40.5)
Accumulation ratio (Day 29/Day 1)		
C_{max}	1.2 ± 0.6[b] (0.67–2.6)	1.5 ± 0.4[c] (0.94–2.2)
AUC_{0-24}	1.4 ± 0.5[b] (0.90–2.3)	1.8 ± 0.5[d] (1.2–2.5)

[a] Days 28 to 31
[b] n = 8
[c] n = 7
[d] n = 6

Table 3. Confirmed best responses to imatinib in patients with GIST

Best response	N (% [95% CI])		
	400 mg (n = 28)	600 mg (n = 46)	Either dose (n = 74)
Complete response	0	0	0
Partial response	17 (60.7 [40.6–78.5])	34 (73.9 [58.9–85.7])	51 (68.9 [57.1–79.1])
Stable disease	11 (39.3 [21.5–59.4])	8 (17.4 [7.8–31.4])	19 (25.7 [16.2–37.2])
Progressive disease	0	2 (4.3)	2 (2.7)
Not evaluated	0	2 (4.3)	2 (2.7)

CI, Confidence interval

3.45 μg/ml in the 400-mg and 600-mg dose groups, respectively. Although there was some interpatient variability, dose-dependency was observed for AUC and Cmax. After repeated administrations, accumulation of imatinib was observed, and the AUC was 1.4 to 1.8-fold higher than after the first dose. This accumulation ratio corresponded well to the value estimated from half-lives on day 1, i.e., a 1.5- to 1.7-fold accumulation.

Efficacy

Of the 74 patients registered, 51 patients (69%) achieved a PR, 19 patients (26%) had SD, and 2 patients had PD, while no CR was observed (Table 3). All the PRs were confirmed by repeated imaging after at least 28 days. Although the median time to response was 12 weeks, the cumulative incidence of response (Fig. 1) showed that 3 patients achieved a clinical response after 48 weeks.

Disease progression during treatment occurred in 48 patients, 34 of whom were withdrawn from the study. Two patients died during the study, and a follow-up after study discontinuation indicated that 20 further patients had died. The median PFS of all patients was 96 weeks (Fig. 2). There was no significant difference between the two dose-groups (400-mg vs 600-mg) in the intention-to-treat analysis Log-rank P = 0.1943). The estimated 3-year overall survival (Kaplan-Meier) rate for all patients was 73.6%. Median survival time was not reached during the study (Fig. 3). It could not be said that the OS of the 600 mg dose-group was better than that of the 400 mg dose-group (log-rank P = 0.0679).

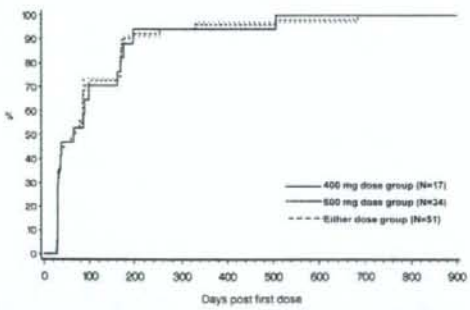


Fig. 1. Cumulative incidence of response by dose level of imatinib. Cumulative response incidence of the 400-mg dose group (*solid line*), 600-mg dose group (*dotted line*), and either dose group (*dashed line*) are shown

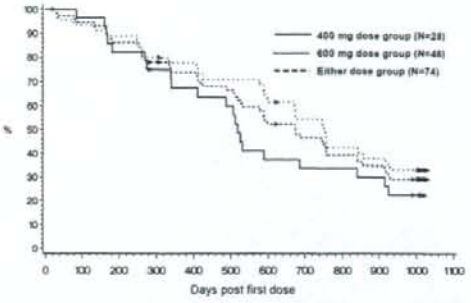
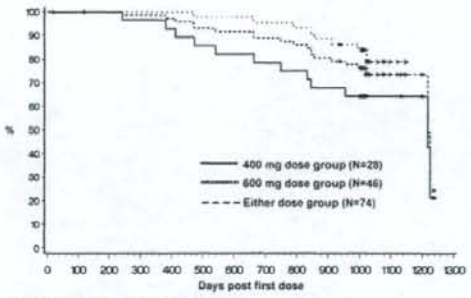


Fig. 2. Kaplan-Meier estimates of progression free survival (PFS) by dose level of imatinib. The PFS times of the 400-mg dose group (*solid line*), 600-mg dose group (*dotted line*), and either dose group (*dashed line*) are shown. The median PFS of the 400-mg dose group (74.1 weeks) was not significantly different from that of the 600-mg dose group (107.3 weeks; log-rank $P = 0.1943$)



Risk sets of Kaplan-Meier plot (Overall survival)

Day	200	400	600	800	1000	1200
400 mg	28	26	23	21	18	4
600 mg	44	44	43	41	36	0
Either dose	72	70	66	62	54	4

Fig. 3. Kaplan-Meier estimates of overall survival (OS) times by dose level of imatinib. The OS of the 400-mg dose group (*solid line*), 600-mg dose group (*dotted line*), and either dose group (*dashed line*) are shown. There was no significant difference in the overall survival rates of the two dose groups (log-rank $P = 0.0679$)

Safety

In this study, imatinib was generally well tolerated. Although all patients had adverse effects related to the drug (Table 4), most of these effects were of grades 1 or 2. The most frequently affected systems were the skin and subcutaneous tissue (94.6%), gastrointestinal system (91.9%), and general disorders and administration-site conditions (85.1%). Troublesome symptoms included nausea not otherwise specified (NOS; 78.4%), diarrhea NOS (70.3%), dermatitis NOS (62.2%), facial edema (60.8%), edema of the lower limbs (58.1%), vomiting NOS (54.1%), and eyelid edema (51.4%). Grade 3 or 4 adverse effects occurred in 40 patients (54.1%) and the most frequent were neutropenia (21.6%), anemia

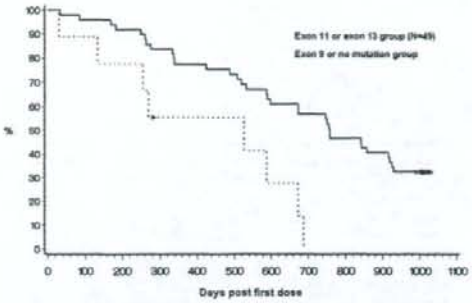


Fig. 4. Kaplan-Meier estimates of progression-free survival (PFS) by kinase genotype. Patients with gastrointestinal stromal tumor (GIST) with KIT mutations in exon 11 or exon 13 (*solid line*) showed significantly better PFS than those with KIT mutations in exon 9 or no mutation (*dotted line*; log-rank $P = 0.0014$)

NOS (17.6%), dermatitis NOS (6.8%), and anorexia (5.4%). A total of 8 patients (10.8%) were reported as having gastrointestinal or intratumoral hemorrhage, which included tumor hemorrhage (6.8%), intraperitoneal hemorrhage (4.1%), gastrointestinal hemorrhage NOS (2.7%), and melena (2.7%). There was no evidence of tumor lysis syndrome, even in patients with very rapid decreases in tumor volume.

Genetic analysis

Somatic mutation analyses were available in 58 patients. In others, this could not be performed because of lack of amplification products by PCR. Among the 58 tumors, c-*kit* gene mutations were found in 53, including 48 in exon 11, 4 in exon 9, and 1 in exon 13 (Table 5). Five patients had no mutation in the c-*kit* gene. The proportions of mutations found in each exon were similar to those in previous reports.[14] Subgroup analysis of tumor response according to the c-*kit* genotype indicated that 39 (81%) of the 48 patients with exon 11 mutations achieved a PR, while 2 (50%) of the 4 patients with an exon 9 mutation showed a PR. None of the 5 patients who had no mutation in the c-*kit* gene achieved a PR (Table 5).

As shown in Fig. 4, the median PFS in patients with exon 11 or 13 mutations (median PFS, 108 weeks) was significantly longer than that in patients with exon 9 or no mutations (75 weeks; log-rank $P = 0.0014$).

Discussion

Most GISTs are caused by gain-of-function mutations of the c-*kit* or *PDGFRα* genes, which induce tumor-cell proliferation.[1,3-7] Imatinib mesylate efficiently inhibits BCR-ABL, KIT, and PDGFRα proteins in vitro, and is clinically active against GIST and CML, which express mutated KIT and/or

Table 4. Adverse effects possibly related to imatinib

	n (%)					
	Any grade			Grade 3 or 4		
	400 mg (*n* = 28)	600 mg (*n* = 46)	Either dose (*n* = 74)	400 mg (*n* = 28)	600 mg (*n* = 46)	Either dose (*n* = 74)
Any adverse effect possibly related to the study drug	28 (100.0)	46 (100.0)	74 (100.0)	13 (46.4)	27 (58.7)	40 (54.1)
Skin and subcutaneous tissue disorders	25 (89.3)	45 (97.8)	70 (94.6)	1 (3.6)	6 (13.0)	7 (9.5)
Dermatitis NOS*	15 (53.6)	31 (67.4)	46 (62.2)	0	5 (10.9)	5 (6.8)
Facial edema	16 (57.1)	29 (63.0)	45 (60.8)	1 (3.6)	1 (2.2)	2 (2.7)
Eyelid edema	14 (50.0)	24 (52.2)	38 (51.4)	1 (3.6)	0	1 (1.4)
Periorbital edema	5 (17.9)	8 (17.4)	13 (17.6)	0	0	0
Alopecia	1 (3.6)	9 (19.6)	10 (13.5)	0	0	0
Pigmentation disorder NOS*	2 (7.1)	8 (17.4)	10 (13.5)	0	0	0
Pruritus NOS*	2 (7.1)	8 (17.4)	10 (13.5)	0	0	0
Skin discoloration	1 (3.6)	6 (13.0)	7 (9.5)	0	0	0
Hemorrhage, subcutaneous	3 (10.7)	2 (4.3)	5 (6.8)	0	0	0
Gastrointestinal disorders	27 (96.4)	41 (89.1)	68 (91.9)	6 (21.4)	6 (13.0)	12 (16.2)
Nausea NOS*	21 (75.0)	37 (80.4)	58 (78.4)	1 (3.6)	1 (2.2)	2 (2.7)
Diarrhea NOS*	20 (71.4)	32 (69.6)	52 (70.3)	0	2 (4.3)	2 (2.7)
Vomiting NOS*	16 (57.1)	24 (52.2)	40 (54.1)	1 (3.6)	1 (2.2)	2 (2.7)
Abdominal pain NOS*	8 (28.6)	11 (23.9)	19 (25.7)	2 (7.1)	0	2 (2.7)
Flatulence	9 (32.1)	9 (19.6)	18 (24.3)	0	0	0
Ascites	4 (14.3)	5 (10.9)	9 (12.2)	1 (3.6)	0	1 (1.4)
Abdominal distension	4 (14.3)	4 (8.7)	8 (10.8)	0	0	0
Stomatitis	2 (7.1)	6 (13.0)	8 (10.8)	0	0	0
Abdominal pain, upper	1 (3.6)	5 (10.9)	6 (8.1)	0	0	0
Constipation	1 (3.6)	5 (10.9)	6 (8.1)	0	0	0
Loose stools	3 (10.7)	2 (4.3)	5 (6.8)	0	0	0
General disorders and administration-site conditions	25 (89.3)	38 (82.6)	63 (85.1)	3 (10.7)	5 (10.9)	8 (10.8)
Edema, lower limb	19 (67.9)	24 (52.2)	43 (58.1)	0	1 (2.2)	1 (1.4)
Malaise	8 (28.6)	18 (39.1)	26 (35.1)	0	1 (2.2)	1 (1.4)
Edema NOS*	7 (25.0)	16 (34.8)	23 (31.1)	0	1 (2.2)	1 (1.4)
Fatigue	9 (32.1)	12 (26.1)	21 (28.4)	1 (3.6)	1 (2.2)	2 (2.7)
Pyrexia	3 (10.7)	2 (4.3)	5 (6.8)	1 (3.6)	0	1 (1.4)
Musculoskeletal and connective tissue disorders	14 (50.0)	30 (65.2)	44 (59.5)	0	1 (2.2)	1 (1.4)
Muscle cramps	12 (42.9)	24 (52.2)	36 (48.6)	0	0	0
Arthralgia	4 (14.3)	4 (8.7)	8 (10.8)	0	0	0
Myalgia	0	6 (13.0)	6 (8.1)	0	0	0
Blood and lymphatic system disorders	10 (35.7)	22 (47.8)	32 (43.2)	7 (25.0)	20 (43.5)	27 (36.5)
Neutropenia	4 (14.3)	16 (34.8)	20 (27.0)	3 (10.7)	13 (28.3)	16 (21.6)
Anemia NOS*	6 (21.4)	9 (19.6)	15 (20.3)	5 (17.9)	8 (17.4)	13 (17.6)
Eye disorders	7 (25.0)	21 (45.7)	28 (37.8)	0	0	0
Lacrimation, increased	1 (3.6)	11 (23.9)	12 (16.2)	0	0	0
Eye redness	3 (10.7)	4 (8.7)	7 (9.5)	0	0	0
Metabolic and nutritional disorders	9 (32.1)	17 (37.0)	26 (35.1)	3 (10.7)	3 (6.5)	6 (8.1)
Anorexia	6 (21.4)	15 (32.6)	21 (28.4)	2 (7.1)	2 (4.3)	4 (5.4)
Nervous system disorders	7 (25.0)	15 (32.6)	22 (29.7)	0	0	0
Headache NOS*	4 (14.3)	4 (8.7)	8 (10.8)	0	0	0
Dysgeusia	1 (3.6)	5 (10.9)	6 (8.1)	0	0	0
Respiratory, thoracic, and mediastinal disorders	3 (10.7)	9 (19.6)	12 (16.2)	0	1 (2.2)	1 (1.4)
Pleural effusion	2 (7.1)	7 (15.2)	9 (12.2)	0	0	0

Data are for categories of events that occurred in at least 10 % of the patients in at least one of the two groups
*NOS, not otherwise specified

Table 5. Correlation of tumor response by kinase genotype

	Best response *n* (%)			
	Exon 9 (*n* = 4)	Exon 11 (*n* = 48)	Exon 13 (*n* = 1)	No mutation (*n* = 5)
Complete response	0	0	0	0
Partial response	2 (50.0)	39 (81.3)	1 (100)	0
Stable disease	2 (50.0)	8 (16.7)	0	4 (80.0)
Progressive disease	0	1 (2.1)	0	1 (20.0)