

Cyclophosphamide augments the anti-tumor efficacy of uracil and tegafur by inhibiting dihydropyrimidine dehydrogenase

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Abstract. The present study assesses the effects of neo-adjuvant chemotherapy (NAC) with uracil and tegafur (UFT) alone vs UFT plus cyclophosphamide (CPA), on the activity of thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) in breast cancer tissues. Breast cancer patients were randomly assigned to 3 groups; the control (no-treatment) group (n=13), the UFT (5-8 mg/kg/day) alone group (n=10) and the UFT plus CPA (1 mg/kg/one day interval) (UC) group (n=9), and they received NAC for 2-4 weeks. A total of 32 invasive ductal breast carcinomas were used to assay for TS and DPD activity. There were no statistically significant differences in tumor size or stage classification between the 3 groups. The DPD activity was inversely and significantly correlated with the tumor size and pT, but the TS activity was not correlated with these clinicopathological factors. The TS activity was decreased by NAC with UFT, and the addition of CPA resulted in an increased inhibition of TS activity. In contrast, DPD activity was increased by NAC with UFT administration, but its increased activity was significantly inhibited by the addition of CPA. Multiple regression analyses demonstrated that the total dose of UFT was a significant variable for inhibiting TS activity, and that CPA was a significant variable for inhibiting DPD activity. The DPD activity increased by UFT can be inhibited by CPA, and this may represent one of the possible mechanisms responsible for the anti-tumor activity of 5-FU or its derivatives as enhanced by CPA.

Introduction

Cyclophosphamide (CPA) is widely used for chemotherapy against breast cancer in combination with adriamycin (ADR),

methotrexate (MTX) or 5-fluorouracil (5-FU). We are interested in the combination effects of CPA with 5-FU or its derivatives, because CPA frequently shows remarkable augmenting effects on the anti-tumor effects of 5-FU or its derivatives.

In Japan, instead of intravenous (iv) 5-FU, an oral fluoropyrimidine, uracil-tegafur (UFT), has been widely used for the treatment of various malignancies (1). UFT is as effective as a continuous infusion of 5-FU, but with a better toxicity profile (2,3). Furthermore, one particular pharmacokinetic study demonstrated that the 5-FU concentration in the blood after oral UFT was comparable to that after a continuous iv infusion of 5-FU (4).

Several previous clinical reports have suggested that the combination regimen of UFT+CPA (UC) showed a good anti-tumor activity against breast cancer, sometimes in combination with anthracyclines (5,6). We have also used oral chemotherapy with UFT plus oral CPA (oral UC regimen) for adjuvant chemotherapy after pancreatic cancer surgery, and a retrospective analysis demonstrated that this UC regimen was beneficial in improving survival after pancreatic cancer surgery (7). Furthermore, there were also some patients, whose recurrent pancreatic or ampullary carcinomas responded to oral UC therapy (8,9).

Although it has been suggested that CPA can augment the antitumor activity of 5-FU or its derivatives, the mechanisms responsible remain unclear. However, several reports have suggested that CPA can augment the anti-tumor activity of 5-FU derivatives by modulating the activity of pyrimidine-metabolizing enzymes. It has also been reported that CPA augmented the activity of ribonucleotide reductase (RNR), which metabolizes 5-FU to fluoro-deoxyuridine monophosphate (FDUMP) (10); other researchers have also reported that CPA enhanced the efficacy of capecitabine and 5'-deoxyfluorouridine, which are also 5-FU derivatives, by inducing thymidine phosphorylase (TP) in a human breast tumor xenograft model (11). Although these modulatory effects of CPA on RNR or TP may represent some of the mechanisms responsible for the effects of CPA on the anti-tumor activity of 5-FU or its derivatives, no studies to date have investigated the effects of CPA on the key enzymes in pyrimidine metabolism,

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thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD).

TS is an enzyme, which catalyzes the reductive methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) by 5,10-methylene-5,6,7,8-tetrahydrofolate (CH_2H_4 folate) to produce thymidine-5'-monophosphate (dTMP) and 7,8-dihydrofolate (H_2 folate). TS is a dimer of identical 30-35 kDa subunits, and two major pathways are known in the metabolism of pyrimidine during DNA synthesis, the *de novo* pathway and the salvage pathway. TS is a key enzyme in the *de novo* pathway of pyrimidine synthesis, and is a very important target for chemotherapy with 5-FU or its derivatives (12).

DPD degrades fluoropyrimidine to 2-fluoro- β -tetrahydrofolate (5,10- CH_2FH_2), and is the initial and rate-limiting enzyme in this process. Fluoropyrimidines are degraded by DPD mainly in the liver, but the DPD activity in the tumor is correlated with the clinical response to 5-FU-based chemotherapies (13,14).

The present study was designed to assess the effects of neoadjuvant chemotherapy (NAC) with UFT alone vs UFT plus CPA (UC regimen) on the activity of TS and DPD in human breast cancer tissues.

Patients and methods

Patient enrolment. The study included 32 invasive ductal carcinomas of the breast. Two basic criteria had to be met before NAC administration: i) Histological or cytological proof of breast cancer and a preoperative diagnosis that the tumor could be curatively resectable, and ii) a performance status (PS) of 0-3 (ECOG score). The contraindications included: i) Total disability (PS=4, ECOG score), ii) prior chemotherapy, radiotherapy or immunotherapy within 4 weeks, iii) an active infectious disease, iv) severe anemia (hemoglobin <9 g/dl), leukopenia (<3,000/mm³), thrombocytopenia (<70,000/mm³), azotemia (creatinine >2 mg/dl), or liver dysfunction (GOT, GPT and alkaline phosphatase >4-fold the normal limits), v) severe heart disease or a concomitant malignant disease, and vi) pregnancy.

All patients and their families in the treatment group were fully informed with regard to the study aim, treatment program, expected side-effects and clinical benefits of the study, and informed consent was obtained in all cases. After surgery, the stage of breast cancer was classified according to the UICC (TNM) stage classification system (15).

Neoadjuvant chemotherapy (NAC). With their informed consent, the breast cancer patients were randomly assigned into 3 groups: The control (no-treatment) group, the UFT alone group and the UFT plus CPA (UC) group. The CPA alone group was not set-up, because in Japan, under the universal health insurance system, the Japanese Ministry of Health, Labor and Welfare strictly regulates the use of anticancer agents, and they do not approve of chemotherapy with CPA alone against breast cancer. UFT was orally administered at 5-8 mg/kg/day daily, and CPA was orally administered at 50 mg/body every other day. The NAC usually started from the day of the first visit to the outpatient ward until 2 days before the surgery for 2-4 weeks. The administration period was dependent on the waiting period

for surgery. The dose of UFT is usually expressed as the dose of FT and the dose of UFT was set at 8 mg/kg/day for the patients under 70 years of age, and at 6 mg/kg/day for those over 71 years old, but it was difficult to administer an accurate dose in terms of /kg or /m². UFT is an oral agent and is usually administered in a capsule or granule package, and one capsule contains 100 mg UFT whereas one granule package contains 150 mg UFT.

Evaluation of side-effects. In the patients in the NAC groups, hematology, serum biochemistry, tumor markers, the symptomatic status and PS, were examined before surgery routinely at biweekly intervals, or sometimes more frequently. Toxicity was evaluated according to the National Cancer Institute - Common Toxicity Criteria (16). If toxicity occurred, then the first CPA administration was interrupted and the dose of UFT for each patient was decreased according to the patient's condition (PS, body weight, age, hematology and serum biochemistry).

Evaluation of objective response (OR). If possible, the size of the primary lesion was assessed by ultrasonography (USG) or mammography (MMG) before and after NAC administration (usually 1 or 2 days before surgery). The response was evaluated by the RECIST (unidimensional) guideline (17). In brief, the largest diameter of the lesion was taken from bi-dimensional measurements. The responses were categorized as follows: i) A complete response (CR) indicating a complete disappearance of the lesion, ii) a partial response (PR) indicating a >30% decrease in tumor diameter, iii) progressive disease (PD) indicating a >20% increase in tumor diameter, or iv) stable disease (SD) (neither CR, PR nor PD).

If the tumor was evaluated to be progressive, then NAC was suspended. The duration of the response was not included in the evaluation of the OR, because all patients underwent surgery.

Pathological response. The histopathological effect was evaluated based on the grade (0-3), according to the histopathological criteria for the assessment of therapeutic response in breast cancer by the Japanese Breast Cancer Society (18). In brief, grade 3 (complete response) represented the necrosis or disappearance of all tumor cells, and the replacement of all tumor cells by granuloma-like and/or fibrous tissue. In the case of a complete disappearance of the tumor cells, pre-treatment pathological evidence of the presence of a tumor is necessary; grade 2 (marked response) represented marked changes in two thirds or more of the tumor cells; grade 1 (slight response); grade 1a (mild response) represented mild changes in the cancer cells regardless of the area, or marked changes seen in less than one third of the cancer cells; grade 1b (moderate response) represented marked changes in one third or more but less than two thirds of the tumor cells; and grade 0 (no response) represented almost no changes in the tumor cells after treatment.

Measurement of TS and DPD activity in tissues. The tissues were homogenized with 3 volumes of 50 mM Tris-HCl (pH 7.6) containing 10 mM 2-mercaptoethanol, 25 mM KCl and 5 mM MgCl_2 , centrifuged at 105,000 g for 60 min, and



	Control (n=13)	UFT (n=10)	UFT+CPA (n=9)
Age	52.3±8.8 ^a	56.6±14.4	64.7±13.8 ^a
Clinical stage	2.22±0.99	2.30±0.75	2.19±0.71
Tumor size (cm)	2.55±1.23	2.89±1.85	3.20±1.64
pT	1.69±0.75	1.80±0.63	2.00±0.71
pN	0.92±1.19	0.90±0.88	0.56±1.01
Number of involved nodes	3.77±6.70	2.80±5.77	3.22±8.93
ER status (+:-)	10:3	8:2	6:3
Administration period (days)		26.7±6.5	26.1±8.4
Total dose of UFT (g)		9.09±3.10	8.28±3.51
Total dose of CPA (mg)			653±211

^ap=0.0180.

then the resulting supernatant was used to measure the enzyme activity.

TS activity was measured by the [6-3H]-FdUMP-binding assay based on the method of Spears *et al* (19).

DPD was assayed, by a modification of the method of Naguib *et al* (20) as described previously (21). Briefly, a reaction mixture containing 10 mM potassium phosphate (pH 8.0), 0.5 mM EDTA, 0.5 mM 2-mercaptoethanol, 2 mM dithiothreitol, 5 mM MgCl₂, 20 μM [6-¹⁴C]-5-FU, 0.1 mM NADPH, and 25 μl of the enzyme extract in a total volume of 50 μl was incubated at 37°C for 30 min. The DPD activity was determined as the sum of the products formed from 5-FU: Dihydrofluorouracil (DHFU), 2-fluoro-β-ureidopropionic acid, and 2-fluoro-β-alanine. After the addition of 25 μl 0.36 mM KOH, the reaction mixture was allowed to stand at room temperature for at least 30 min to hydrolyze the DHFU formed, and was then neutralized with 25 μl 0.36 mM HClO₄ and immediately centrifuged at 14,000 rpm for 10 min. A 5 μl aliquot of the supernatant was applied to a silica gel 60F254 plate (2.5x20 cm, Merck, Darmstadt, Germany), and developed with a mixture of methanol and 1 M ammonium acetate (5:1, v/v). Each product was visualized and quantified using an imaging analyzer (BAS-2000, Fujix, Tokyo).

Statistics. The Chi-square test and Student's t-test were used for comparison of the patients' clinicopathological backgrounds. The correlation between enzyme activity and clinicopathological factors was examined using Pearson's correlation analysis. A p-value of <0.05 was considered to be statistically significant.

Results

The background data of the 3 groups are summarized in Table I. The age of the UC group was significantly higher than the control group. Although the tumor size in the UC

Table II. Objective and pathological responses.

	Objective response (RECIST)	
	UFT	UFT+CPA
% inhibition (mean ±SD)	12.8±8.9	11.2±13
CR or PR	0	1
SD	8	7
PD	0	1
Not evaluated	2	0
Total	10	9
Pathological response		
Grade		
1b	1	0
1a	7	8
0	2	1
Total	10	9

group was the largest among the 3 groups, there were no significant differences in tumor size or clinical stage among the 3 groups.

The ORs and pathological responses are summarized in Table II. NAC with UFT or UC resulted in an average 10% decrease in unidimensional tumor size, and there were no differences in the percentage decrease in tumor size between the two groups. One PR was observed in the UC group, and the changes in the MMG are shown in Fig. 1. The pathological responses were evaluated as 1a or 1b in most cases.

The TS and DPD activity is summarized in Fig. 2. The TS activity was highest in the control group, followed by the UFT and UC groups, and the TS activity in the UC group was significantly lower than that in the control group (p=0.0230). In contrast, the DPD activity was highest in the UFT group, followed by the control and UC group, and the DPD activity in the UC group was significantly lower than that in the UFT group (p=0.0355).

The correlations between the TS or DPD activity with the clinicopathological factors are summarized in Table III. The DPD activity was inversely correlated with the tumor size and pT, and these correlations reached statistical significance (p=0.0108 and 0.0051, respectively). The DPD activity was also inversely correlated with ER expression, but this was only a statistical trend (p=0.086). In contrast, the TS activity was not correlated with these clinicopathological factors. Furthermore, multiple regression analyses indicated that the total dose of UFT administered and the administration period were significant variables for the TS activity, and that the total dose of CPA administered and the administration period, as well as pT, were significant variables for the DPD activity (Table IV).

Discussion

The present study did not aim to produce CRs or pCRs by NAC with UFT alone or the UC regimen. The OR in the present study was SD and the pathological responses were

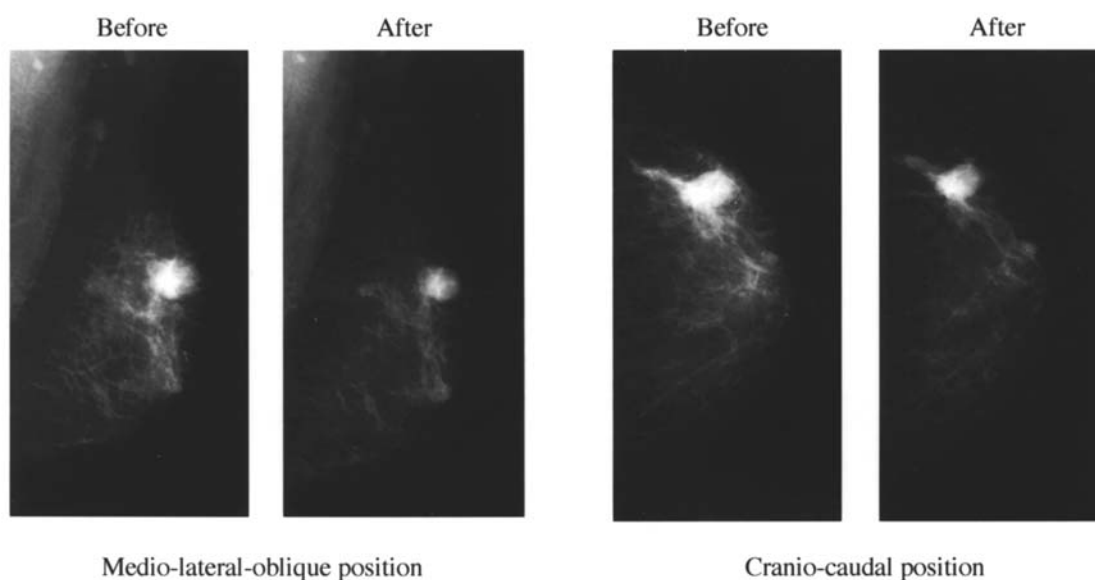


Figure 1. Comparative mammography before and after NAC with UFT+CPA in a patient with breast cancer (55-year-old female, invasive ductal carcinoma). The patient received UFT at 300 mg/day daily and CPA at 50 mg at one day intervals for 28 days. The tumor size decreased from 29 mm to 20 mm in longitudinal diameter (30% decrease), which was evaluated as a PR according to the RECIST guideline.

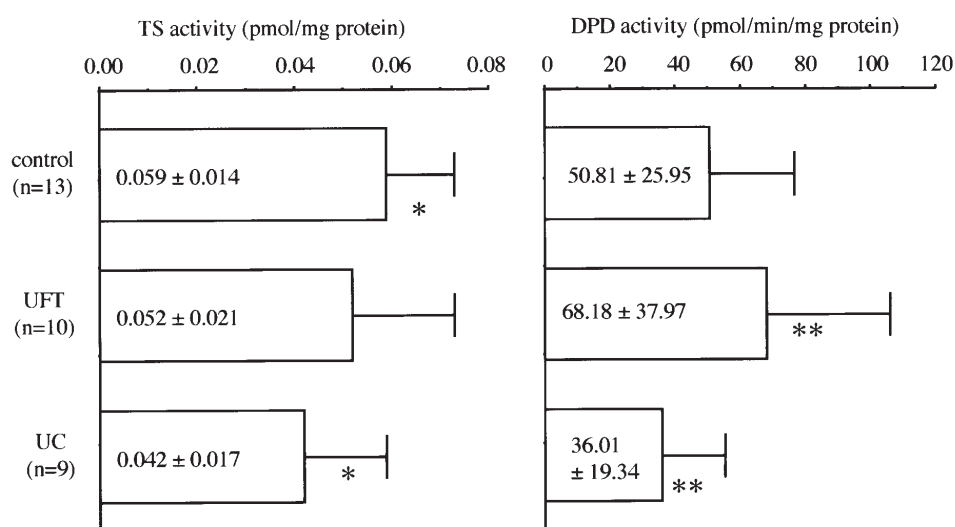


Figure 2. Comparative activities of TS and DPD after NAC with UFT or the UC regimen. The values represent means \pm SD. The cancer group included the control, UFT and UC groups. * $p=0.0230$, ** $p=0.0355$.

Table III. Correlation between TS or DPD activity and clinico-pathological factors.

Variables	TS (p-value)	DPD (p-value)
DPD	-0.078 (0.6729)	-
Age	-0.038 (0.8392)	0.087 (0.6387)
Clinical stage	-0.059 (0.7507)	-0.166 (0.3677)
Tumor size	-0.132 (0.4742)	-0.441 (0.0108)
pT	-0.080 (0.6666)	-0.478 (0.0051)
pN	-0.017 (0.9260)	0.082 (0.6575)
ER expression	0.040 (0.8310)	-0.327 (0.0674)

The values indicate r-values and the parentheses indicate p-values.

slight in most cases. These results suggest that, in the present study, the administration dose might be too low and the administration period might be too short to induce an obvious OR against breast cancer, because a previous study in gastric cancer demonstrated that NAC with oral UFT resulted in a 33.3% OR, and the mean totally administered dose of UFT was 7.67 g, respectively, and the mean administration period was 20.8 days (22). In the present study, the mean totally administered doses of UFT were 9.09 g in the UFT group and 8.28 g in the UC group, and the mean administration periods were 26.7 and 26.1 days, respectively (Table I). Accordingly, in order to induce an obvious OR in breast cancer, UFT should be administered in larger doses and for longer periods. The present study aimed to assess the changes in intratumoral TS or DPD activities after NAC, and

Variables	TS activity		DPD activity	
	r	p-value	r	p-value
Age	3.635×10^{-4}	0.1970	-0.117	0.7814
%ER	-3.535×10^{-5}	0.6788	-0.169	0.1697
Administration day	-0.002	0.0395	3.785	0.0301
UFT (total dose)	6.551×10^{-6}	0.0384	-0.009	0.0570
CPA (total dose)	-1.527×10^{-5}	0.2649	-0.048	0.0124
Tumor size	-0.002	0.4368	-6.109	0.0524
DPD activity	-1.719×10^{-5}	0.9004	-	-
TS activity	-	-	-37.187	0.9004

particularly to assess the effects of CPA. With respect to this issue, the present study demonstrated that UFT inhibited TS activity, but increased DPD activity. On the contrary, CPA augmented the inhibition of TS by UFT, and inhibited the DPD activity augmented by UFT. These results may clarify part of the mechanisms responsible for the combination effects of 5-FU derivatives and CPA.

TS is a key enzyme in the *de novo* pathway of pyrimidine synthesis, and is a very important target for chemotherapy with 5-FU or its derivatives. On the contrary, DPD degrades fluoropyrimidine to 2-fluoro- β -tetrahydrofolate (5,10- CH_2FH_2), and is the initial and rate-limiting enzyme in this process. Fluoropyrimidines are degraded by DPD mainly in the liver, but the DPD activity in the tumor is correlated with the clinical response or survival after 5-FU-based chemotherapies in colorectal, lung and breast cancers (23-26). The present study demonstrated that in human breast cancers, NAC with UFT inhibited the TS activity in the tumor, whereas CPA augmented the inhibitory effects of UFT on TS through inhibiting DPD activity in the tumor.

In chemotherapy protocols against breast cancer, the combination of 5-FU and CPA has been widely used with other anti-cancer agents, such as doxorubicin or methotrexate. Furthermore, in other malignancies, CPA has been reported to augment the anti-tumor activity of 5-FU or its derivatives including UFT, and we have also reported the usefulness of the UC regimen against digestive organ cancers such as pancreatic cancer and ampullary cancer of the papilla of Vater (7-9). Several previous clinical reports have also suggested that this combination regimen of UFT+CPA (UC) showed anti-tumor activity against breast cancer, sometimes in combination with anthracyclines (5,6). In addition, it has been reported that CPA augments the activity of RNR, which metabolizes 5-FU to fluoro-deoxyuridine monophosphate (FdUMP) in a human breast cancer xenograft line, and this may represent one of the mechanisms responsible for the beneficial effects of CPA on the anti-tumor activity of UFT (10). Other researchers have also reported that CPA enhanced the efficacy of capecitabine and 5'-deoxy-fluorouridine, which are also 5-FU derivatives, by inducing TP in a human breast tumor xenograft model (11). Therefore,

CPA can modulate the activity of various enzymes, which are associated with pyrimidine metabolism. Accordingly, the present study reports for the first time that CPA inhibits the intratumoral activity of DPD, resulting in increased anti-tumor activity for UFT.

TS also plays a very important role in tumor growth, but previous studies have reported conflicting results with respect to the clinicopathological significance of TS. Some reports have indicated that the overexpression of TS in a tumor is a significant indicator for a poor prognosis in patients with colorectal cancer (27,28), ovarian cancer (29), and gastric cancer (30-33), whereas others have reported that a high TS activity is not always associated with a poor prognosis in colon cancer (34), breast cancer (35) and gastric cancer (36). In breast cancer, it was reported that high TS levels were correlated with advanced disease stages and nodal involvement (37), but other reports have indicated no correlation (38,39). Furthermore, it was reported that a high TS activity was associated with a slow disease progression (35), although another study reported that extremely high TS levels were accompanied by an unfavorable prognosis (40). In the present study, TS activity did not show any correlation with tumor size or clinical stage. Therefore, correlations between the level of TS expression and the clinicopathological factors or prognosis remain unclear.

As discussed above, TS is the main target of 5-FU or its derivatives. After 5-FU is metabolized to fluoro-deoxyuridine monophosphate (FdUMP), FdUMP forms a ternary complex with TS and folic acid, resulting in an inhibition of TS activity. With regard to the role of TS in the efficacy of 5-FU based chemotherapy, although early studies indicate that the TS inhibition was significantly correlated with the response to 5-FU (19,41), the effects of the TS level on the efficacy of 5-FU or its derivatives-based chemotherapy is unclear. Many studies indicate that the low TS levels in the tumor are an indicator for favorable response to 5-FU-based chemotherapy in colorectal or gastric cancer (30,42-44), whereas other reports indicate that the benefits of 5-FU-based chemotherapy are most evident in the high TS group in colorectal (27,28) and breast cancer patients (35,38), or that the TS levels have no effect on the efficacy of 5-FU-based

chemotherapy in gastric (32,33) and colorectal cancers (45). Therefore, this issue remains controversial.

The DPD activity increased by UFT can be inhibited by CPA. This may be one of the possible mechanisms responsible for the enhanced anti-tumor activity of 5-FU or its derivatives induced by CPA.

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