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*Genome Informatics 14: 472–473 (2003)*

# Analysis of 3'/5' Ratio of Actin and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)

**Yasuo Oshima** **Akio Fujimura**
oshima@jichi.ac.jp akiofuji@jichi.ac.jp
Jichi Medical School, 3311 Yakushiji, Minamikawachimachi, Kawachigun, Tochigi 329-0498, Japan

**Keywords:** clinical sample, quality control, microarray

## 1 Introduction

The GeneChip system (Affymetrix) utilizes polyadenylation complementary oligonucleotides as a primer for labeling of ribonucleic acid (RNA). The polyadenylation usually locates at a downstream of the 3' end of transcripts. When RNA is somehow degenerated, 5' moiety of a transcript will not be well labeled. On the other hand, its 3' moiety may relatively well be labeled even though RNA is degenerated. Thus 3'/5' ratio of housekeeping genes may represent RNA quality. In the previous study, we experienced difficulties in confirming the microarray data by real-time polymerase chain reaction. During the analysis of this problem using 3'/5' ratio of the housekeeping genes, we found suggestive and general concern which should be considered before an analysis of microarray data using any clinical samples.

## 2 Methods and Results

### 2.1 Materials and Methods

Samples in previous study were obtained and treated as reported before [1]. In brief, bone marrow cells from acute myeloid leukemia patients were collected, purified to mononuclear cells using a density-gradient centrifugation and then purified to CD-133 anigen-positive cells. The microscopic observation and flowcytometoric analysis with CD-133 antibody staining confirmed more than 95% cells were cells with leukemia blasts like morphology. Since an analysis using purified cells should generate background matched results, we call it BAMP (back ground matched population) screening. The RNA from the CD-133 antigen-positive cells were purified immediately or after storage in a deep freezer (approx. -70°C). Since the RNA amount obtained were not enough to analyze, we amplified the RNA using T7-RNA polymerase. Clinical samples in recent study were obtained from surgical operation. Renal and hepatic tissues were treated with dispase and trypsin. Resulting cells were cultured for a few weeks before purification of RNA. The culture is intended to recover cell viability from some kinds of injury during the cell purification. RNA labeling and scanning were performed using GeneChip system (Affymettrix) as manufacture's instructions. In the previous study with BAMP screening and the recent study with cell viability recovery (CVR), we utilized HG-U95 and HG-U133 GeneChip, respectively. If available, we also obtained data from test chip (Affymetrix) in both studies. Since both GeneChip can yield 3'/5' ratio of actin and GAPDH, we compared these data.

### 2.2 Results

#### 2.2.1 Location of Probes

Figures 1 and 2 are potential secondary structures of human actin and human GAPDH RNA, respectively. Both transpcipts are supporsed to have polyadenylation at their 3' ends where oligo dT primer for RNA labeling are adhere. The 5'-, medium and 3'-probes of actin are designed to adhere to the position 81 615, 675 1204 and 1232 1769, respectively. Similarly the 5'-, medium and 3'-probes of GAPDH are designed to detect the position 101 401, 507 846 and 922 1270, respectively.

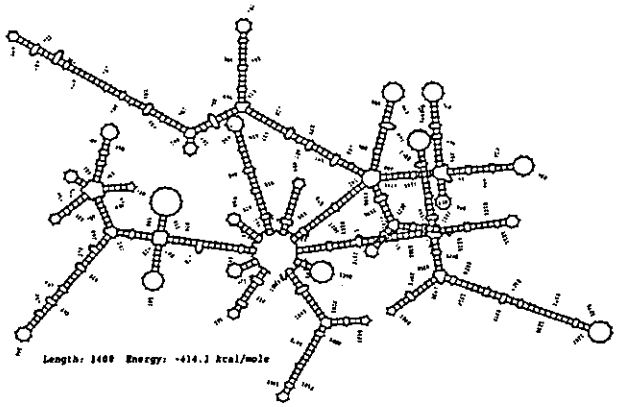

Figure 1: Human actin RNA.

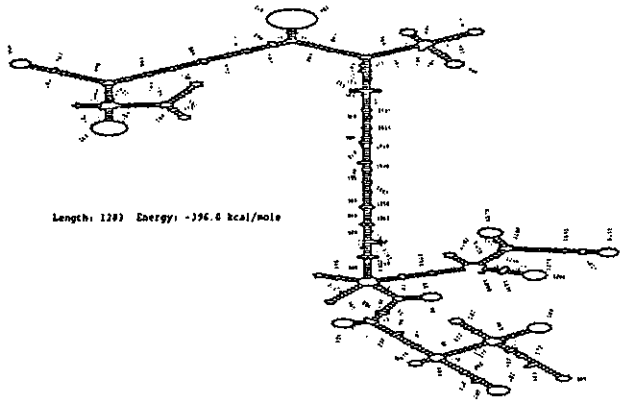

Figure 2: Human GAPDH RNA.

### 2.2.2 Descriptive Statistical Summary

Tables 1 and 2 are the descriptive results of BAMP screened and CVR samples, respectively. The 3'/5' ratio of transcripts in cell lines and model animals, whose RNA are prepared in timely manner, usually fall between 1.0 and 3.0 though they should ideally be 1.0.

Table 1: BAMP screened samples.

| | GAPDH | Actin |
|---|---|---|
| Number | 30 | 30 |
| Average | 57.07 | 23.89 |
| Standard Deviation | 10.42 | 4.362 |
| Maximum | 243.8 | 132.8 |
| Minimum | 3.49 | 0.91 |

Table 2: CVR samples.

| | GAPDH | Actin |
|---|---|---|
| Number | 60 | 60 |
| Average | 1.559 | 1.662 |
| Standard Deviation | 0.388 | 1.094 |
| Maximum | 2.32 | 2.23 |
| Minimum | 0.68 | 1.08 |

## 3 Discussion

In clinical situation, medical doctors are required to perform their clinical duty first, even if samples should be treated immediately. Moreover, since previous BAMP screened samples were the collection of multicenter derived cells, they usually experienced a few-hours warm ischemia before beginning of cell purification procedures which also took a few hours. At that time, we confirmed cell quality using microscopic observation of their morphology. The analysis of BAMP screened samples with GeneChip indicated low RNA quality, although their morphology showed great quality. Based on these observations, we hypothesized that the RNA were degenerated during a sample purification. Moreover, our RNA amplification procedure utilize polyadenylation complementary primer, the amplification could enhance 3'-moiety of transcripts compared to 5'-moiety. To resolve this problem, we reduced warm ischemic time in the recent study and recovered cell viability by culturing for a few weeks. In this study, we presented that the new procedure led to great RNA quality by GeneChip. We note that as a nature of clinical human samples, their RNA may be degenerated during sample treatment especially artificial amplification of RNA and long warm ischemia, and their sample quality may not meet our needs, though scientists make efforts to maintain their research quality. Those who analyze clinical sample data should be careful for their sample quality. When the samples are analyzed for their mRNA expression, sample quality should be monitored by their expression data.

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P-1

# Primary cell preraration and genome-wide gene expression analysis of human renal tubular cells

Yasuo Oshima[¶]*, Shinsuke Kurokawa[§], Akihiko Tokue[§], Hiroyuki Mano[‡], Ken Saito[†], Makoto Suzuki[¶], Masashi Imai[¶], Akio Fujimura[¶]

Departments of [¶]Pharmacology, [§]Urology and [†]Pathology, [‡]Division of Functional Genomics, Jichi Medical School, Tochigi, 329-0498 Japan

Background

Toxicological experiments using model animals and immortalized cell lines have provided a lot of useful information. Since the availability of human samples are limited, methods, which distinguish real toxicological events in human subjects from just artifacts generated under the artificial conditions or differences between species, are desired. As a practical approach to this issue, we are conducting exposure experiments to chemical agents and gene expression analysis using human primarily cultured cells and GeneChip. Gene expression analysis using DNA microarray is getting more and more popular tool for clinical and biomedical researches. For performing experiments with the new technology, adequate sample preparation is essential. Even if samples are prepared according to previously reported method, it is important to evaluate whether samples are suitable for such technology.

Methods

In this study, we prepared cell culture using surgically resected human kidney. They were treated with dispase, trypsin and sodium ethylenediamine tetraacetate and then, cultured for a few weeks. We analyzed these primarily cultured cells by GeneChip.

Results

We suceeded to purify human cortical cells. In addition, we found their origin as proximal renal tubule by GeneChip analysis. We also compared the gene expressions of the cells before and after cryopreservation. Selected 567 housekeeping genes were analyzed, and their Pearson's correlation coefficients and p-value were 0.980 and less

than 0.0001, respectively.

Conclusions

These results suggest that we can obtain adequate primary cell culture of renal tublar origin. In addition, essential gene expressions of these cells might not be significantly influenced by cryopreservation.

Oncology

Laboratory/Clinical Translational Research

Oncology 2003;65:125–131
DOI: 10.1159/000072337

# Quantitative Analysis of Thymidine Phosphorylase and Dihydropyrimidine Dehydrogenase in Renal Cell Carcinoma

Tatsuo Morita Atsushi Matsuzaki Akihiko Tokue

Department of Urology, Jichi Medical School, Tochigi, Japan

**Key Words**
Dihydropyrimidine dehydrogenase · Fluoropyrimidines · Prognosis · Renal cell carcinoma · Thymidine phosphorylase

**Abstract**
***Objective:*** The purpose of the present study was to clarify the clinicopathological significance of both thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD) in renal cell carcinoma (RCC) based on a quantitative analysis of RCC patients. ***Methods:*** Levels of TP and DPD in RCC and/or uninvolved renal tissues from 65 RCC patients were measured by enzyme-linked immunosorbent assay. ***Results:*** The TP level and TP/DPD ratio were significantly higher in RCC than in adjacent uninvolved renal tissues ($p < 0.0001$). There was no significant difference in DPD levels between RCC and uninvolved renal tissues. The ratio of the highest to the lowest level was 623 in TP level, 28.9 in DPD level, and 985 in TP/DPD ratio. In the univariate analysis, patient's age ($p = 0.04$), tumor stage ($p < 0.0001$), tumor size ($p = 0.007$), TP expression ($p = 0.03$), and DPD expression ($p = 0.04$) were significantly associated with increased risk of death. Multivariate analysis showed that patient's age, tumor stage, and TP expression were independent prognostic factors. ***Conclusions:*** TP and DPD in RCC provide prognostic information although DPD was not an independent prognostic factor. The present finding of a wide range in these enzyme expressions in RCC suggests that a certain subpopulation with a high TP/DPD ratio has potential responsiveness to fluoropyrimidines, especially 5′-deoxy-5-fluorouridine and capecitabine.



## Introduction

Monotherapy with hormones, anticancer drugs, interleukin 2 (IL2) or interferon (IFN) achieves responses in 10–20% of renal cell carcinoma (RCC) patients [1–3]. Combination of 5-fluorouracil (5-FU) and IFN with or without IL2 given in various schedules showed higher response rates of 30–48.6% [4–9] as compared to monotherapy although conclusive evaluation of the efficacy awaits further studies in randomized trials. Recently, combination of 5-FU or capecitabine with gemcitabine has significant anticancer activity against RCC [10, 11]. These reports suggest that a certain subpopulation of RCC patients might respond to fluoropyrimidine-containing regimens. Thus, it seems of value to identify the subpopulation who may respond to fluoropyrimidines or fluoropyrimidine-containing regimens in terms of tailor-made therapy for RCC patients.

Anticancer efficacy of fluoropyrimidines such as 5-FU [12], 5′-deoxy-5-fluorouridine (5′-DFUR) [13] and capecitabine [14, 15] primarily depends on expression levels of various enzymes in metabolic pathways of pyrimidine

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Tatsuo Morita, MD
Department of Urology, Jichi Medical School
Minamikawachi-machi, Kawachi-gun
Tochigi 3290498 (Japan)
Tel. +81 285 58 7379, Fax +81 285 40 6595, E-Mail moritatu@jichi.ac.jp

**Table 1.** Association of expression levels of TP and DPD and the TP/DPD ratio in RCC with clinicopathological factors

| Factor | Patients | TP | | | DPD | | | TP/DPD ratio | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | high | low | p | high | low | p | high | low | p |
| *Age* | | | | | | | | | | |
| ≤65 years | 43 | 21 | 22 | 0.663 | 20 | 23 | 0.337 | 22 | 21 | 0.929 |
| >65 years | 22 | 12 | 10 | | 13 | 9 | | 11 | 11 | |
| *Gender* | | | | | | | | | | |
| Male | 47 | 24 | | 0.938 | 26 | 21 | 0.235 | 24 | 23 | 0.938 |
| Female | 18 | 9 | 9 | | 7 | 11 | | 9 | 9 | |
| *Tumor stage* | | | | | | | | | | |
| I + II | 55 | 27 | 28 | 0.525 | 25 | 30 | 0.044 | 29 | 26 | 0.459 |
| III + IV | 10 | 6 | 4 | | 8 | 2 | | 4 | 6 | |
| *Tumor grade* | | | | | | | | | | |
| G1 | 22 | 8 | 14 | 0.096 | 11 | 1 | 0.929 | 9 | 13 | 0.255 |
| G2 + G3 | 43 | 25 | 18 | | 22 | 21 | | 24 | 19 | |
| *Tumor size* | | | | | | | | | | |
| <5 cm | 41 | 16 | 25 | 0.013 | 16 | 25 | 0.013 | 22 | 19 | 0.542 |
| ≥5 cm | 24 | 17 | 7 | | 17 | 7 | | 11 | 13 | |

The $\chi^2$ test was employed for statistical analysis. The numbers of the patients are listed. Tumor stage was classified according to Robson.

nucleosides which exist in both normal cells and cancer cells. Thymidine phosphorylase (TP) is a key enzyme for the anabolic pathway of 5-FU prodrugs such as 5′-DFUR and capecitabine and converts them to 5-FU and to fluorodeoxyuridine which is further converted to fluorodeoxyuridine monophosphate by thymidine kinase. Fluorodeoxyuridine monophosphate binds to both 5, 10-methylenetetrahydrofolate ($CH_2THF$) and thymidylate synthase to form a ternary complex which inhibits thymidylate synthase activity, resulting in the inhibition of DNA synthesis. TP plays important roles not only in nucleic acid metabolism, but also in angiogenesis [16, 17] because TP is an angiogenic factor identical to platelet-derived endothelial cell growth factor [18]. On the other hand, dihydropyrimidine dehydrogenase (DPD), which is predominantly distributed in the liver, is the first and rate-limiting enzyme in the catabolic pathway of 5-FU with regulation of the availability of 5-FU for anabolism [19], potentially determining the resultant anticancer efficacy and/or toxicity of 5-FU. Several clinical studies demonstrated that TP and DPD activities in tumor cells were related to the responsiveness to fluoropyrimidines [20–25]. Clinicopathological significance of TP and/or DPD in tumor tissues has been also reported in various kinds of cancers [26]. With respect to RCC, we [27] and Imazono et al. [28] examined TP expression in RCC by immunohistochemistry, and showed that TP is an unfavorable independent prognostic factor in RCC patients. However, clinicopathological evaluation of both TP and DPD in RCC based on the quantitative analysis has not been reported in the literature. The purpose of the present study was to clarify the clinicopathological significance of both TP and DPD in tumor tissues based on the quantitative analysis to predict not only biologic behaviors of RCC but also potential responsiveness to the fluoropyrimidines in RCC patients. In the present study, we measured quantitatively the expression levels of TP and DPD using enzyme-linked immunosorbent assay (ELISA) in specimens from RCC and adjacent uninvolved renal tissues, and examined their association with clinicopathological factors and prognosis in RCC patients.

## Patients and Methods

*Patients*

Sixty-five patients with RCC who underwent radical nephrectomy were entered into the study. Detailed characteristics of the patients are shown in table 1. Tumor stage and tumor grade were

**Table 2.** Levels of TP and DPD, and TP/DPD ratio in RCC and adjacent uninvolved renal tissues

| Factor | RCC (n = 65) | Uninvolved renal tissues (n = 50) | p value |
|---|---|---|---|
| *TP, U/mg protein* | | | |
| Mean ± SD | 97.5 ± 98[b] | 10.2 ± 6.9 | <0.0001 |
| Median (range) | 79.3[c] (1–623)[d] | 7.5 (2.7–36.7) | |
| Ratio[a] | 623[e] | 13.6 | |
| *DPD, U/mg protein* | | | |
| Mean ± SD | 32.0 ± 26 | 25.3 ± 12 | 0.572 |
| Median (range) | 23.4 (4.4–127) | 22.3 (13–72) | |
| Ratio[a] | 28.9 | 5.5 | |
| *TP/DPD ratio* | | | |
| Mean ± SD | 4.0 ± 5.2 | 0.4 ± 0.2 | <0.0001 |
| Median (range) | 2.9 (0.04–39.4) | 0.4 (0.07–0.88) | |
| Ratio[a] | 985 | 12.6 | |

[b] Wilcoxon signed rank test.
[a] Ratio of the highest to the lowest level.

defined according to the classification of Robson et al. [29] and the TNM classification system [30], respectively. Treatments consist of radical nephrectomy and adjuvant IFN therapy. No patient received any therapy before radical nephrectomy. The mean follow-up period was 42.5 months (range, 12.3–88.5). For data analysis, patients were classified into two groups in each factor as shown in table 1. Surgical specimens of RCC and adjacent uninvolved renal tissues were removed during the operation, frozen and stored at –80 °C.

*ELISA for TP and DPD*

Specimens were homogenized in 10 m*M* Tris buffer (pH 7.4) containing 15 m*M* NaCl, 1.5 m*M* $MgCl_2$, and 50 μ*M* potassium phosphate with a glass homogenizer. The homogenate was then centrifuged at 10,000 *g* for 15 min at 4 °C, and the supernatants were frozen and stored at –80 °C until analyzed. The protein levels were determined by the DC protein assay™ kit (Bio-Rad). The assays for TP and DPD were performed by sandwich ELISAs as described elsewhere [31, 32]. Briefly, samples and the serially diluted standard were dispensed into anti-TP monoclonal antibody (MoAb) 104B- or anti-DPD MoAb 4B9-coated microplate wells. The plates were incubated at 37 °C for 1 h and then washed with 0.05% Tween 20 in 10 m*M* phosphate-buffered solution (pH 7.6), incubated with anti-TP MoAb 232-2 or anti-DPD MoAb 3A5 at 37 °C for 1 h and washed. Incubation was also performed with horseradish peroxidase conjugated-goat anti-mouse IgG for TP assay or horseradish peroxidase conjugated-rat anti-mouse IgM for DPD assay. A substrate reaction was performed with a substrate solution of the TMB substrate system™ (KPL, Gaithersburg, Md., USA). After terminating the peroxide reaction with 1 *M* phosphate solution, absorbance was measured at 450 nm, and the enzyme level was calibrated with that measured for the standard solution, and expressed as U/mg protein where one unit was defined as the TP level that phosphorolyzes 5′-DFUR to 5-FU at the rate of 1 μg 5-FU/h [31], and the DPD level that catabolyzes 1 pmol of 5-FU/min [32].

*Statistics*

The $\chi^2$ test, Wilcoxon's signed rank test, and Pearson's correlation analysis were used for statistical analysis. Survival times were measured from the date of radical nephrectomy. Survival curves were calculated by the Kaplan-Meier method and compared by log-rank test. The effect of different factors on the survival was studied in multivariate analysis using the Cox proportional hazards model. The TP/DPD ratio was calculated and used for combined analysis of the expression status of TP and DPD. Median values of TP and DPD levels and the TP/DPD ratio were used as a cutoff point to divide the patients into two groups for statistical analysis. The expression levels greater than the median value were regarded as 'high' and those less than the median value were regarded as 'low'. $p < 0.05$ was considered statistically significant.

## Results

### *Levels of TP and DPD, TP/DPD Ratio in RCC and Adjacent Uninvolved Renal Tissues*

As shown in table 2, TP level and the TP/DPD ratio were significantly higher in RCC than in adjacent uninvolved renal tissues ($p < 0.0001$). There was no significant difference in DPD levels between RCC and adjacent uninvolved renal tissues. The same statistical results were obtained when the 50 paired samples were analyzed (data not shown). The median values of TP and DPD levels and the TP/DPD ratio were, respectively, 10.6-fold, 1.1-fold and 7.3-fold higher than those in adjacent uninvolved renal tissues. The ranges of TP and DPD levels and the TP/DPD ratio in RCC were much wider than those in adjacent uninvolved renal tissues. Furthermore, TP and DPD levels and the TP/DPD ratio varied considerably among patients in RCC. The ratio of the highest to the lowest level in RCC was 623 in TP expression, 28.9 in DPD expression, and 985 in the TP/DPD ratio. As shown in table 3, TP expression correlated significantly with DPD expression ($p = 0.0249$) and the TP/DPD ratio ($p < 0.0001$) in RCC while there was no significant correlation between DPD expression and the TP/DPD ratio in RCC.

### *Association of Expression Levels of TP and DPD and the TP/DPD Ratio in RCC with Clinicopathological Factors*

Associations of expression levels of TP and DPD and the TP/DPD ratio in RCC with clinicopathological factors is shown in table 1. For this analysis, enzyme expression levels were divided into two groups ('high' and 'low'). A significant association was found between TP expression and tumor size ($p = 0.013$). On the other hand, DPD expression was significantly associated with tumor stage ($p = 0.044$) and tumor size ($p = 0.013$).

Thymidine Phosphorylase/
Dihydropyrimidine Dehydrogenase

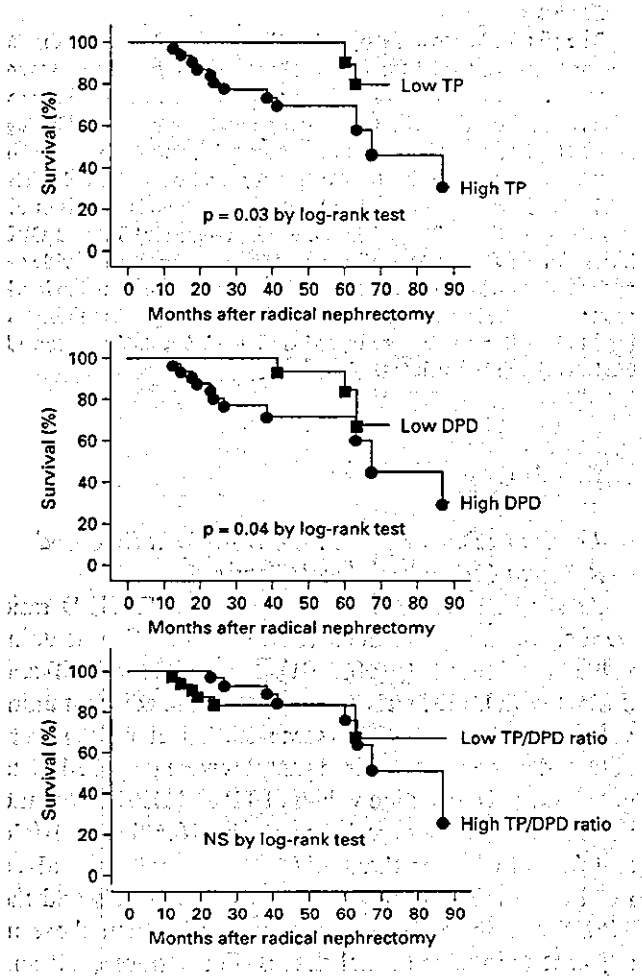


**Fig. 1.** Kaplan-Meier survival curves according to the expression levels of TP and DPD and the TP/DPD ratio in RCC. Median values of TP and DPD levels and the TP/DPD ratio were used as a cutoff point to divide the patients into two groups for statistical analysis. The expression levels greater than the median value were regarded as 'high' and those below the median value were regarded as 'low'.

**Table 3.** Correlation between TP and DPD levels and TP/DPD ratio in RCC (n = 65)

| | TP | DPD |
|---|---|---|
| TP | – | – |
| DPD | r = 0.277<br>(p = 0.0249) | – |
| TP/DPD ratio | r = 0.749<br>(p < 0.0001) | r = –0.231<br>(p = 0.064) |

Pearson's correlation analysis.

*Prognosis*

Figure 1 shows Kaplan-Meier survival curves according to the expression levels of TP and DPD and the TP/DPD ratio in RCC. Patients with high TP expression had significantly poorer survival compared with those with low TP expression (p = 0.03). The same applied to DPD expression (p = 0.04) but not to the TP/DPD ratio. Additionally, patient's age (p = 0.04), tumor stage (p < 0.0001), and tumor size (p = 0.007) were also significantly associated with increased risk of death by univariate analysis (table 4). In the Cox multivariate regression analysis, patient's age, tumor stage and TP expression were unfavorable independent prognostic factors while DPD expression or the TP/DPD ratio was not an independent prognostic factor (table 4).

## Discussion

The present study with the use of ELISA showed that the expression level of TP in RCC was significantly higher than that in adjacent uninvolved renal tissues while there was no significant difference in DPD expression levels between RCC and adjacent uninvolved renal tissues. The expression levels of TP and DPD obtained by ELISA have been reported to correlate well with TP and DPD activities measured by conventional enzyme activity assays, respectively [31, 32]. Higher TP expression in tumor tissues than in adjacent non-tumorous tissues has been reported in various kinds of cancers, as reviewed elsewhere [26]. Imazono et al. [28] examined TP activity in 18 RCCs and 19 non-neoplastic renal tissue specimens, and reported that the median value of TP activity in RCC was ninefold higher than in non-neoplastic renal tissues, in agreement with the present study. On the other hand, the DPD expression level in RCC has not been compared with that in adjacent uninvolved renal tissues. Higher DPD activity in tumor tissues than in adjacent non-tumorous tissues has been demonstrated in gastric [33] and pancreatic cancer [34]. However, other studies have found DPD expression in hepatocellular cancer [35] and epithelial ovarian cancer [36] to be significantly lower than that in adjacent non-tumorous tissues. Etienne et al. [23] reported no significant difference in DPD activity between uninvolved tissues and tumor tissues in patients with head-and-neck cancer, in agreement with the present study in RCC. Regarding the TP/DPD ratio, Fujiwaki et al. [36] examined TP and DPD expression by reverse transcription and polymerase chain reaction in epithelial ovarian cancer, and showed that the TP/DPD ratio was

**Table 4.** Univariate and multivariate analyses of prognostic factors in 65 RCC patients

| Factor | | Univariate[a] | Multivariate[b] | | |
|---|---|---|---|---|---|
| | | p | relative risk | 95% CI | p |
| Age (years) | ≤65 vs. >65 | 0.04 | 27.3 | 2.83–263.1 | 0.004 |
| Gender | Male vs. female | 0.87 | 1.1 | 0.233–4.93 | 0.928 |
| Tumor stage[c] | I + II vs. III + IV | <0.0001 | 63.1 | 6.56–606.9 | 0.0003 |
| Tumor grade | G1 vs. G2 + G3 | 0.14 | 1.18 | 0.16–8.81 | 0.871 |
| Tumor size (cm) | ≤5 vs. >5 | 0.007 | 2.66 | 0.16–43.6 | 0.494 |
| TP | Low vs. high | 0.03 | 40 | 1.99–802.3 | 0.016 |
| DPD | Low vs. high | 0.04 | 1.2 | 0.12–12.2 | 0.876 |
| TP/DPD ratio | Low vs. high | 0.86 | 0.21 | 0.03–1.32 | 0.095 |

TP = Thymidine phosphorylase; DPD = dihydropyrimidine dehydrogenase; RCC = renal cell carcinoma.
[a] log-rank test.
[b] Cox proportional hazards model.
[c] Robson's classification.

significantly higher in epithelial ovarian cancer specimens than in normal ovary specimens. The discrepancy among the reports might be ascribed to the differences in the histological types of cancer, the numbers of patients examined, assays for the enzyme expression, and/or evaluation of immunohistochemistry results. Correlation between TP and DPD in tumor tissues has been examined in various kinds of solid cancers by Mori et al. [32]. They reported a significant correlation between TP and DPD in colorectal, pancreatic, esophageal, bladder, cervical, hepatic and gastric cancer, but not in RCC. The present study on 65 RCC patients showed a significant positive correlation between TP and DPD in RCC. Although the mechanisms of regulation of TP and DPD expression remain unclear, our present results suggest that TP and DPD expression might be regulated by common factors present in the microenvironment of RCC.

We [27] and Imazono et al. [28] examined TP expression in RCC by immunohistochemistry and showed that TP expression in RCC was an unfavorable independent prognostic factor in RCC patients. The present results obtained by ELISA were in accord with those obtained by immunohistochemistry. However, in RCC, the prognostic significance of DPD expression has not been investigated in the literature. The present study showed that elevated DPD expression was significantly associated with poor survival by univariate analysis although the prognostic significance of DPD expression was not evident by multivariate analysis, probably because of the strong correlation between TP and DPD expression. In gastric cancer [33] and epithelial ovarian cancer [36], tumoral DPD expression had no influence on prognosis. Although the biological role of DPD remains unclear in tumor progression, TP plays important roles not only in angiogenesis but also in apoptosis inhibition [26]. Taken together, the present finding that TP and DPD expression in RCC were associated with tumor size and/or tumor stage, and poor prognosis suggests that elevated expression of these enzymes indicates a predisposition to aggressive RCC.

It is a reasonable strategy to target TP and DPD in the treatment of RCC with fluoropyrimidines since elevated TP and DPD expression was significantly associated with a poor prognosis in RCC, as described above. We have already reported that transfection of TP cDNA made the human RCC line KU2 susceptible to 5-FU and 5′-DFUR in vitro, and to 5′-DFUR and capecitabine in vivo [37]. Furthermore, we examined the biomodulation of 5-FU by IFN-α in RCC and reported that IFN-α upregulates TP expression and modulates 5-FU anabolism enhancing 5-FU cytotoxicity in RCC [38]. This biomodulation was more frequently observed in the cell line showing higher TP expression. In a human cancer xenograft model, Ishikawa et al. [39] reported that TP expression or the TP/DPD ratio in the tumors correlated well with the efficacy of 5′-DFUR and capecitabine. Furthermore, another study showed that DPD levels significantly correlated with 5-FU sensitivity, with high DPD activity and high DPD mRNA level resulting in a low sensitivity to 5-FU in a human cancer xenograft model [40]. In the clinical setting, several studies reported an association of TP or DPD expression in tumor tissues with the clinical outcome following treatment with fluoropyrimidines or fluoropyri-

midine-containing regimens [20–25]. This evidence suggests that TP and DPD would be useful parameters to predict the sensitivity of tumor cells to fluoropyrimidines, especially 5′-DFUR and capecitabine. However, expression levels of these enzymes show significant differences among cancer types and patients. Mori et al. [32] measured expression levels of TP and DPD by ELISA in 10 different human cancer tissues, and showed that the interpatient variance of tumor levels of TP, DPD, and TP/DPD were much larger than the variability among cancer types, suggesting that it would be difficult to predict the cancer type susceptible to fluoropyrimidines only on the basis of tumor levels of either TP, DPD, or TP/DPD. Furthermore, they showed that the interpatient variance in the TP/DPD ratio in RCC is highest among the 10 kinds of cancers examined: highest/lowest ratios for TP, DPD, and TP/DPD in RCC were 204, 25, and 293, respectively. Likewise, levels of TP, DPD, and TP/DPD varied widely in the present study. These conditions would present different clinical efficacies of fluoropyrimidines among the patients. Namely, certain subpopulations of RCC patients might respond well to fluoropyrimidines. In terms of tailor-made therapy, assessment of expression levels of these enzymes in RCC might be useful for selecting the RCC patients who are likely to respond to fluoropyrimidines. Conversely, it will be also important to avoid unnecessary therapies for patients who will not respond to fluoropyrimidines alone.

In conclusion, the present study showed that evaluation of the TP and DPD expression in RCC provides prognostic information on RCC patients although DPD was not an independent prognostic factor. The wide range of enzyme expression in RCC suggests that a certain subpopulation with a high TP/DPD ratio might respond to fluoropyrimidines, especially 5′-DFUR and capecitabine. In terms of tailor-made therapy, to correlate the expression levels of TP and DPD with the response to fluoropyrimidines or fluoropyrimidine-containing regimens in RCC patients, further studies are needed in order to examine whether or not measurement of expression levels of these enzymes in RCC is useful for selecting RCC patients who are likely to respond to fluoropyrimidines.


## Acknowledgment

The authors thank Nippon Roche KK (Tokyo, Japan) for providing part of the enzyme assays.


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Oncology

Laboratory/Clinical Translational Research

Oncology 2003;65:267–274
DOI: 10.1159/000074480

# Forced Expression of Cytidine Deaminase Confers Sensitivity to Capecitabine

Tatsuo Morita Atsushi Matsuzaki Shinsuke Kurokawa Akihiko Tokue

Department of Urology, Jichi Medical School, Tochigi, Japan

**Key Words**
Capecitabine · Cytidine deaminase · Gemcitabine · Sensitivity · Transfection

**Abstract**
***Objective:*** Cytidine deaminase (CDD) is involved in the metabolism of new pyrimidine analogues, capecitabine ($N^4$-pentyloxycarbonyl-5′-deoxy-5-fluorocytidine) and gemcitabine (2′,2′-difluorodeoxycytidine). The purpose of the present study was to directly examine the role of CDD in tumor cells themselves in mediating the sensitivity to capecitabine compared with gemcitabine. ***Methods:*** The human bladder cancer cell line T24 was transfected with human *CDD2* cDNA by the lipofectin method. ***Results:*** Transfection of *CDD2* cDNA did not change the levels of thymidine phosphorylase, dihydropyrimidine dehydrogenase and thymidylate synthase (TS) but increased the CDD activity significantly ($p < 0.01$). Forced expression of CDD made T24 sensitive to 5′-deoxy-5-fluorocytidine (5′DFCR) in vitro and capecitabine in vivo, but resistant to gemcitabine both in vitro and in vivo. Tetrahydrouridine, a specific CDD inhibitor, abrogated the changes in the in vitro sensitivity to 5′DFCR and gemcitabine by transfection of *CDD2* cDNA. Transfection of *CDD2* cDNA resulted in a significant increase in cellular 5-fluorouracil level ($p < 0.01$) and inhibition of TS activity ($p < 0.01$) after treatment with 5′DFCR in vitro. ***Conclusions:*** The present study clearly showed direct evidence for the contribution of CDD in tumor cells themselves to the sensitivities to capecitabine and gemcitabine.



## Introduction

Cytidine deaminase (CDD), which is an enzyme of the pyrimidine salvage pathway that catalyzes the deamination of cytidine and deoxycytidine to uridine and deoxyuridine, respectively, is also involved in the metabolism of capecitabine ($N^4$-pentyloxycarbonyl-5′-deoxy-5-fluorocytidine) [1] and gemcitabine (2′,2′-difluorodeoxycytidine: dFdC) [2]. Capecitabine, a novel fluoropyrimidine carbamate which mimics continuous 5-fluorouracil (5FU) infusion, has shown significant antitumor activity in a variety of solid cancers including breast cancer [3] and colorectal cancer [4]. Capecitabine given orally is first converted to 5′-deoxy-5-fluorocytidine (5′DFCR) by carboxylesterase mainly located in the liver, to 5′-deoxy-5-fluorouridine (5′DFUR) by CDD in the liver and tumors, and finally to 5FU and its active metabolites by thymidine phosphory-

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Dr. Tatsuo Morita
Department of Urology, Jichi Medical School
Minamikawachi-machi, Kawachi-gun
Tochigi 3290498 (Japan)
Tel./Fax +81 285 40 6595 (or +81 285 44 2495), E-Mail moritatu@jichi.ac.jp

lase (TP), which is identical to platelet-derived endothelial cell growth factor [5, 6]. Gemcitabine, another pyrimidine analogue, has shown significant antitumor activity in solid cancers including non-small cell lung cancer, pancreatic cancer, breast cancer, ovarian cancer, bladder cancer, and head-and-neck cancer [7–9]. Gemcitabine is inactive in the parental form but is first phosphorylated by deoxycytidine kinase and then converted to active forms of its di- and triphosphate metabolites by other kinases [10]. The former inhibits ribonucleotide reductase [11]; the latter is incorporated into actively replicating DNA, resulting in masked-chain termination [12] through self-potentiation [10, 13] whereby di- and triphosphate metabolites contribute to the reduction in cellular deoxycytidine triphosphate levels facilitating increased phosphorylation and decreased elimination of gemcitabine from the cell. On the other hand, gemcitabine is deaminated to 2′, 2′-difluorodeoxyuridine and inactivated by CDD [10, 14]. It has been reported that forced expression of CDD confers resistance to gemcitabine in the human lymphoid leukemic cell line CCFR-CEM [15] but not in the human colon cancer cell line HCT116 [16]. However, there has been no report on the role of CDD in tumor cells themselves with respect to the sensitivity to capecitabine.

The purpose of the present study was to directly elucidate the role of CDD in tumor cells themselves in mediating the sensitivity to capecitabine as compared with gemcitabine. In the present study, we examined whether forced expression of CDD by the transfection of CDD gene changes the cellular sensitivity to capecitabine and gemcitabine.

## Materials and Methods

*Animals and Cell Line*

Six-week-old male Balb/c nu/nu mice were purchased from CLEA Japan (Tokyo, Japan). The human bladder cell line T24, which has endogenous CDD activity <0.013 nmol/mg protein/min [16, 17] and is reportedly refractory to capecitabine [18], was supplied by the Japanese Center Research Resources Bank and maintained in vitro in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum and 2 m*M* *L*-glutamine at 37°C in a 5% $CO_2$ incubator.

*Reagents*

5′DFUR, 5′DFCR, and capecitabine were supplied by Nippon Roche K.K. (Tokyo, Japan). 5FU and gemcitabine were provided by Kyowa Hakko Kogyo (Tokyo, Japan) and Eli Lilly (Indianapolis, Ind., USA), respectively. Tetrahydrouridine (THU) was purchased from Calbiochem-Novabiochem (La Jolla, Calif., USA).

*Subcloning of Human* CDD2 *cDNA*

Human *CDD2* cDNA in PGEX4TI containing human *CDD2* cDNA, which was kindly provided by Drs. J. Schröder and S. Secber (Department of Internal Medicine, University of Essen Medical School, Germany), was ligated into the *Bam*H1/*Xho*1 site in the multiple cloning site of the plasmid pcDNA3.1/hygro(+) (Invitrogen, San Diego, Calif., USA). CDD2 is one of the natural variants of CDD and has higher deamination activity than CDD1 [19]. The constructed plasmid pcDNA3.1/hygro(+)/*CDD2* was transformed into competent cells (*Escherichia coli*, DH5α), and the plasmid DNA was purified with MagExtractor™ MFX-2000 (Toyobo, Tokyo, Japan). The correct orientation of the *CDD2* cDNA in the multiple cloning site of pcDNA3.1/hygro(+) was confirmed by restriction enzyme digestion.

*Transfection*

The pcDNA3.1/hygro(+) with or without human *CDD2* cDNA was transfected by the lipofectin method with Lipofectin™ reagent (Invitrogen Co., Carlsbad, Calif., USA) into T24. Hygromycin™-resistant clones were randomly selected from the surviving colonies.

*CDD Assay*

CDD activity was measured by the method reported by Eda et al. [16]. Briefly, cells were sonicated and centrifuged at 105,000 *g* for 90 min. The supernatants were dialyzed overnight at 4°C. The protein concentration was determined by the method of Lowry et al. [20]. CDD activity in the dialyzed supernatants was determined by measuring 5′-dFUrd and 5FU generated from the enzyme substrate 5′-deoxy-5-fluorocytidine using a high-performance liquid chromatography column and expressed as nanomoles 5′-deoxy-5-fluorocytidine 5′-dFCyd deaminated per minute per milligram protein.

*Reverse Transcription and Polymerase Chain Reaction*

mRNA of the cultured cells was isolated using the Micro-Fast-Track™ mRNA isolation kit (Invitrogen, San Diego, Calif., USA). Two micrograms of mRNA were reverse transcribed in 50 μl reaction volume using the cDNA Cycle™ kit (Invitrogen). PCR was performed as previously described by Schröder et al. [21] in 30 μl final volume containing 5 μl of RT reaction mix and 10 and 20 pmol each of GAPDH and CDD-specific primers, respectively. PCR consisted of 29 amplification cycles at 94°C for 1 min, 62°C for 1 min, 72°C for 2 min, and a final extension step for 5 min at 72°C. The primer sets used were as follows: CDD [22], 5′-CTGAATTCATGGCCCA-GAAGCGTCCTGCCTGC-3′ (forward) and 5′-CTGAATTCGC-TCGGAACAGGATAGAACCT-3′ (reverse); GAPDH [23], 5′-CC-ACCCATGGCAAATTCCATGGCA-3′ (forward) and 5′-TCTAGA-CGGCAGGTCAGGTCCACC-3′ (reverse). Amplification products were separated on 1.5% agarose gel, visualized by 0.5 μg/ml ethidium bromide staining and photographed on Polaroid films.

*Enzyme-Linked Immunosorbent Assay for TP and Dihydropyrimidine Dehydrogenase*

Levels of TP and dihydropyrimidine dehydrogenase (DPD) in the supernatants of cell homogenates were measured using a 2-step sandwich ELISA as reported by Nishida et al. [24] and Mori et al. [25], respectively. ELISA was partly performed by Nippon Roche K.K. The protein levels were determined by the DC protein assay kit (Bio-Rad, Hercules, Calif., USA). TP was expressed as units per milligram protein, where one unit was defined as the TP level that phosphorolyzes 5′DFUR to 5FU at the rate of 1 μg 5FU/h. DPD was expressed as units per milligram protein where one unit is equivalent

to the amount of DPD protein that catabolizes 1 pmol of 5FU/min. Protein levels of TP or DPD measured by ELISA correlated well with those measured by a conventional enzyme activity assay [24, 25].

*TS Assay*

TS activity was determined as [6-$^3$H] FdUMP binding sites in the supernatants of cell homogenates based on the method of Spears et al. [26] and Shirasaka et al. [27]; $TS_{total}$ (TSt) and $TS_{free}$ (TSf) were determined by measuring total FdUMP binding sites and unoccupied FdUMP binding sites, respectively. The cells were homogenized by sonication and centrifuged at 10,5000 *g* for 60 min, and the supernatant was used for the TS assay. The TSt samples were prepared by causing the ternary complex present in the supernatant to fully dissociate to unbound TS in a preincubation period. Dissociation was achieved by the addition of the buffer including 0.6 *M* $NH_4HCO_3$, pH 8.0, 100 m*M* 2-mercaptoethanol, 100 m*M* NaF, 15 m*M* 5′-CMP, to the supernatant and incubating the mixture for 3 h at 26°C. In the case of TSf samples, the dissociation process in a preincubation period was omitted. TSt and TSf samples were then incubated with [6-$^3$H] FdUMP and cofactor solution (50 m*M* potassium phosphate buffer, pH 7.4, 20 m*M* 2-mercaptoethanol, 100 m*M* NaF, 15 m*M* 5′-CMP, 2% bovine serum albumin, 2 m*M* tetrahydrofolic acid, 16 m*M* sodium ascorbate and 9 m*M* formaldehyde) for 20 min at 30°C, and stopped by cooling on ice. The radioactivity in the acid-insoluble fraction was measured with a liquid scintillation counter. Results were expressed as picomoles per milligram protein. TS inhibition rate (TSIR) was calculated using the following equation [27]: TSIR (%) = [1 – TSf (pmol/mg protein)/TSt (pmol/mg protein)] × 100.

*Determination of 5FU Levels*

5FU levels in the supernatants of cell homogenates were measured based on the method of Marunaka et al. [28]. Briefly, the samples were homogenated with physiological saline containing [1,3-$^{15}N_2$]-5FU as an internal standard. After extraction from the supernatants of cell homogenates with chloroform, the aqueous layer was further extracted with ethyl acetate, subjected to silylation and then analyzed by a gas chromatograph-mass spectrometer. The calibration curve for the determination of 5FU was prepared by plotting the ratio of the peak height of 5FU to that of the internal standard against concentration. The calibration plots were linear. The results were expressed as nanograms per milligram protein.

*Drug Sensitivity in vitro*

The AlamarBlue™ assay was used to evaluate drug sensitivity as described previously [29, 30]. AlamarBlue™ contains an oxidation-reduction (REDOX) indicator that changes color from blue (oxidized form) to red (reduced form) in response to chemical reduction of growth medium induced by cellular growth. Cells were seeded in 96-well flat-bottomed plates at a density of 1,000 cells/well. After 1 day in culture, cells were cultured at various concentrations of the drug solution for 4 additional days. AlamarBlue solution (20 µl) was added to each well and cultured for 6 h. Absorbances at 570 and 600 nm were measured with a microplate reader model 450 (Bio-Rad Laboratories, Richmond, Calif., USA) and the percent survival was calculated with the formula: % survival = [sample ($OD_{570} - OD_{600}$) – blank ($OD_{570} - OD_{600}$)]/[control ($OD_{570} - OD_{600}$) – blank ($OD_{570} - OD_{600}$)] × 100. Dose-response curves were plotted, and the 50 or 10% inhibitory concentration ($IC_{50}$ or $IC_{10}$) was determined graphically as the dose of drug causing a 50 or 10% reduction in absorbance compared with the control.

**Table 1.** Effect of *CDD2* cDNA transfection on the levels of CDD, TP, DPD, and TSt

| Enzyme | T24 | T24-C2 | T24-21 |
|---|---|---|---|
| CDD, nmol/min/mg protein | 0.09 ± 0.06 | 0.07 ± 0.06 | 119 ± 25* |
| TP, U/mg protein | 2.5 ± 1.1 | 1.6 ± 0.6 | 1.6 ± 0.4 |
| DPD, U/mg protein | 87 ± 16 | 95 ± 8.3 | 91 ± 4.2 |
| TSt, pmol/mg protein | 1.2 ± 0.1 | 1.2 ± 0.2 | 1.3 ± 0.1 |

Expression levels of TP and DPD in the supernatants of cell homogenates were measured by ELISA, and activities of CDD and TSt were measured by enzyme activity assays. Means ± SD of triplicate analyses are given. * $p < 0.01$ compared with T24 or T24-C2.

*In vivo Study*

Treatment was started when the subcutaneous tumors established in the backs of Balb/c nu/nu mice reached a volume of 100–200 $mm^3$. Gemcitabine was injected intraperitoneally every 3 days for 4 injections at the maximum tolerated dose (MTD) of 120 mg/kg/day [31]. Veerman et al. [31] reported that gemcitabine at 120 mg/kg i.p. (q3dx4) was effective as a single agent, and that pharmacokinetic parameters of gemcitabine and 2′,2′-difluorodeoxyuridine in mice treated with intraperitoneal infusion of gemcitabine were similar to those observed in humans treated with intravenous infusion. Capecitabine was dissolved in 5% gum arabic as the vehicle and administered to the mice orally via a stomach tube 5 days a week for 3 weeks at the MTD of 2.1 mmol/kg/day [32]. Vehicle alone was given intraperitoneally or per os to the control mice. Gastrointestinal toxicity was estimated by observing the feces, and body weight was measured twice a week. Estimated tumor volume (V) was calculated by the following formula: $V = \text{width}^2 \times \text{length} \times 0.5$. Relative tumor volumes were calculated as values which are relative to the tumor volume observed at the beginning of the treatment.

*Statistics*

Student's two-tailed t test and ANOVA were used to assess the statistical significance of differences between data. Differences were considered to be statistically significant when a probability value was less than 0.05.

## Results

*Levels of CDD, TP, DPD, and TS*

Among the clones established from stable transfectants by the selection with Hygromycin, a pcDNA3.1/hygro(+)-transfected clone designated as T24-C2 and a pcDNA3.1/hygro (+)/*CDD2*-transfected clone designated as T24-21 were selected for further analysis based on the CDD activities (data not shown). As shown in table 1, CDD activity in T24-21 was significantly higher than that in T24 or T24-C2 ($p < 0.01$). In agreement with the results obtained

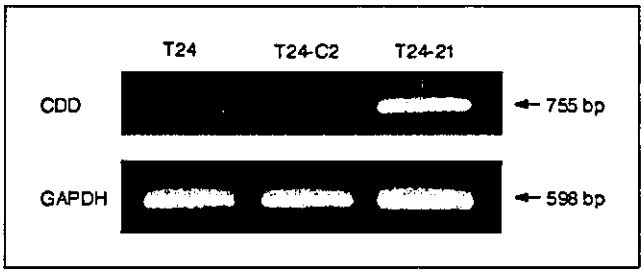


**Fig. 1.** Expression of CDD by RT-PCR in T24, T24-C2 and T24-21. RT-PCR was performed using primer sets for CDD mRNA detection after the isolation of mRNA from each cell line. Amplification products were separated on 1.5% agarose gel and visualized by 0.5 μg/ml ethidium bromide. Primer sets for GAPDH were used as a positive control. Strong expression of CDD mRNA was only detected in T24-21 by RT-PCR.

by an enzyme activity assay, strong expression of CDD mRNA was only detected in T24-21 by RT-PCR in the condition employed (fig. 1). We next examined the levels of TP, DPD, and TSt which might affect the sensitivities to fluoropyrimidines in T24, T24-C2, and T24-21 (table 1). No significant differences in the levels of TP, DPD, and TSt were found among them. Furthermore, there were no significant differences in biological properties including growth rates and morphological appearances in vitro among T24, T24-C2, and T24-21 (data not shown).

*Sensitivities to 5FU, 5'DFUR, 5'DFCR, and Gemcitabine in vitro*

Capecitabine itself does not show antitumor efficacy in vitro because carboxylesterase, CDD, and TP are needed to activate capecitabine as described before [1]. Therefore, we compared the sensitivities of T24, T24-C2, and T24-21 to 5FU, 5'DFUR, 5'DFCR, and gemcitabine in vitro (table 2). Transfection of *CDD2* cDNA increased the sensitivity to 5'DFCR (fig. 2a) but decreased the sensitivity to gemcitabine (fig. 2b). Because $IC_{50}$ for 5'DFCR in T24 or T24-C2 was not obtained in the conditions employed as shown in figure 2a, $IC_{10}$ for 5'DFCR was used for the statistical comparison in the remainder of the study. $IC_{10}$ for 5'DFCR in T24-21 was significantly lower than that in T24 or T24-C2 ($p < 0.0001$) while there was no significant difference in $IC_{10}$ for 5'DFCR between T24 and T24-C2 (table 2). On the other hand, $IC_{50}$ for gemcitabine in T24-21 was significantly higher than that in T24 or T24-C2 ($p < 0.01$) while there was no significant difference in $IC_{50}$ for gemcitabine between T24 and T24-C2 (table 2, fig. 2b). There was no significant difference in the in vitro sensitivity to 5FU or 5'DFUR among T24, T24-C2, and T24-21 (table 2). We then examined whether the modulation of sensitivity to 5'DFCR or gemcitabine by the transfection of *CDD2* cDNA as described above is due to the induction of CDD activity in T24 (table 2). THU, a CDD-specific inhibitor which had no influence on the growth of T24, T24-C2, and T24-21 (data not shown), abrogated significantly the changes in the sensitivity to 5'DFCR ($p < 0.0001$) or gemcitabine ($p < 0.01$) by the transfection of *CDD2* cDNA while there was no significant change in the sensitivity of T24 or T24-C2 to 5'DFCR or gemcitabine by the treatment with THU.

*TS Activity and Cellular 5FU Level after Treatment with Fluoropyrimidines*

To examine whether FdUMP-mediated events are responsible for the modulation of sensitivity to 5'DFCR by the transfection of *CDD2* cDNA, TS activity and cellular 5FU level were measured in cells treated for 24 h with 5'DFUR or 5'DFCR in vitro. The concentrations of 5'DFUR and 5'DFCR used for culture were 400 and 3 m*M*, respectively, which were around their $IC_{50}$. As shown in table 3, there was no significant difference in TSt activity between T24-C2 and T24-21 after treatment with or without 5'DFCR. TSf activity in T24-21 was significantly lower than that in T24-C2 after treatment with 5'DFCR ($p < 0.01$), resulting in a significant increase in TSIR in T24-21 as compared with T24-C2 ($p < 0.01$). As expected, cellular 5FU level in T24-21 was significantly higher than that in T24-C2 after treatment with 5'DFCR ($p < 0.01$) while there was no significant difference in the cellular 5FU level between T24-C2 and T24-21 after treatment with 5'DFUR (table 4).

*Sensitivities to Capecitabine and Gemcitabine in vivo*

The in vivo growth rate of T24-21 treated with vehicle alone (p.o. or i.p.) was not significantly different from that of T24 or T24-C2 treated with vehicle alone (p.o. or i.p.) by ANOVA (data not shown). Furthermore, there was no significant difference in in vivo sensitivity to capecitabine or gemcitabine between T24 and T24-C2 (data not shown). As shown in figure 3a, capecitabine significantly inhibited the in vivo growth of T24-21 ($p < 0.05$) but not T24-C2 as compared with the controls (T24-C2) treated with vehicle alone; T24-21 was more sensitive to capecitabine than T24-C2 ($p < 0.05$). On the other hand, gemcitabine inhibited the in vivo growth of T24-C2 and T24-21 significantly ($p < 0.01$) as compared with the controls (T24-C2) treated with vehicle alone (fig. 3b). T24-21 was more resistant to gemcitabine than T24-C2 ($p < 0.05$).

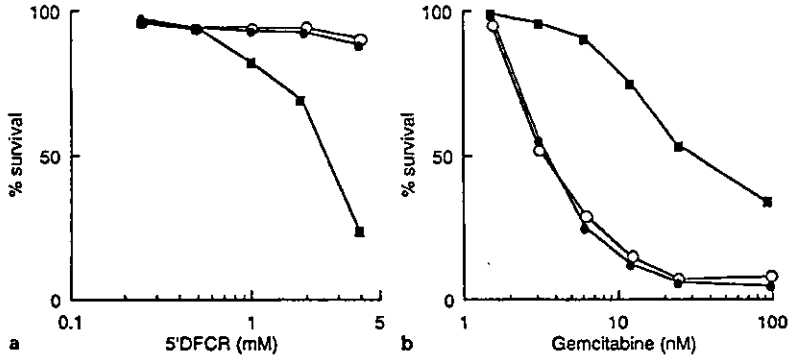


**Fig. 2.** In vitro sensitivities to 5'DFCR and gemcitabine. Cells were cultured with 5'DFCR (**a**) or gemcitabine (**b**) at various concentrations for 4 days. Percent survival was measured using the AlamarBlue™ assay and dose-response curves of T24 (○), T24-C2 (●) and T24-21 (■) were plotted. Each point represents the mean value from triplicate analysis. SDs of the means were less than 15% and were omitted.

**Table 2.** Effect of *CDD2* cDNA transfection on the sensitivities to 5FU, 5'DFUR, 5'DFCR, and gemcitabine

| | $IC_{50}$ | | |
|---|---|---|---|
| | T24 | T24-C2 | T24-21 |
| 5FU, μ*M* | 9.3±3.1 | 8.3±2.5 | 10.3±1.5 |
| 5'DFUR, μ*M* | 416±49 | 453±130 | 433±49 |
| 5'DFCR, m*M* | | | |
| −THU | >4 (4.0±0.3)[1] | >4 (3.8±0.3) | 2.8±0.6 (0.6±0.1)* |
| +THU | >4 (4.1±0.2) | >4 (4.2±0.2) | >4 (4.1±0.3) |
| Gemcitabine, n*M* | | | |
| −THU | 3.5±0.7 | 3.8±0.9 | 27.7±8.7** |
| +THU | 3.4±0.5 | 3.9±0.6 | 3.3±1.2 |

Cells were cultured with drugs at various concentrations for 4 days and the viability was evaluated by the AlamarBlue™ assay. Means ± SD of triplicate analyses.

* $p < 0.0001$, ** $p < 0.01$, vs. T24, T24-C2 or T24-21 treated with THU.

[1] $IC_{10}$ was used for the statistical comparison because $IC_{50}$ was not obtained in 5'DFCR concentrations up to 4 m*M* as shown in figure 2a.

**Table 3.** TS activity after treatment with or without 5'DFCR

| | T24-C2 | T24-21 |
|---|---|---|
| TSt activity, pmol/mg protein | | |
| −5'DFCR | 1.2±0.2 | 1.3±0.1 |
| +5'DFCR | 1.2±0.1 | 1.4±0.1 |
| TSf activity, pmol/mg protein | | |
| −5'DFCR | 1.2±0.2 | 1.4±0.1 |
| +5'DFCR | 1.0±0.1 | 0.4±0.1* |
| TSIR, % | | |
| +5'DFCR | 15±2.1 | 66±3.5* |

TS activity was measured in cells treated for 24 h with or without around $IC_{50}$ of 5'DFCR in vitro. The concentration of 5'DFCR used was 3 m*M*. Means ± SD of triplicate analyses.

* $p < 0.01$, vs. T24-C2 treated with 5'DFCR. TSIR was determined as described.

**Table 4.** Cellular 5FU level after treatment with fluoropyrimidines

| | T24-C2 | T24-21 |
|---|---|---|
| 5FU level, ng/mg protein | | |
| After 5'DFUR treatment | 1.5±0.1 | 1.4±0.1 |
| After 5'DFCR treatment | 0.3±0.1 | 12.3±2.5* |

Cellular 5FU level was measured in cells treated for 24 h with around $IC_{50}$ of 5'DFUR or 5'DFCR in vitro. The concentrations of 5'DFUR and 5'DFCR used were 400 μ*M* and 3 m*M*, respectively. Means ± SD of triplicate analyses.

* $p < 0.01$, vs. T24-C2 treated with 5'DFCR.

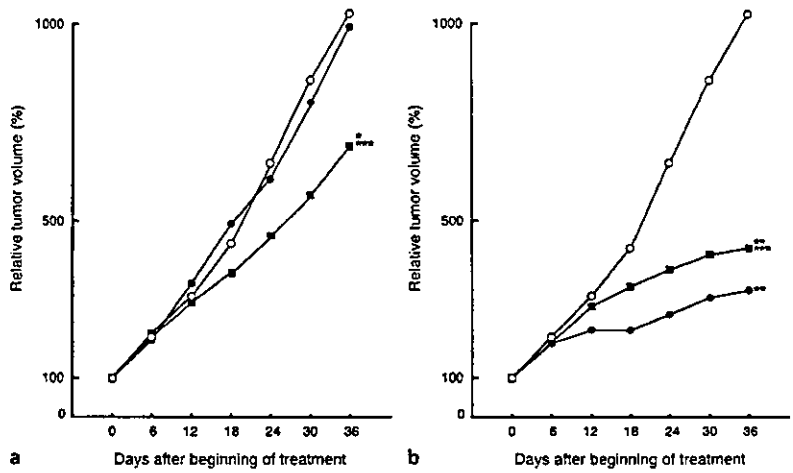


**Fig. 3.** In vivo sensitivities to capecitabine and gemcitabine. MTD of capecitabine (**a**) was administered per os 5 days a week for 3 weeks to mice (7 mice in each group) bearing subcutaneous tumors of T24-C2 (●) and T24-21 (■). MTD of gemcitabine (**b**) was given intraperitoneally every 3 days for 4 injections to mice (7 mice in each group) bearing subcutaneous tumors of T24-C2 (●) and T24-21 (■). Representative relative mean tumor volume in the control group (T24-C2) treated with vehicle alone is shown (○) since there was no significant difference in the in vivo growth rate by the treatment with vehicle alone between T24-C2 and T24-21 by ANOVA. Each point represents the relative mean tumor volume. The SDs of the means were less than 20% and were omitted. * $p < 0.05$, ** $p < 0.01$, vs. the control group by ANOVA. *** $p < 0.05$, vs. T24-C2 by ANOVA.

These in vivo results showed that transfection of *CDD2* cDNA made T24 sensitive to capecitabine but resistant to gemcitabine, similar to in vitro studies. No significant weight loss or diarrhea was observed in any of the treatment groups.

## Discussion

Pharmacokinetic study in cancer patients after oral administration of capecitabine showed a rapid gastrointestinal absorption of capecitabine followed by an efficient conversion to the two main metabolites, 5′DFCR and 5′DFUR [33, 34]. However, saturation of the metabolism during first pass through the liver, where carboxylesterase and CDD are located, leads to lower clearances of capecitabine and 5′DFCR, resulting in the detection of 5′DFCR in plasma; mean maximum plasma concentration ($C_{max}$) and mean area under the plasma concentration time curve (AUC) of 5′DFCR after oral administration of capecitabine 1,657 mg/m$^2$/day were 1.941 μg/ml and 4.438 μg·h/ml, respectively, while those for 5FU were 0.177 μg/ml and 0.327 μg·h/ml, respectively [34]. These evidences suggest that conversion of 5′DFCR to 5′DFUR by CDD in tumor cells themselves might influence their sensitivity to capecitabine even though 5′DFCR is deaminated mainly in the liver. As expected, the present study showed that forced expression of CDD by the transfection of *CDD2* cDNA made T24, which has low endogenous CDD activity [16, 17], sensitive to 5′DFCR in vitro and capecitabine in vivo. The following findings suggest that increased sensitivity to 5′DFCR in vitro would be ascribed to the increased expression of CDD by the transfection of *CDD2* cDNA: transfection of *CDD2* cDNA did not change the levels of TP, DPD, and TS which are involved in the fluoropyrimidine metabolism and related to the sensitivity to fluoropyrimidines; in vitro sensitivity to 5FU or 5′DFUR in which CDD activity is not essential for the activation was not changed by the transfection of *CDD2* cDNA although that to 5′DFCR was increased significantly; THU, a specific CDD inhibitor, abrogated the changes in the sensitivity of T24-21 to 5′DFCR in vitro. Transfection of *CDD2* cDNA resulted in a significant increase in cellular 5FU level and inhibition of TS activity in T24-21 after treatment with 5′DFCR but not with 5′DFUR in vitro. Furthermore, the proposed mechanism of increased sensitivity to capecitabine in vivo by the transfection of *CDD2* cDNA based upon the in vitro findings would be as follows: 5′DFCR in plasma which is converted from capecitabine in the liver is efficiently deaminated to 5′DFUR in T24-21 tumor cells themselves with increased CDD activity, resulting in the increase in cellular 5FU levels and inhibition of TS activity, and inhibition of DNA synthesis. However, changes in sensitivities to capecitabine and gemcitabine in vivo by the transfection of *CDD2* cDNA were not as dramatic as those observed in vitro. The possible explanation for this is that capecitabine and gemcitabine administered in vivo were deaminated not only in tumor cells themselves but also in the liver.

Several studies with the transfection of TP cDNA into various cancer cell lines showed that TP plays an important role in the activation of 5'DFUR [35] and capecitabine [36]. We previously transfected TP cDNA into the human renal cancer cell line KU2 to examine its sensitivity to fluoropyrimidines, and showed that transfection of TP cDNA made the cells sensitive to 5'DFUR and capecitabine [36]. Our previous study suggests that capecitabine exerts an antitumor effect primarily on cells with high TP expression. In a human cancer xenograft model, Ishikawa et al. [32] reported that TP expression or the TP/DPD ratio in the tumors correlated well with the efficacy of 5'DFUR and capecitabine. Although TP and DPD are key enzymes for the sensitivity to capecitabine as described above, the present study suggests that CDD in tumor cells themselves also contributes to the sensitivity to capecitabine to some extent.

In vitro resistance to gemcitabine by the transfection of the CDD gene has been already reported by Neff and Blau [15]. They showed that CDD gene transfection conferred a 2.4-fold increased in vitro resistance to gemcitabine in the human lymphoid leukemic cell line CCFR-CEM. The present study showed that transfection of *CDD2* cDNA made T24 resistant to gemcitabine not only in vitro but also in vivo in which the latter has not been reported previously. However, Eda et al. [16] reported no significant change in the sensitivity to gemcitabine by the CDD gene transfection into the human colon cancer cell line HCT116, and explained that their result may be ascribed to insufficient CDD expression or high activity of deoxycytidine kinase, which has a higher affinity for gemcitabine than CDD. Taken together, the present study suggests that CDD in tumor cells themselves is indeed involved in gemcitabine sensitivity although CDD is not a sole determinant for gemcitabine sensitivity [10].

Clinical trials have demonstrated the anticancer efficacy of gemcitabine as a single agent [7–9] or in combination with 5FU [37–39] or capecitabine [40, 41]. In the present study, sensitivity to gemcitabine and capecitabine was oppositely affected by tumoral CDD activity. Namely, cells with high CDD expression which are resistant to gemcitabine are potentially sensitive to capecitabine. In terms of tailor-made therapy, it seems to be of value to examine whether or not measurement of CDD expression levels in tumors is useful for predicting the tumor sensitivities to capecitabine and gemcitabine. This point should be examined in further studies to correlate the CDD expression in tumors with the response to capecitabine and/or gemcitabine.

In conclusion, the present study clearly showed direct evidence for the contribution of CDD in tumor cells themselves to the sensitivities to capecitabine and gemcitabine.

## Acknowledgments

We thank Drs. J. Schröder and S. Seeber (Department of Internal Medicine, University of Essen Medical School, Germany) for providing *CDD2* cDNA. This work was supported in part by a Grant-in-Aid for Scientific Research (No. 13671673) from the Ministry of Education, Science, Sports and Culture of Japan (to T.M.).

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