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ORIGINAL ARTICLE

Can the 2-^{13}C-uracil breath test be used to predict the effect of the antitumor drug S-1?

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Abstract
Purpose S-1 is an oral anticancer drug containing tegafur (FT), a pro-drug of fluorouracil, combined with two modulators, 5-chloro-2,4-dihydroxypyridine and potassium oxonate (Oxo), at a molar ratio of 1:0.4:1. *CYP2A6* genetic polymorphism and dihydropyrimidine dehydrogenase (DPD) inhibition are important for the antitumor effect of S-1. Exploiting the usefulness of the 2-^{13}C-uracil breath test (UrBT) as an indicator of DPD activity, we examined whether the results of *CYP2A6* genetic polymorphism analysis and UrBT could be used to predict the antitumor effect of S-1.
Methods Thirty-four patients with advanced or recurrent cancer (15, 16 and 3 with gastric, colorectal and pancreatic cancer, respectively) were orally administered 40 mg/m^2 S-1 twice daily in the morning and evening. Eighteen patients with a complete response (CR)/partial response (PR) (2 with CR, 16 with PR) and 16 with progressive disease (PD) were compared with respect to *CYP2A6* genetic polymorphisms (1- vs. 2-allele mutation), UrBT results, and plasma FT and 5-fluorouracil levels at 3 h after S-1 ingestion in the morning.
Results On multivariate analysis between the CR/PR and PD groups, only the UrBT results was an independent factor of CR/PR to S-1 (95% CI 1.02–1.10).
Conclusion These results suggest that the anticancer effect of S-1 can be predicted by performing UrBT 3 h after the initial oral S-1 administration.

Keywords S-1 · CYP2A6 · Uracil · ^{13}C · Breath test

Introduction

S-1 is a novel oral fluorouracil antitumor drug that combines three pharmacological agents: tegafur (FT), which is a pro-drug of 5-fluorouracil (5-FU); 5-chloro-2,4-dihydroxypyridine (CDHP), which inhibits dihydropyrimidine dehydrogenase (DPD) activity; and potassium oxonate (Oxo), which reduces gastrointestinal toxicity. In Yoshida sarcoma-bearing rats, the anticancer effect was the highest, the 5-FU level was high over time, and body weight loss as an adverse effect was the smallest when the FT content was fixed at 1 M and that of CDHP was set at 0.4. The FT:CDHP ratio was fixed at 1:0.4, Oxo was combined at various molar ratios to investigate the composition ratio, and the compounds were combined at a molar ratio of FT:CDHP:Oxo = 1:0.4:1 based on the balance between the anticancer effect and toxicity [1, 2].

It was pharmacokinetically demonstrated that S-1 is not only an oral drug that shows a similar effect to that of long-term treatment by 5-FU continuous venous infusion but also a next-generation drug with a self-rescuing character.

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FT is a pro-drug for cytotoxic 5-FU. The biotransformation of FT to 5-FU has been demonstrated to be catalyzed by the liver drug-metabolizing enzyme CYP2A6 [3–6]. The addition of CDHP increases the plasma level of 5-FU, as CDHP prevents the degradation of 5-FU by competitively inhibiting DPD [7], which is a rate-limiting enzyme responsible for 5-FU detoxification [8].

CYP2A6 is a polymorphic enzyme that shows considerable interindividual variability in its activity [9–16]. *CYP2A6*1* is defined as the wild-type allele. *CYP2A6*4* is a complete deletion of the *CYP2A6* gene [9–11]. Among the *CYP2A6*4* variants, *CYP2A6*4A* is a major variant seen in the Japanese population [9]. Fujita et al. [17] reported that *CYP2A6* is a determinant of the pharmacokinetic variability of tegafur in Japanese patients with cancer given S-1. Kaida et al. [18] reported that genotyping of CYP2A6 polymorphisms may provide vital information for effective cancer therapy using S-1.

On the other hand, it was demonstrated that the 2-^{13}C-uracil breath test (UrBT) results were significantly different between healthy and DPD-deficient individuals [19]. Inada et al. [20] reported that UrBT results provide a good marker of hepatic DPD activity in vivo.

Based on these reports, we considered that the anticancer and adverse effects of S-1 might be predicted from the analysis of *CYP2A6* gene polymorphism and UrBT results. This study investigated the possibility of predicting the anticancer and adverse effects of S-1 from the analysis of *CYP2A6* gene polymorphism and the UrBT results.

Materials and methods

Patients

All patients of 20 years or older with metastatic or recurrent, histologically confirmed solid tumors who received S-1 had a World Health Organization (WHO) performance status of 0–2, and no history of chemotherapy within 4 weeks was eligible. Each patient was confirmed to have adequate bone marrow function (neutrophil count: at least 1.5×10^9 L^{-1}; platelet count: at least 100×10^9 L^{-1}), liver function (plasma bilirubin level: <3.0 mg/dL; transaminases: <2.0 times the upper limit of normal), and renal function (plasma creatinine level: <2.0 mg/dL).

A total of 34 cases with progressive/recurrent digestive organ cancers were investigated. The primary lesion was stomach cancer in 15, colon cancer in 16, and pancreatic cancer in 3. The median age was 67 years (range 39–88 years). The patient characteristics are summarized in Table 1.

Table 1 Patient characteristics

Characteristics	Number of patients
Age (years)	
67 (39–88)	34
Sex	
Male	20
Female	14
Performance status	
0	22
1	8
2	4
BMI	20.4 (16.4–34.4)
Creatinine clearance (mL/min)	94.9 (54.2–130)
T.Bil (mg/dL)	0.49 (1.05–0.28)
Alb (g/dL)	4.1 (3.6–4.8)
PT (%)	85 (72–100)
Tumor type	
Stomach	15
Colon	16
Pancreas	3
Prior chemotherapy regimens	
0	24
1	8
2	2

Values represent median (range)

BMI body mass index, *T.Bil* total bilirubin (normal range 0.2–1.2 mg/dL), *Alb* albumin (normal range 3.8–5.0 g/dL), *PT* prothrombin time (normal range ≥70%)

Treatment

S-1 was given orally twice daily for 28 consecutive days, followed by 2 weeks of rest. The dose of S-1 was fixed based on the patients' body surface area (BSA) according to the manufacturer's package insert, as distributed in Japan. The dose was 80 mg/day for patients with a BSA of <1.25 m^2, 100 mg/day for those with a BSA of 1.25–1.5 m^2, and 120 mg/day for those with a BSA of more than 1.5 m^2.

Judgment of therapeutic effects

The response was classified based on the Response Evaluation Criteria in Solid Tumor Guidelines (RECIST criteria) [21]. Effectiveness was judged in in-patients achieving a complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). Toxicity was evaluated based on the National Cancer Institute Common Toxicity Criteria (NCI-CTC) ver. 3.

CYP2A6 genotyping

Genomic DNA was extracted from 200 μL of peripheral blood with a QIAamp Blood Kit (Qiagen, Hilden, Germany). *CYP2A6*7* (T1412C) and *CYP2A6*9* (T-48G) were analyzed with the PCR-Invader assay described by Nevilie et al. [22] with some modifications. The region containing the polymorphic site T1412C or T-48G was amplified by PCR using a pair of forward and reverse oligonucleotide primers [23, 24] (Table 2). The signal probes and the Invader oligonucleotides for detection of the T1412C and T-48G polymorphisms were designed using the Invader Creator software (Third Wave Technologies, Madison, WI, USA) (Table 2). Invader reactions were performed using 384-well plates with reagents containing Cleavase XI enzyme for amplified DNA, both FAM dye and Redmond Red (RED) dye (Epoch Biosciences, Redmond, WA, USA) FRET cassettes and *CYP2A6*-specific PCR products. The plate was incubated at 63°C for 10 min in a PTC-100 thermal cycler (MJ Research, Waltham, MA, USA), and then directly read in a CytoFlour 4000 fluorescence plate reader (Applied Biosystems, Foster City, CA, USA). Fold over zero (FOZ) is used to confirm the validity of each assay of the samples [22].

*CYP2A6*4* (deletion) was analyzed with the Invader copy number assay by comparing the target gene signal (*CYP2A6*) with that of a reference gene (α-actin). The *CYP2A6* probe set was designed to react specifically with only the *CYP2A6* sequence (Table 2). Invader reactions were performed with reagents containing Cleavase XI enzyme for Genomic DNA, both FAM dye and RED dye FRET cassettes and Genomic DNA. The plate was incubated at 63°C for 3 h in a PTC-100 thermal cycler, and then read in a CytoFlour 4000 fluorescence plate reader. For the copy number assay, the FOZ values (for *CYP2A6* and α-actin) were calculated and the ratio of the *CYP2A6* to α-actin FOZ was calculated to identify *CYP2A6* copy number [22].

FT and 5-FU concentrations

The analysis of FT and 5-FU was conducted according to the method of Matsushima et al. [25].

Blood samples for assessing 5-FU and FT concentrations were obtained on the first day of treatment. The blood samples were taken directly before the administration of S-1 and 3 h after the first administration in the morning. The samples were immediately centrifuged, and plasma was stored at −80°C until analysis.

Plasma concentrations of FT were analyzed using an HPLC method, and 5-FU was analyzed using negative ion chemical ionization gas chromatography mass spectrometry (GC-NICI-MS).

HPLC was performed using an L7100 pump system with an L7250 (Hitachi, Tokyo, Japan) auto sample injector and SPD-10A variable-wavelength UV detector (Shimadzu, Kyoto, Japan) operated at 254 nm. HPLC separation was achieved at an ambient temperature with a reversed-phase Tsk-gel ODS 80Ts column (15 cm × 4.6 mm i.d., 5 μm particle size; Tosoh) and a 0.6 mL/min flow-rate of 15% methanol in 10 mM phosphate buffer (pH 5.5).

GC-NICI-MS was carried out using a Hewlett-Packard 5890 gas chromatograph interfaced with TRIG-1000 quadrupole mass spectrometry (VG Masslab, Manchester,

Table 2 PCR and Invader signal probes and Invader oligonucleotides sequence

Target	Reaction type	Oligonucleotide type	Sequence
*CYP2A6*7* (T1412C)	PCR	PCR forward (2A6ex8F)	CACTTCCTGAATGAGAAGG
	PCR	PCR reverse (2A6R3)	GGAATAGGTGCTTTTTAAGAATC
	Invader	Invader Oligo	CCACGTGTTTGGGGGACACGTCAT
		Signal Probe 1	cgcgccgaggATGTCCTTAGGTGACTGG
		Signal Probe 2	acggacgcggagGTGTCCTTAGGTGACTG
*CYP2A6*9* (T-48G)	PCR	PCR forward (*9/s1)	CAGGATTCATGGTGGGGCATGT
	PCR	PCR reverse (ex1R)	CTTCATGAGGGAGTTGTACATC
	Invader	Invader Oligo	TGACGGCTGGGGTGGTTTGCCTTTA
		Signal Probe 1	cgcgccgaggATACTGCCTGAAAAAGAGG
		Signal Probe 2	acggacgcggagCTACTGCCTGAAAAAGAGG
*CYP2A6*4* (deletion)	Invader copy number assay	2A6 Invader Oligo	GTATCTAGGGGTCTCAGAGCAGGAAATGATAGT CCGAATAG
		α-Actin Invader Oligo	AAGAGTAGCCACGCTCGGTGAGGATCTTCATT
		2A6 Signal Probe	acggacgcggagGCAAAATGGGGTGG
		α-Actin Signal Probe	cgcgccgaggCAGGTAGTCGGTGAGATC

Lower case letters indicate the flap sequences of signal probes

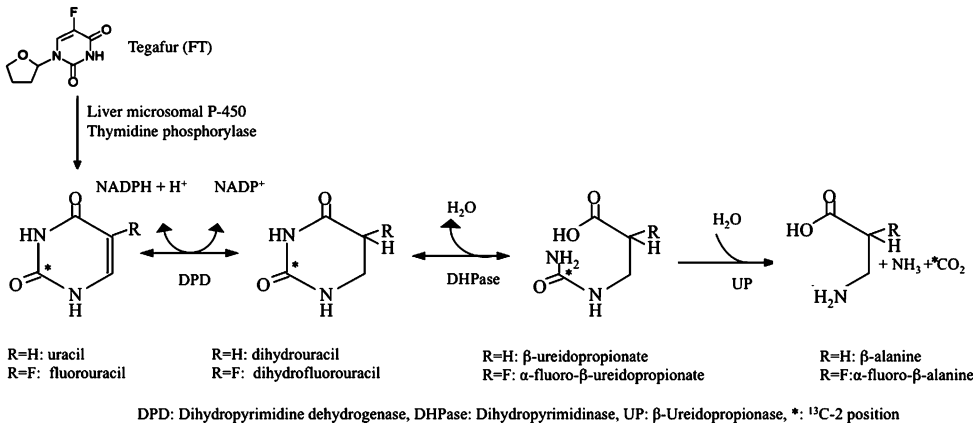

Fig. 1 The principle of the UrBT is based on the metabolism of 2-^{13}C-uracil by the enzymes of the pyrimidine-catabolic pathway to produce $^{13}CO_2$

UK). Negative ion chemical ionization was performed with isobutane as the reagent gas. The source pressure was adjusted and optimized to achieve maximum sensitivity. The GC column was interfaced directly with the ion source and the interface temperature was maintained at 250°C. Gas chromatographic separation was performed on a DB-5 chemical-bonded capillary column (15 m × 0.32 mm i.d., film thickness: 0.25 μm) (J&W Scientific, Folsom, CA, USA).

To 0.25 mL of plasma diluted (1:10, v/v) with water, 0.1 mL of 0.2 M phosphate buffer (pH 7.0) and 0.1 mL of internal standard solution ([$^{15}N_2$]-5-fluorouracil; 100 ng/mL) were added. After adding 4 mL of dichloromethane, the mixture was shaken for 10 min, and then centrifuged at 2,000*g* for 5 min. The organic layer was transferred to another test tube, and this extraction was repeated once. The combined organic layer was evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was dissolved in 0.2 mL of the HPLC mobile phase, and an aliquot (20 μL) was injected into an HPLC column for the chromatographic separation of FT.

To the residual aqueous layer, 0.4 mL of 1 M potassium dihydrogen phosphate and 4 mL of ethyl acetate were added. The mixture was shaken for 10 min, and then centrifuged at 2,000*g* for 5 min. The organic layer was transferred to another test tube, and this extraction was repeated once. The combined ethyl acetate layer was evaporated to dryness at 40°C under a stream of nitrogen and then the residue was dissolved in acetonitrile (30 μL), and this was followed by the addition of pentafluorobenzyl bromide (10 μL) and triethylamine (10 μL). The solution was allowed to stand at room temperature (30 min), then ethyl acetate (0.05 mL) followed by *n*-hexane (0.5 mL) was added, and the solution was vortex-mixed and centrifuged. The separated upper layer was evaporated to dryness under a stream of nitrogen; the residue was dissolved in 0.2 mL of ethyl acetate, and then an aliquot (1 μL) was injected into the GC–MS system.

Uracil breath test

Figure 1 shows metabolic pathway of uracil and 5-FU. The principle of the UrBT is based on metabolism of 2-^{13}C-uracil by the enzymes of the pyrimidine-catabolic pathway to produce $^{13}CO_2$. At 8 a.m. on the day of testing, fasting subjects were weighed and an aqueous solution containing 2 mg/kg 2-^{13}C-uracil (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) was prepared. UrBT was performed twice, before and 3 h after oral administration of S-1. $^{13}CO_2$ and $^{12}CO_2$ in exhaled breath samples were measured by IR spectrometry using a Breath ID system (Breath ID, Exalenz Co. Ltd., Israel).

The amount of $^{13}CO_2$ present in breath samples was expressed as the ratio of the change divided by the baseline, i.e., $\Delta^{13}CO_2/^{12}CO_2$ of breath samples collected before and after [2-^{13}C] uracil ingestion.

$$\Delta^{13}CO_2‰ = \{[^{13}CO_2/^{12}CO_2]_{\text{post-dose sample}} - [^{13}CO_2/^{12}CO_2]_{\text{pre-dose sample}}\} \times 1,000$$

The results of the breath test were expressed as $\Delta^{13}CO_2‰$, and the area under the curve ($AUC_{60\ min}$: Σ60) was calculated using $\Delta^{13}CO_2$ values.

This study was performed according to the guidelines of the Declaration of Helsinki as amended in Edinburgh, Scotland, October 2000. According to the regulations of the ethics committee of Nihon University Nerima Hikarigaoka Hospital, peripheral blood samples, *CYP2A6* genotyping, and the uracil breath test were performed after obtaining informed consent from all patients.

Statistical analysis

The results of the UrBT are expressed as mean ± SD and were compared using the Mann–Whitney *U* test. The Wilcoxon signed rank test and/or Chi-square test was used as appropriate ($P < 0.05$). The relationship of the UrBT results with 5-FU and FT concentrations was examined

using Spearman's correlation coefficient ($P < 0.05$). Logistic-regression analysis was employed for factor analysis of the anticancer effect. Receiver operating characteristic (ROC) curves of factors selected by logistic-regression analysis were prepared (using SPSS 17.0). The cut-off value for obtaining a tumor size-reducing effect was determined using the curve, and the sensitivity, specificity, and likelihood ratio at the cut-off value were calculated.

Results

Uracil breath test

Figure 2 shows the results of the UrBT performed before and after oral S-1 administration in all patients. The values after S-1 administration were significantly lower at all time points than those before administration, and the Σ60 value was also lower after administration [Σ60 before vs. Σ60 after administration (mean ± SD‰): 322.6 ± 88.3 vs. 64.3 ± 40.3, respectively] (Fig. 2a, b).

Antitumor effect of oral S-1

There were 2 CR and 16 PR cases (CR + PR group: responder group, 18 cases), whereas 16 had PD (PD group: non-responder group), and the response rate was 52.9% (18/34). There were no significant differences in the age, sex ratio, performance status, BMI, creatinine clearance, prior chemotherapy regimens, total bilirubin, albumin, or prothrombin time between the responder and non-responder groups (Table 3).

The plasma FT level 3 h after the oral administration of S-1 was not significantly different between the responder and non-responder groups, but the plasma 5-FU level was significantly higher in the responder group (Table 3).

Results of the UrBT with and without antitumor effects

There was no significant difference in the UrBT value at any time point or Σ60 between the groups in UrBT performed before oral S-1 administration (Fig. 3a, b). However, in UrBT performed 3 h after S-1 administration, the values at 10 and 60 min and Σ60 in the PD group were significantly higher than those in the CR + PR groups (Fig. 4a, b; Table 3).

CYP2A6 alleles and antitumor effect

The distribution of *CYP2A6* genotypes was as follows: **1/*4A* in 8 cases, **1/*7* in 3, **1/*9* in 6, **1/*11* in 1, **4A/*4A* in 2, **4A/*9* in 1, **4A/*10* in 1, **7/*9* in 5, **9/*9* in 5, and **9/*10* in 2. In the CR + PR groups (18 cases), a single-variant allele of *CYP2A6* (**1/*X*) was detected in 12, and two-variant alleles (**X/*X*) in 6. In the PD group (16 cases), a single-variant allele was detected in 6, and two-variant alleles in 10. The frequency of a single variant was higher in the CR + PR groups, while that of two variants was higher in the PD group, but the differences were not significant (Table 4). The presence or absence of an antitumor effect associated with the single-variant alleles was investigated, but no significant difference was noted (Table 4).

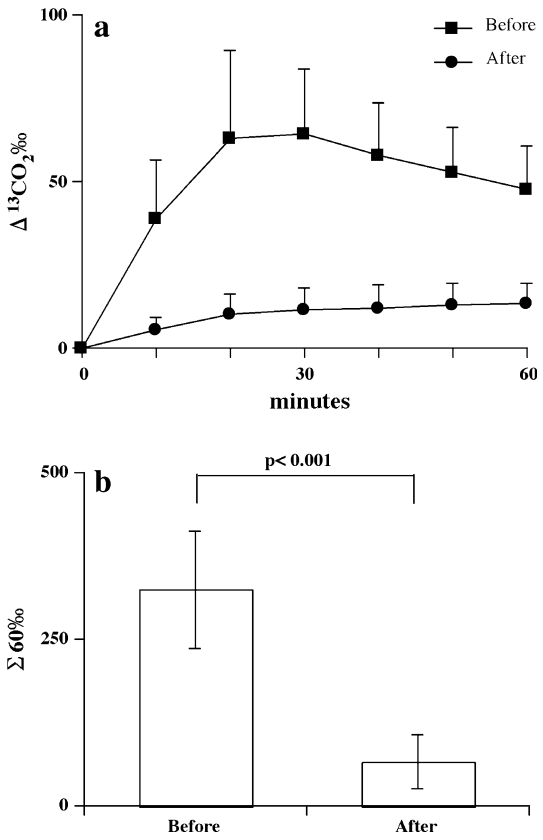

Fig. 2 *Before* Result of uracil breath test before S-1 administration. *After* Result of uracil breath test performed 3 h after S-1 administration. *Σ60* The area under the curve ($AUC_{60\ min}$) was calculated using $\Delta^{13}CO_2$ values. The values after S-1 administration were significantly lower at all time points than those before administration (**a**) and the Σ60 value was also lower after administration (**b**)

CYP2A6 allele and plasma FT and 5-FU concentrations

Plasma FT level was significantly lower in **1/*X* than in **X/*X*, while plasma 5-FU level was significantly higher in **1/*X* than in **X/*X*. However, there were no significant differences in plasma FT or 5-FU level between **X/*4* and *X/*X* (Table 4).

Table 3 Characteristics of responder and non-responder patients

	CR + PR (responders)	PD (non-responders)	*P*
Number of patients	18	16	
Age (years)	67 (39–88)	65 (42–76)	0.326
Sex			
Male	11	9	
Female	7	7	0.105
Performance status	1 (0–2)	0 (0–2)	0.625
BMI	21.6 (16.4–34.4)	19.2 (14.7–24.3)	0.061
Tumor type			
Stomach	10	5	
Colon	7	9	
Pancreas	1	2	
Prior chemotherapy regimens	0 (0–2)	0 (0–2)	0.638
Concentration after 3 h (ng/mL)			
FT (ng/mL)	1,689.9 (1,137.3–4,798.3)	1,724.4 (1,068.8–3,827)	0.904
5-FU (ng/mL)	161.3 (71.9–243.3)	75.55 (38.0–248.3)	0.001*
Σ60 (‰)			
Before	314.524 (±108.001)[a]	336.068 (±80.840)[a]	0.512
After	36.60 (±21.474)[a]	92.815 (±38.093)[a]	<0.0001*
Creatinine clearance (mL/min)	94.9 (54.2–123.8)	98.4 (58.6–130)	0.610
T.Bil (mg/dL)	0.47 (1.0–1.05)	0.37 (0.35–1.05)	0.785
Alb (g/dL)	4.1 (3.8–4.6)	4.0 (3.6–4.8)	0.621
PT (%)	88 (79–100)	82 (72–91)	0.112

Values represent median (range)

CP complete response, *PR* partial response, *PD* progressive disease, *BMI* body mass index, *Σ60* the area under the curve ($AUC_{60\ min}$) was calculated using $\Delta^{13}CO_2$ values, *before* Σ60 of uracil breath test before the S-1 administration, *after* Σ60 of uracil breath test after 3 h S-1 administration, *FT* tegafur, *5-FU* 5-fluorouracil

* Significant difference

[a] Values represent mean (±SD)

CYP2A6 allele and adverse effects

Differences in the frequency of adverse effects due to the presence or absence of **1/*X* and **X/*4* were investigated. There was no difference in the frequency of adverse effects associated with any single-variant allele (Table 5).

Results of UrBT, plasma FT and 5-FU concentrations and adverse effects

Plasma FT and 5-FU levels 3 h after oral administration of S-1 were not significantly different between with and without hematotoxicity, non-hematotoxicity and either. Σ60 was compared between cases with and without hematotoxicity and non-hematotoxicity before and after oral S-1 administration, but no significant difference was detected (Table 6).

Correlation results of the UrBT and plasma FT and 5-FU concentrations

Table 7 shows the correlations between UrBT Σ60 and the plasma FT and 5-FU levels. A negative correlation was present only between Σ60 after S-1 administration (after Σ60) and the plasma 5-FU level at 3 h (Table 7; Fig. 5).

Results of multivariate analysis

Logistic analysis was performed with four factors: the presence or absence of *CYP2A6* allele **1/*X*, plasma FT and 5-FU levels, and after Σ60, and significance was noted only in the latter (Table 8).

Receiver operating characteristic (ROC) curve of the UrBT after oral S-1 administration

The area under the ROC curve of the presence of an anticancer effect against after Σ60 was 0.904 ($P = 0.0017$) (Fig. 6). When the cut-off value of Σ60 was set to 43.2‰ based on the ROC curve, sensitivity was 83.3%, specificity was 93.7%, likelihood ratio was 13.3, and pre- and post-test odds ratios were 1.1 and 15, respectively (Table 9).

Discussion

S-1 elevates the plasma 5-FU level by the CDHP-induced inhibition of DPD, through which exertion of the antitumor effect of 5-FU is enhanced. Response rates of 49% [26], 35% [27], and 20% [28] to S-1 in progressive recurrent stomach, colon, and pancreatic cancers, respectively, have been reported.

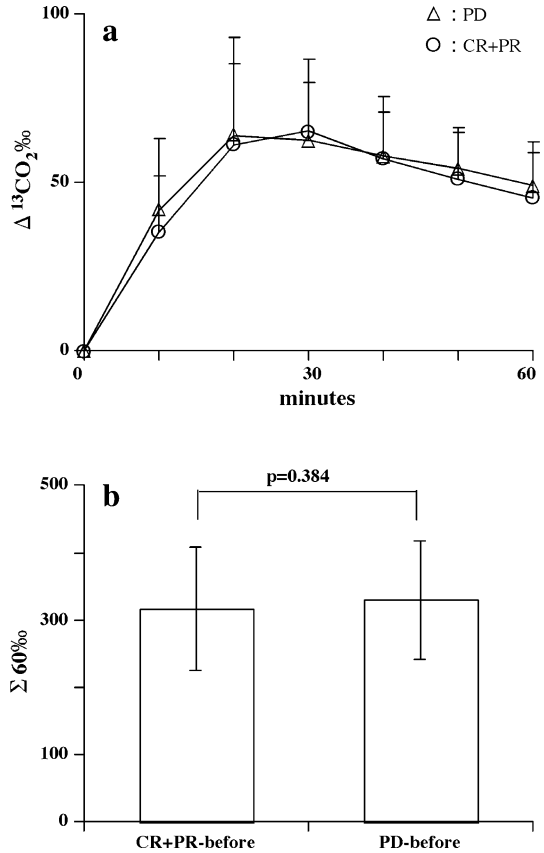

Fig. 3 *CR* complete response, *PR* partial response, *PD* progressive disease, *open triangles* PD (non-responders), *open circles* CR + PR (responders). *Before* Result of uracil breath test before S-1 administration. There was no significant difference in UrBT value at any time point (**a**) or Σ60 (**b**) between the responders (CR + PR) and the non-responders (PD) on UrBT performed before oral S-1 administration

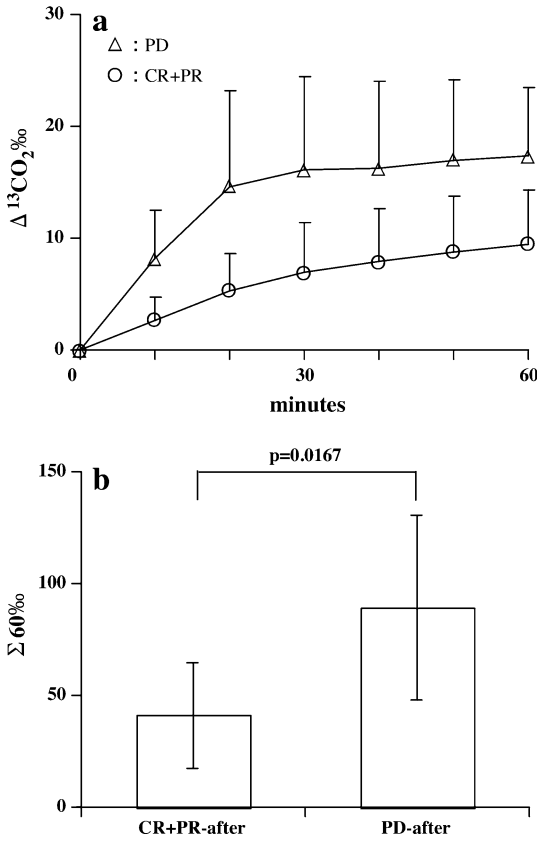

Fig. 4 *Open triangles* PD (non-responders), *open circles* CR + PR (responders). *After* Result of uracil breath test 3 h after S-1 administration. In the UrBT performed 3 h after S-1 administration, the values at 10 and 60 min in the non-responders group were significantly higher than those in the responders group (**a**) and so was the Σ60 value (**b**)

There are polymorphisms in the *CYP2A6* gene. Fujita et al. reported that the *CYP2A6* genotype statistically correlated with tegafur pharmacokinetics. Kaida et al. reported that patients with the *CYP 2A6*4C* allele had a significantly lower maximum plasma concentration of 5-FU than that in patients without the *CYP 2A6*4C* allele [18]. In our study, the plasma FT level 3 h after oral S-1 administration was significantly decreased, while the 5-FU level was significantly increased, in the presence of the *CYP2A6*1/*X* allele, compared to **X/*X*, but there was no significant difference in the plasma FT or 5-FU level in the presence of **X/*4*. These findings suggested that FT was efficiently converted to 5-FU in patients with the *1/*X allele, supporting the findings reported by Fujita et al.

Several days were necessary for the FT and 5-FU levels to reach a steady state after oral S-1 administration. Kaida et al. investigated the pharmacokinetic (PK) of FT and 5-FU in the steady state. We measured the levels only once at 3 h after the first oral S-1 administration, which may have been the reason for the absence of a significant difference. Accordingly, this finding does not indicate that the PK of S-1 is not influenced by the presence of the CYP2A6*4 allele. Actually, we measured the FT and 5-FU levels in two healthy wild-type (*1/*1) adults and two *4A/*4A patients under the same conditions and obtained a finding similar to that reported by Kaida, although these were not included in this study (unpublished data). CYP2A6 played a major role in 5-FU formation from FT. It has been reported that thymidine phosphorylase is involved in the production of 5-FU from FT [3–6]. Komatsu et al. [6] reported that 5-FU formation from tegafur in the human liver was mainly catalyzed by microsomal P450 at low concentrations of tegafur, but the contribution of cytosolic 5-FU formation by thymidine phosphorylase would be important at high concentrations. These reports suggest that investigation of not only the CYP2A6 allele but also thymidine phosphorylase is necessary with regard to the PK after oral S-1 administration.

Regarding the antitumor effect, there was no significant difference associated with **1/*X* or **X/*4*. Kaida et al. [18] reported that genotyping of *CYP 2A6* polymorphisms may

Table 4 Antitumor effect and plasma levels in patients with single-variant allele *CYP2A6*, **1/*X* or **X/*4*

	**1/*X*	**X/*X*	*P*	**X/*4*	**X/*X*	*P*
CR + PR (18 cases)	12	6		6	12	
PD (16 cases)	6	10	0.1679	6	10	1.00
After 3 h concentration (ng/mL)						
FT	1,506.2 (1,068.8–4,798.3)	2,072 (1,427.9–3,827)	0.0309*	2,072 (1,137.3–3,451)	1,627.9 (1,068.8–4,798.3)	0.396
5-FU	161.3 (38–248.3)	81.4 (49.0–211.4)	0.0025*	120 (49–211.4)	152.2 (38–248.3)	0.613

Values represent median (range)

CP complete response, *PR* partial response, *PD* progressive disease

* Significant difference

Table 5 Frequency of adverse effects according to *CYP2A6 allele* with or without **1/*X* and **X/*4*

	**1/*X*	**X/*X*	*P*	**X/*4*	**X/*X*	*P*
Hematotoxicity						
≥G1						
Leukopenia/ neutropenia	3	2		2	5	
Anemia	2	1		1	4	
Non-hematotoxicity						
Liver dysfunction	0	1			0	
Renal dysfunction	0	1		0	0	
General fatigue	1	0		0	1	
Diarrhea	1	0			1	
Pigmentation/ dermatitis	2	0		1	2	
Nausea/vomiting	1	0				
Total	10/18	5/16	0.185	4/12	13/22	0.151

provide vital information for effective cancer therapy using S-1. Regarding the prediction of responses to S-1, our study suggested that the conditions to achieve an anticancer effect are a low after Σ60 value and high plasma 5-FU level, and the absence of **1/*X* or **X/*4*. For S-1 to increase the antitumor effect, maintenance of high plasma and tissue levels of 5-FU is necessary. Inhibition of DPD may be important to increase the anticancer effect, rather than the *CYP2A6* alleles.

The DPD gene is located at chromosome 1 p22, and consists of 23 exons. The gene has been reported to exhibit 14 mutations and deletions in its base sequence associated with adverse effects, with a frequency of 0.47–2.2% in Europeans/Americans, and 0% in Japanese [29]. There are many polymorphisms of intron 14 [29]. In patients with this heterozygous allele, DPD activity in peripheral blood mononuclear cells is markedly low, and patients with the homozygous allele develop total DPD deficiency [30]. The UrBT is capable of identifying partial and marked DPD deficiency [19]. In addition, the correlation of UrBT results with liver DPD activity has been reported [20]. We performed UrBT before and after oral S-1 administration, and investigated the changes in UrBT results due to CDHP-induced inhibition of DPD, and the relationship of the UrBT findings with the antitumor and adverse effects of S-1.

Since $T_{1/2}$ of CDHP is about 3 h, UrBT was performed 3 h after oral S-1 administration. To set the measurement time, UrBT was performed in healthy adults (8 cases) and patients at 5 or more years after gastrectomy with no recurrence or metastasis (total gastrectomy: 15, distal gastrectomy: 8), and the measurement time was set at

Table 6 Plasma FT, 5-FU levels and Σ60 before and after oral S-1 administration in patients with and without adverse effects

	FT (ng/mL)	*P*	5-FU (ng/mL)	*P*	UrBT before Σ60 (‰)	*P*	UrBT after Σ60 (‰)	*P*
Hematotoxicity								
Yes	2,191.73 (±688.14)	0.80	125.0 (±72.22)	0.45	324.4 (±55.4)	0.87	66.6 (±38.3)	0.47
No	1,934.75 (±1,121.94)		137.327 (±55.34)		324.7 (±112.6)		61.1 (±43.6)	
Non-hematotoxicity								
Yes	2,191.73 (±1,121.94)	0.64	128.50 (±54.85)	1.00	322.6 (±65.5)	0.96	71.3 (±52.6)	0.78
No	1,934.75 (±688.14)		133.748 (±62.88)		325.0 (±100.5)		61.6 (±40.0)	
Either								
Yes	2,117.92 (±1,029.15)	0.88	133.447 (±66.78)	0.97	317.8 (±119.0)	0.80	60.3 (±37.4)	0.95
No	1,952.44 (±716.70)		132.605 (±57.94)		330.5 (±55.8)		65.1 (±45.0)	

Values represent mean (±SD)

Table 7 Correlation of UrBT Σ60 with FT and 5-FU levels

	FT 3 h		5-FU 3 h	
	Correlation coefficient	*P*	Correlation coefficient	*P*
Before Σ60	0.060	0.707	−0.192	0.277
After Σ60	−0.162	0.360	−0.505	0.002*

Before Σ60 Σ60 of uracil breath test before the S-1 administration, *after Σ60*: Σ60 of uracil breath test after three h S-1 administration

* Significant difference

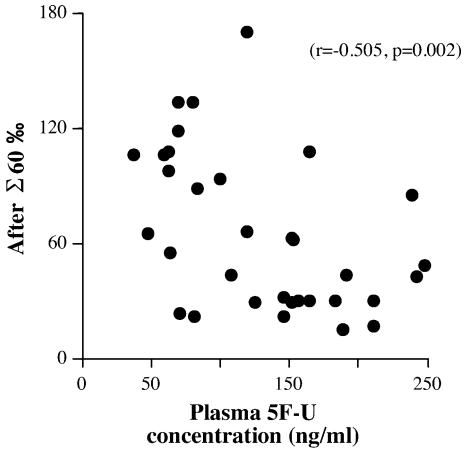

Fig. 5 *After Σ60* ‰ the area under the curve ($AUC_{60\ min}$) was calculated using $\Delta^{13}CO_2$ values of the uracil breath test performed 3 h after S-1 administration. A negative correlation was present only between Σ60 after S-1 administration (after Σ60) and the plasma 5-FU level at 3 h

60 min, at which no significant difference was noted between the two groups (unpublished data).

The results of UrBT were not affected by the presence or absence of *1/*X or *X/*4, and the after Σ60 value was negatively correlated with the plasma 5-FU level at 3 h after administration (Fig. 5). CDHP inhibited DPD, which inhibited 5-FU metabolism and increased the plasma level. At the same time, the inhibition of DPD may have reduced uracil metabolism, decreasing the UrBT value. These findings suggested that UrBT reflects the DPD-inhibitory effect of CDHP, and may be useful as a non-invasive test to evaluate the DPD-inhibitory effect of CDHP. Fujita et al. [17] investigated the PK of CDHP after oral S-1 administration, and found that the plasma CDHP concentration was affected by the renal function and the variability of the plasma CDHP concentration was likely to be affected by the PK of 5-FU. Investigation of the relationships between the PK of CDHP and UrBT may promote the use of UrBT as the first tailor-made medicine if the dose of CDHP can be changed individually after UrBT.

In multivariate analysis between the responders and non-responders, only the after Σ60 value was an independent factor of responses to S-1 (Table 8). Thus, the cut-off value was set based on the ROC curve of after Σ60, and the sensitivity, specificity, and likelihood ratio were investigated. The odds ratio after the test compared to that before the test. These results suggested that the anticancer effect of S-1 can be predicted by performing UrBT 3 h after the initial oral S-1 administration.

Regarding adverse effects, the frequency was not high in patients with *1/*X or *X/*4. In patients in whom DPD is strongly inhibited by CDHP after S-1 administration, the plasma 5-FU level rises and they may exhibit hematotoxicity and non-hematotoxicity. Thus, we investigated whether adverse effects can be predicted from the UrBT results, but there was no significant difference in Σ60 in UrBT between before and after S-1 administration (Table 7), showing that adverse effects cannot be predicted from UrBT performed 3 h after S-1 administration. Since the involvement of thymidylate synthase (TS) gene alleles and orotate phosphoribosyltransferase (OPRT) gene polymorphisms in adverse effects of pyrimidine fluoride anticancer agents has also been reported [30–32], it may be difficult to predict the development of adverse effects by measuring the inhibitory effect of DPD alone.

It has been reported that at least 27 genes are involved in the therapeutic effect of 5-FU [33]. In the field of effect prediction, a global approach has recently been proposed, in which gene expression is comprehensively investigated using a gene array that simultaneously detects the expression of several thousands to several tens of thousands of genes to search for those useful for the prediction of effects. A similar approach has been employed to predict the adverse effects of pyrimidine fluoride anticancer agents [30–32].

In conclusion, our results suggest that the anticancer effect of S-1 can be predicted by performing UrBT 3 h

Table 8 Multivariate analysis results

	Univariate *P*	Non-adjusted odds ratio	95% CI	Multivariate *P*	Adjusted odds ratio	95% CI
*1/*X	0.093	1.82	0.917–3.811	0.441	1.72	0.406–7.474
3 h FT	0.624	0.99	0.998–1.001	0.462	0.99	0.997–1.001
3 h 5-FU	0.003*	0.97	0.956–0.990	0.306	0.98	0.962–1.009
After Σ60	0.001*	1.05	1.027–1.107	0.009*	1.05	1.020–1.106

* Significant difference

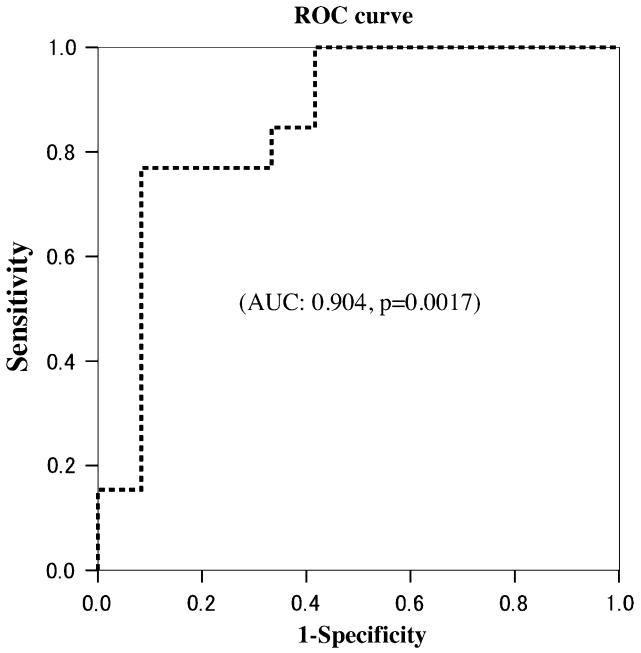

Fig. 6 The area under the ROC curve against the presence of an anticancer effect against after Σ60 was 0.904 ($P = 0.0017$)

Table 9 Sensitivity, specificity and likelihood ratio of after Σ60 at 43.2‰

Σ60 cut-off value (‰)	43.2
Sensitivity (%)	83.3
Specificity (%)	93.7
Likelihood ratio	13.3
Odds ratio before examination	1.1
Odds ratio after examination	15

after the initial oral S-1 administration. Based on this study, UrBT may be used as a simple test to select a DPD inhibitor for anticancer treatment, and it might become the first tailor-made medicine if the dose of CDHP can be changed individually after UrBT.

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