Cancer Chemother Pharmacol (2008) 63:85–89
DOI 10.1007/s00280-008-0714-x

ORIGINAL ARTICLE

High intratumoral dihydropyrimidine dehydrogenase mRNA levels in pancreatic cancer associated with a high rate of response to S-1

Hidekazu Kuramochi · Kazuhiko Hayashi · Kazumi Uchida · Go Nakajima · Takashi Hatori · Kathleen D. Danenberg · Peter V. Danenberg · Masakazu Yamamoto

Received: 19 December 2007 / Accepted: 17 February 2008 / Published online: 29 February 2008

Abstract
Purpose Although the prognosis in patients with pancreatic cancer has been poor, we recently reported unusually high response rate and survival benefit of S-1 treatment in patients with pancreatic cancer. The aim of this study was to reveal genetic background of this unique activity of S-1 against pancreatic cancer. S-1 is a novel oral fluoropyrimidine derivative consisting of Tegafur (FT) and dihydropyrimidine dehydrogenase (DPD) inhibitor (5-chloro-2,4-dihydroxypyridine; CDHP). Accordingly, intratumoral DPD mRNA expression level was measured to reveal whether the level in pancreatic cancer was different from other GI cancer and whether it was relevant to chemosensitivity.
Methods Thirty-three recurrent pancreatic cancer patients treated with S-1 were studied. We obtained 15 responders and 13 non-responders according to the change of serum CA19-9. The mRNA was extracted from paraffin-embedded surgical specimens using laser captured microdissection, and relative expression levels of each DPD/β-actin were measured using a quantitative reverse transcription polymerase chain reaction (RT-PCR) (Taqman) system. Forty-four colorectal cancer patients and 20 gastric cancer patients treated with S-1 were enrolled as control groups. Thymidylate synthase (TS) mRNA expression levels were also measured.
Results Intratumoral DPD mRNA expression level was significantly higher in pancreatic cancer than that in colorectal cancer ($P = 0.0003$; median level, 1.38 vs. 0.44) and gastric cancer ($P = 0.0061$; 1.38 vs. 0.82). No difference in TS mRNA expression levels was observed among cancer types. DPD expression among responded pancreatic cancer was significantly lower than non-responded. ($P = 0.012$, Mann–Whitney U test).
Conclusions Intratumoral DPD mRNA expression level in pancreatic cancer was significantly higher than the other malignancies. This result may elucidate possible reasons for the high effectiveness of S-1 in pancreatic cancer.

Keywords Pancreatic cancer · DPD · S-1 · Gene expression

H. Kuramochi · K. Hayashi (✉) · K. Uchida · G. Nakajima · T. Hatori · M. Yamamoto
Department of Gastroenterology,
Tokyo Women's Medical University, 8-1 Kawadacho,
Shinjukuku, Tokyo 162-8666, Japan
e-mail: hayashi@hosp.net

K. D. Danenberg
Response Genetics Inc., 1640 Marengo Street HRA 620,
Los Angeles, CA 90033, USA

P. V. Danenberg
Department of Biochemistry and Molecular Biology,
University of Southern California, 1441 East Lake Avenue,
Los Angeles, CA 90033, USA

Introduction

The outcomes of patients with pancreatic cancer remain very poor. Even after curative resection, the 5-year survival rate is only 7% [19]. 5-Fluorouracil (5-FU) had been the mainstay of treatment for pancreatic cancer, although, its response rate as a single agent is less than 20% [9]. Clinical trials have shown that 5-FU-based combination chemotherapy is no more effective than single-agent treatment, with greater toxicity [4, 5, 7]. In recent studies, gemcitabine showed a survival benefit over 5-FU [3] and is now used as a standard treatment for pancreatic cancer.

S-1 is a novel oral fluoropyrimidine derivative consisting of tegafur (FT) and two modulators, 5-chloro-2,4-dihydoxypyridine (CDHP) and potassium oxonate (Oxo) [24]. Antitumor effect is provided by the 5-FU prodrug FT. CDHP competitively inhibits the 5-FU degradative enzyme dihydropyrimidine dehydrogenase (DPD), resulting in prolonged active concentrations of 5-FU in blood [12, 26]. Nowadays, S-1 is widely used to treat many types of cancer, including gastric cancer [23], colorectal cancer [20], breast cancer [22], and head and neck cancer [18].

We have used S-1 in patients with pancreatic cancer and obtained a high response rate and prolonged survival [10]. Although, our previous study was small and retrospective, the response rate was 20% in patients given S-1 alone, as compared with 57.1% in those given S-1 plus cisplatin. To elucidate possible reasons for the high effectiveness of S-1 in pancreatic cancer, which is generally resistant to 5-FU-based therapy, we studied intratumoral DPD expression. We hypothesized that DPD expression levels were higher in pancreatic cancer than in other types of gastrointestinal cancers. We measured the DPD gene expression levels of pancreatic cancers by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) and compared the results with the levels in colorectal cancer and gastric cancer. Since thymidylate synthase (TS) is the other key enzyme for 5-FU metabolism, TS gene expression levels were also measured in these samples.

Patients and samples

Thirty-three patients with recurrent pancreatic cancer were studied (21 men and 11 women; median age 61.5 years, range 37–80). All patients had undergone surgical resection between 1998 and 2001 at the Department of Gastroenterology, Tokyo Women's Medical University, Tokyo, Japan. Diagnoses were individually confirmed by histopathological examination. All patients received S-1 and cisplatin after confirmation of recurrence. S-1 was given orally twice daily for 21 days, and cisplatin 30 mg/m^2 was given on days 1 and 8, followed by a 2 week period of no treatment. The dose of S-1 was based on body surface area (BSA) as follows: BSA < 1.25 m^2, 40 mg; BSA > 1.25 but < 1.5 m^2, 50 mg; and BSA > 1.5 m^2, 60 mg. All of the patients were Japanese, and written informed consent was obtained from each patient according to institutional regulations. No patient had preoperatively received neoadjuvant chemotherapy. Serum CA19–9 tumor marker levels were measured every 2 weeks during chemotherapy. Forty-four patients with advanced colorectal cancer and 20 with advanced gastric cancer were studied as controls.

Microdissection

FFPE tumor specimens were cut into serial sections 10 μm in thickness. For pathological diagnosis, one slide was stained with hematoxylin and eosin and evaluated by a pathologist. Other sections were stained with nuclear fast red (NFR, American MasterTech Scientific Inc., Lodi, CA) to facilitate visualization of histologic features. All tumor samples underwent laser capture microdissection (P.A.L.M. Microlaser Technologies AG, Munich, Germany) to ensure that only tumor cells were dissected.

RNA isolation and cDNA synthesis

RNA was extracted and cDNA was prepared from each sample as described previously [15, 16].

Reverse transcription-PCR

Quantification of TS, DPD and an internal reference gene (β-actin) was done using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System [Taqman]; Applied Biosystems, Foster City, CA) as described previously [11]. The primers and probe sequences used are listed in Table 1. The PCR reaction mixture consisted of 1,200 nM of each primer, 200 nM probe, 0.4 U of AmpliTaq Gold Polymerase, 200 nM each of dATP, dCTP, dGTP, and dTTP, 3.5 mM $MgCl_2$ and 1× Taqman Buffer A containing a reference dye, to a final volume of 20 μl (all reagents from PE Applied Biosystems, Foster City, CA, USA). Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 46 cycles at 95°C for 15 s and 60°C for 1 min. Gene expression values (relative mRNA levels) are expressed as ratios (differences between the Ct values) between the gene of interest (TS, DPD) and an internal reference gene (β-actin), which provides a normalization factor for the amount of RNA isolated from a specimen.

Statistical analysis

Median DPD mRNA levels were compared among pancreatic, colorectal, and gastric cancers by the Mann–Whitney U test, and multiple testing was corrected using the Benjamini and Hochberg False Discovery Rate. Median DPD mRNA levels were compared between responders and non-responders with the use of Mann–Whitney's U test. TS mRNA levels were compared by the Kruskal–Wallis test. P-values of less than 0.05 were considered to indicate statistical significance. All values were two-sided.

Table 1 Primers and probes

Thymidylate synthase (TS)		
Gen bank accession: NM_001071		
Forward primer	TS-764F	5′-GCCTCGGTGTGCCTTTCA-3′
Reverse primer	TS-830R	5′-CCCGTGATGTGCGCAAT-3′
Probe	TS-785T	5′-TCGCCAGCTACGCCCTGCTCA-3′
Dihydropyrimidine dehydrogenase (DPD)		
Gen bank accession: NM_000110		
Forward primer	DPD-51F	5′-AGGACGCAAGGAGGGTTTG-3′
Reverse primer	DPD-134R	5′-GTCCGCCGAGTCCTTACTGA-3′
Probe	DPD-71Tc	5′-CAGTGCCTACAGTCTCGAGTCTGCCAGTG-3′
β-actin		
Gen bank accession: NM_001101		
Forward primer	β-actin-592F	5′-TGAGCGCGGCTACAGCTT-3′
Reverse primer	β-actin-651R	5′-TCCTTAATGTCACGCACGATTT-3′
Probe	β-actin-611T	5′-ACCACCACGGCCGAGCGG-3′

Results

DPD mRNA expression levels in pancreatic, gastric, and colorectal cancers are shown in Table 2 and Fig. 1. The median DPD mRNA level in pancreatic cancer was significantly higher than the levels in colorectal cancer ($P = 0.0003$; median level, 1.38 vs. 0.44) and gastric cancer ($P = 0.0061$; 1.38 vs. 0.82). The median DPD mRNA level also significantly differed between gastric cancer and colorectal cancer ($P = 0.0025$; 0.82 vs. 0.44). No significant difference in TS mRNA levels was observed among pancreatic, colorectal, and gastric cancer ($P = 0.10$) (Table 2).

The serum CA19-9 tumor marker level was above the upper limit of normal (37 U/ml) in 28 of the 33 patients with pancreatic cancer. During chemotherapy the serum CA19-9 level fell by at least 50% in 18 patients (64.3%, responders) and either decreased by less than 50% or increased in 10 (35.7%, non-responders). The median DPD mRNA level in the responders was significantly lower than that in the non-responders ($P = 0.02$; 1.25 vs. 2.20) (Fig. 2).

None of the demographic and clinicopathological variables were significantly associated with the tumor response to S-1 chemotherapy at a P-value of 0.10 on Fisher's exact test (data not shown).

Table 2 Intratumoral DPD mRNA levels in various types of cancer

	Cancer type		
	Gastric	Pancreatic	Colorectal
Number of samples	20	33	44
DPD mRNA levels (median)	0.82	1.38	0.44
Range	0.17–2.21	0.21–4.16	0–2.64
TS mRNA levels (median)	3.26	2.38	2.47
Range	1.69–10.99	0.04–6.68	0.49–19.23

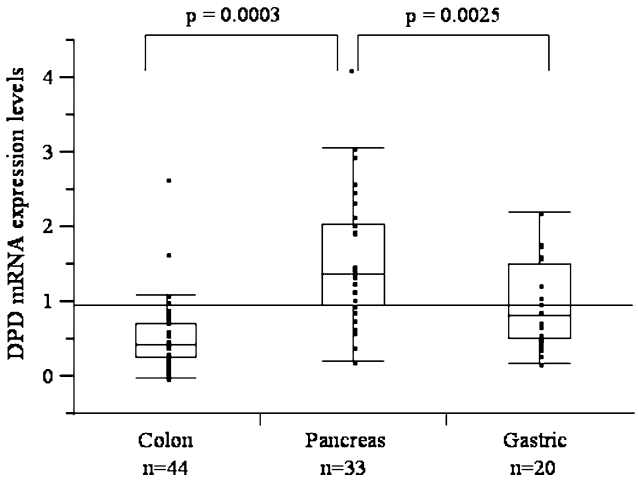

Fig. 1 DPD mRNA expression levels in pancreatic, colorectal, and gastric cancer. DPD expression in pancreatic cancer was significantly higher that that in colorectal [16] and gastric cancer [3]

Discussion

In our study, the DPD mRNA expression levels was significantly higher in pancreatic cancer than in colorectal cancer or gastric cancer, although no difference was seen in TS mRNA expression levels. This difference in DPD expression may explain the discrepancy between the high resistance to 5-fluorouracil and the high sensitivity to S-1 in patients with pancreatic cancer. In patients with colorectal cancer, treatment with 5-fluorouracil alone or a combination of 5-fluorouracil and leucovorin is somewhat effective [1, 6, 8, 21] and had been used as standard therapy before the advent of irinotecan. The response rate of pancreatic cancer is only 7% with 5-fluorouracil alone [5] and is not much higher with 5-fluorouracil plus leucovorin [4]. Ohtsu et al. [20] reported that S-1 had a response rate of 35% in

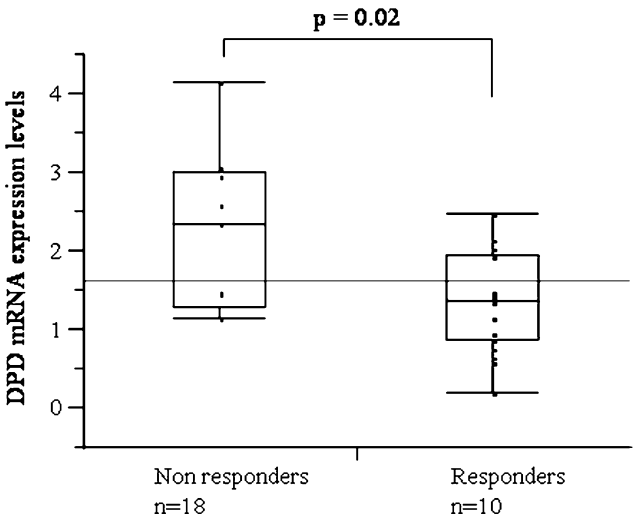

Fig. 2 DPD mRNA expression levels between responders and non-responders. Responders are defined as the serum CA19-9 level fell by at least 50% (18 patients), and non-responders are defined as their serum CA19-9 either decreased by less than 50% or increased (10 patients). DPD expression levels in responders were significantly lower than those in non-responders

phase II clinical trials of patients with colorectal cancer. This rate was not appreciably higher than the response rate with conventional 5-fluorouracil-based combination therapy. In pancreatic cancer, however, we previously obtained a response rate of higher than 50% with a combination of S-1 and cisplatin, which was considerably better than the response rate with conventional 5-fluorouracil treatment. Takechi et al. demonstrated that the in vitro antitumor activity of 5-FU against tumor cells with low DPD expression levels is not appreciably affected by the addition of CDHP. Against tumor cells with high DPD expression, however, the 50% inhibitory concentration differed by about twofold [25]. In tumors with low DPD expression such as colorectal cancer, the presence of CDHP provides no benefit, and the response to S-1 is similar to that with 5-fluorouracil. In tumors with high DPD expression such as pancreatic cancer, S-1 may be more effective than 5-fluorouracil.

Studies of DPD expression in patients with pancreatic cancer are scarce. Mori et al. used enzyme-linked immunosorbent assay [17] to measure DPD protein expression in various types of tumors and reported that pancreatic cancer had high DPD expression levels, similar to cancer of the neck, liver, esophagus, and breast [17]. Kamoshida et al. [14] used immunostaining to measure DPD levels in various types of tumors. They found that DPD expression was high in pancreatic cancer and low in colorectal cancer. To our knowledge, expression of DPD mRNA has not been assessed previously in pancreatic cancer. Uetake et al. reported that DPD gene expression correlated with DPD enzyme activity in colorectal cancer [27]. Mori et al. [17] showed that DPD enzyme activity strongly correlated with DPD protein expression as measured by ELISA. They found that the level of DPD protein expression was about threefold higher in pancreatic cancer and twofold higher in gastric cancer than in colorectal cancer, consistent with the mRNA levels measured by RT-PCR. These results suggest that the mRNA, protein, and activity levels of the DPD gene strongly correlate. DPD mRNA levels can be measured even in small specimens obtained by endoscopy or needle biopsy. The concomitant use of laser-captured microdissection allows DPD mRNA expression of tumors to be assessed quantitatively and objectively, providing clinically significant advantages over conventional procedures.

In our study, DPD levels were significantly lower in the responders to S-1 as assessed by the change in tumor marker levels than in the non-responders (1.25 vs. 2.20; $P = 0.02$). The median DPD level in the non-responders was 2.20, equivalent to about twofold that in the responders and fourfold that in colorectal cancer. Ichikawa et al. measured DPD mRNA levels in patients with gastric cancer and reported that the anticancer activity of S-1 does not depend on the level of DPD expression because CDHP inhibits DPD [13]. Although, the median DPD level did not differ significantly between responders and non-responders to S-1 in their study, patients in whom DPD expression exceeded a certain level did not respond to S-1. The inhibitory activity of CDHP against DPD is about 130-fold higher than that of uracil. However, in patients whose tumors have very high levels of DPD, the inhibitory effect of CDHP on DPD may be limited. In patients with such tumors, more potent DPD inhibitors should be used, or drugs with other mechanisms of action, such as gemcitabine, should be used instead of fluoropyrimidines. As for the former, eniluracil, a new, more potent DPD inhibitor, was developed and evaluated in clinical trials. Eniluracil decreases DPD enzyme activity by more than 99% in the liver and reduces 5-fluorouracil clearance by more than 18-fold [2]. In a phase II study of eniluracil combined with 5-fluorouracil in patients with pancreatic cancer, the median survival was only 3.6 months, indicating an unsatisfactory outcome. Moreover, 68% of the patients had grade 3 or higher toxicity. Subsequent clinical development was therefore terminated Rothenberg, 2002. Because eniluracil itself is free of serious toxicity, the toxic effects in clinical trials were attributed to prolonged, high serum concentrations of 5-fluorouracil. These results suggested the limitations of combination therapy with 5-fluorouracil and DPD inhibitors.

Our data may provide a molecular biologic basis for the high antitumor activity of S-1 in patients with pancreatic cancer. Gemcitabine has been shown to be somewhat effective against pancreatic cancer [3], but its anticancer activity

is far from satisfactory. Further studies of S-1, in patients with pancreatic cancer are awaited.

Acknowledgments K.D is a CEO of Response Genetics Inc. K.D and P.D own a stock of Response Genetics Inc.

References

1. Anonymous (1992) Modulation of fluorouracil by leucovorin in patients with advanced colorectal cancer: evidence in terms of response rate. Advanced Colorectal Cancer Meta-analysis Project. J Clin Oncol 10:896–903
2. Baccanari DP, Davis ST, Knick VC, Spector T (1993) 5-Ethynyluracil (776C85): a potent modulator of the pharmacokinetics and antitumor efficacy of 5-fluorouracil. Proc Natl Acad Sci U S A 90:11064–11068
3. Burris HA 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD (1997) Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol 15:2403–2413
4. Crown J, Casper ES, Botet J, Murray P, Kelsen DP (1991) Lack of efficacy of high-dose leucovorin and fluorouracil in patients with advanced pancreatic adenocarcinoma. J Clin Oncol 9:1682–1686
5. Cullinan S, Moertel CG, Wieand HS, Schutt AJ, Krook JE, Foley JF, Norris BD, Kardinal CG, Tschetter LK, Barlow JF (1990) A phase III trial on the therapy of advanced pancreatic carcinoma. Evaluations of the Mallinson regimen and combined 5-fluorouracil, doxorubicin, and cisplatin. Cancer 65:2207–2212
6. de Gramont A, Bosset JF, Milan C, Rougier P, Bouche O, Etienne PL, Morvan F, Louvet C, Guillot T, Francois E, Bedenne L (1997) Randomized trial comparing monthly low-dose leucovorin and fluorouracil bolus with bimonthly high-dose leucovorin and fluorouracil bolus plus continuous infusion for advanced colorectal cancer: a French intergroup study. J Clin Oncol 15:808–815
7. DeCaprio JA, Mayer RJ, Gonin R, Arbuck SG (1991) Fluorouracil and high-dose leucovorin in previously untreated patients with advanced adenocarcinoma of the pancreas: results of a phase II trial. J Clin Oncol 9:2128–2133
8. Glimelius B, Hoffman K, Graf W, Pahlman L, Sjoden PO (1994) Quality of life during chemotherapy in patients with symptomatic advanced colorectal cancer. The Nordic Gastrointestinal Tumor Adjuvant Therapy Group. Cancer 73:556–562
9. Hansen R, Quebbeman E, Ritch P, Chitambar C, Anderson T (1988) Continuous 5-fluorouracil (5FU) infusion in carcinoma of the pancreas: a phase II study. Am J Med Sci 295:91–93
10. Hayashi K, Imaizumi T, Uchida K, Kuramochi H, Takasaki K (2002) High response rates in patients with pancreatic cancer using the novel oral fluoropyrimidine S-1. Oncol Rep 9:1355–1361
11. Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. Genome Res 6:986–994
12. Hirata K, Horikoshi N, Aiba K, Okazaki M, Denno R, Sasaki K, Nakano Y, Ishizuka H, Yamada Y, Uno S, Taguchi T, Shirasaka T (1999) Pharmacokinetic study of S-1, a novel oral fluorouracil antitumor drug. Clin Cancer Res 5:2000–2005
13. Ichikawa W, Takahashi T, Suto K, Yamashita T, Nihei Z, Shirota Y, Shimizu M, Sasaki Y, Hirayama R (2004) Thymidylate synthase predictive power is overcome by irinotecan combination therapy with S-1 for gastric cancer. Br J Cancer 91:1245–1250
14. Kamoshida S, Shiogama K, Shimomura R, Inada K, Sakurai Y, Ochiai M, Matuoka H, Maeda K, Tsutsumi Y (2005) Immunohistochemical demonstration of fluoropyrimidine-metabolizing enzymes in various types of cancer. Oncol Rep 14:1223–1230
15. Kuramochi H, Hayashi K, Uchida K, Miyakura S, Shimizu D, Vallbohmer D, Park S, Danenberg KD, Takasaki K, Danenberg PV (2006) 5-fluorouracil-related gene expression levels in primary colorectal cancer and corresponding liver metastasis. Int J Cancer 119:522–526
16. Lord RV, Salonga D, Danenberg KD, Peters JH, DeMeester TR, Park JM, Johansson J, Skinner KA, Chandrasoma P, DeMeester SR, Bremner CG, Tsai PI, Danenberg PV (2000) Telomerase reverse transcriptase expression is increased early in the Barrett's metaplasia, dysplasia, adenocarcinoma sequence. J Gastrointest Surg 4:135–142
17. Mori K, Hasegawa M, Nishida M, Toma H, Fukuda M, Kubota T, Nagasue N, Yamana H, Hirakawa YSCK, Ikeda T, Takasaki K, Oka M, Kameyama M, Toi M, Fujii H, Kitamura M, Murai M, Sasaki H, Ozono S, Makuuchi H, Shimada Y, Onishi Y, Aoyagi S, Mizutani K, Ogawa M, Nakao A, Kinoshita H, Tono T, Imamoto H, Nakashima Y, Manabe T (2000) Expression levels of thymidine phosphorylase and dihydropyrimidine dehydrogenase in various human tumor tissues. Int J Oncol 17:33–38
18. Nishimura G, Yanoma S, Mizuno H, Satake K, Taguchi T, Ikeda Y, Tsukuda M (2000) Therapeutic effect of 1 M tegafur-0.4 M 5-chloro-2, 4-dihydroxypridine-1 M potassium oxonate (S-1) on head and neck squamous carcinoma cells. Cancer Lett 159:1–7
19. Nitecki SS, Sarr MG, Colby TV, van Heerden JA (1995) Long-term survival after resection for ductal adenocarcinoma of the pancreas. Is it really improving? Ann Surg 221:59–66
20. Ohtsu A, Baba H, Sakata Y, Mitachi Y, Horikoshi N, Sugimachi K, Taguchi T (2000) Phase II study of S-1, a novel oral fluorophyrimidine derivative, in patients with metastatic colorectal carcinoma. S-1 Cooperative Colorectal Carcinoma Study Group. Br J Cancer 83:141–145
21. Poon MA, O'Connell MJ, Moertel CG, Wieand HS, Cullinan SA, Everson LK, Krook JE, Mailliard JA, Laurie JA, Tschetter LK, et al (1989) Biochemical modulation of fluorouracil: evidence of significant improvement of survival and quality of life in patients with advanced colorectal carcinoma. J Clin Oncol 7:1407–1418
22. Saek T, Takashima S, Sano M, Horikoshi N, Miura S, Shimizu S, Morimoto K, Kimura M, Aoyama H, Ota J, Noguchi S, Taguchi T (2004) A phase II study of S-1 in patients with metastatic breast cancer—a Japanese trial by the S-1 Cooperative Study Group, Breast Cancer Working Group. Breast Cancer 11:194–202
23. Sakata Y, Ohtsu A, Horikoshi N, Sugimachi K, Mitachi Y, Taguchi T (1998) Late phase II study of novel oral fluoropyrimidine anticancer drug S-1 (1 M tegafur-0.4 M gimestat-1 M otastat potassium) in advanced gastric cancer patients. Eur J Cancer 34:1715–1720
24. Shirasaka T, Shimamato Y, Ohshimo H, Yamaguchi M, Kato T, Yonekura K, Fukushima M (1996) Development of a novel form of an oral 5-fluorouracil derivative (S-1) directed to the potentiation of the tumor selective cytotoxicity of 5-fluorouracil by two biochemical modulators. Anticancer Drugs 7:548–557
25. Takechi T, Fujioka A, Matsushima E, Fukushima M (2002) Enhancement of the antitumour activity of 5-fluorouracil (5-FU) by inhibiting dihydropyrimidine dehydrogenase activity (DPD) using 5-chloro-2,4-dihydroxypyridine (CDHP) in human tumour cells. Eur J Cancer 38:1271–1277
26. Tatsumi K, Fukushima M, Shirasaka T, Fujii S (1987) Inhibitory effects of pyrimidine, barbituric acid and pyridine derivatives on 5-fluorouracil degradation in rat liver extracts. Jpn J Cancer Res 78:748–755
27. Uetake H, Ichikawa W, Takechi T, Fukushima M, Nihei Z, Sugihara K (1999) Relationship between intratumoral dihydropyrimidine dehydrogenase activity and gene expression in human colorectal cancer. Clin Cancer Res 5:2836–2839