Significance of Thymidylate Synthase Activity in Renal Cell Carcinoma

Yoichi Mizutani, Hiromi Wada, Osamu Yoshida, Masakazu Fukushima, Mitsuo Nonomura, Masahiro Nakao, and Tsuneharu Miki

Department of Urology, Kyoto Prefectural University of Medicine, Kyoto 602-8566 [Y. M., M. Na., T. M.]; Departments of Thoracic Surgery [H. W.] and Urology [O. Y.]; Graduate School of Medicine, Faculty of Medicine, Kyoto University, Kyoto 606-8507; Cancer Research Laboratory, Taiho Pharmaceutical Co. Ltd., Saitama 357-8527 [M. F.]; and Department of Urology, Kyoto Katsura Hospital, Kyoto 615-8256 [M. No., Japan

ABSTRACT

Purpose: 5-Fluorouracil (5-FU) is an anticancer agent clinically used against various cancers including renal cell carcinoma (RCC). 5-FU inhibits thymidylate synthase (TS) and blocks DNA synthesis. TS is the key enzyme in the catalysis of the methylation from dUMP to dTMP in the DNA synthetic process. Little is known about the significance of TS in RCC. We investigated the activity of TS in 68 RCCs and the association with dihydropyrimidine dehydrogenase (DPD) activities, which is a principal enzyme in the degradation of 5-FU and pyrimidine nucleotides. The relationship between TS/DPD activities in primary cultured RCC cell lines and their sensitivity to 5-FU was also examined.

Experimental Design: The levels of TS and DPD activities in nonfixed fresh-frozen RCC and normal kidney were determined biochemically by the 5-fluoro-2′-deoxyuridine 5′-monophosphate binding assay and the 5-FU degradation assay, respectively. The sensitivity of primary cultured RCC cells to 5-FU was assessed by the microculture tetrazolium dye assay.

Results: The activity of TS was 2.5-fold higher in RCC compared with normal kidney. TS activity in $T_{50}$ RCC was 2.5-fold higher than that in $T_{1/2}$ RCC. TS activity in $M_{1}$ RCC was 2.5-fold higher than that in $M_{0}$ RCC. In addition, TS activity in stage III/IV RCC was 3-fold higher than that in stage I/II RCC. The levels of TS activity in grade 3 RCC were 3-fold and 2-fold higher than that in grade 1 and grade 2 cancer, respectively. TS activity in clear cell RCC were 4-fold higher than that in papillary RCC. Patients with low TS activity had a longer disease-specific survival as compared with those with high activity in the 5-year follow-up. There was no relationship between TS and DPD activities. TS activity in primary cultured RCC cells was positively correlated with their sensitivity to 5-FU. Furthermore, RCC cells with both high TS activity and low DPD activity were more sensitive to 5-FU, compared with those with either low TS activity or high DPD activity.

Conclusions: The present study is the first study to demonstrate that the level of TS activity was correlated with both the progression of the stage and the increase of the grade of RCC, and that higher TS activity in primary cultured RCC predicted higher sensitivity to 5-FU. These results suggest that high TS activity may be associated with malignant potential of RCC, and that it may be possible to use 5-FU for RCC with high TS activity.

INTRODUCTION

TS is the key enzyme that catalyzes the methylation of dUMP to dTMP, which is an important step in the process of DNA synthesis (1). 5-FU is one of the widely used anticancer chemotherapeutic agents for the treatment of various cancers including RCC (2, 3). 5-FU itself is inactive and requires its intracellular conversion to FdUMP. FdUMP exerts its cytotoxic activity through the formation of a ternary complex with TS and 5,10-methylene-tetrahydrofolate, resulting in inhibition of TS and blockade of the DNA synthetic process (4, 5). Previous studies performed on several cancers have demonstrated that the level of TS expression predicted the response to 5-FU-based chemotherapy (6–8). Patients with node-positive breast cancer with high TS expression demonstrated the most significant benefit of adjuvant 5-FU-containing chemotherapy. Adjuvant 5-FU-based chemotherapy in patients with rectal cancers with high TS levels significantly improved disease-free survival, whereas it did not improve prognosis in patients with low levels. Thus, higher TS expression was accompanied with a greater response rate to 5-FU-containing chemotherapy.

Although TS is an important enzyme in pyrimidine nucleotides synthesis, DPD is the initial and rate-limiting enzyme in the three-step pathway of uracil and thymine catabolism, leading to the formation of β-alanine (9, 10). Our previous study showed that DPD activity in RCC (mean, 12.3 pmol/mg protein/min; median, 9.6 pmol/mg protein/min) was lower in normal RCC cell lines and blocks DNA synthesis. Little is known about the significance of TS in RCC. We investigated the activity of TS in 68 RCCs and the association with dihydropyrimidine dehydrogenase (DPD) activities, which is a principal enzyme in the degradation of 5-FU and pyrimidine nucleotides. The relationship between TS/DPD activities in primary cultured RCC cell lines and their sensitivity to 5-FU was also examined.
kidney, and that the level of DPD activity was inversely correlated with both the progression of the stage and the increase of the grade of RCC (11). In addition, DPD is the principal enzyme involved in degradation of 5-FU (12, 13), and its activity is highly correlated with 5-FU pharmacokinetics (14, 15). Our previous reports demonstrated that DPD activity in bladder cancers and RCCs inversely correlated with their sensitivity to 5-FU (11, 16).

Our previous study on bladder cancer demonstrated that TS activity was up-regulated in bladder cancer compared with normal bladder, and that the level of TS activity correlated with the progression of the stage and the increase of the grade of bladder cancer (17). Although 5-FU is clinically used for the therapy of RCC, reported data on TS activity in RCC are limited, and little is known about the significance of TS activity in the biology of RCC. In the present study, we measured the activity of TS in 68 RCCs and evaluated the relationship between the level of TS activity and stage or grade status of RCC. In addition, we investigated the association between TS activity in RCC and the sensitivity to 5-FU. Furthermore, the relationship between TS and DPD activities was also examined.

MATERIALS AND METHODS

Patients. Surgical specimens were obtained from 68 patients with RCC. They included 51 male and 17 female patients, ranging in age from 32 to 82 years. Histological diagnosis revealed that 63 and 5 patients had clear cell carcinoma and papillary RCC, respectively. Their histological classification and staging according to TNM classification were: T1 (n = 47), T2 (n = 11), T3 (n = 9), T4 (n = 1); N0 (n = 64), N1 (n = 2), N2 (n = 2); M0 (n = 62), M1 (n = 6); stage I (n = 47), stage II (n = 9), stage III (n = 5), stage IV (n = 7), and G1 (n = 11), G2 (n = 51), G3 (n = 6), respectively. Samples of normal kidneys were collected from 68 patients with RCC. The specimens were stored frozen at −80°C until use for the assay of TS and DPD activities.

Reagents and Medium. 5-FU (Lot. No. 308033) was kindly supplied by Kyowa Hakko Co. Ltd., Tokyo, Japan. RPMI 1640 (Life Technologies, Inc., Bio-cult, Glasgow, Scotland, United Kingdom) supplemented with 25 mM HEPES (Life Technologies, Inc.), 2 mM l-glutamine (Life Technologies, Inc.), 1% nonessential amino acid (Life Technologies, Inc.), 100 units/ml penicillin (Life Technologies, Inc.), 100 mg/ml streptomycin (Life Technologies, Inc.), and 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.) was used as complete medium.

Tumor Cells. Fresh RCC cells derived from 22 patients were separated from surgical specimens for in vitro primary culture as described previously (18, 19). Although we tried to make primary cultures from all of the RCC specimens, we could make them from 22 RCCs. Their histological staging according to TNM classification were: stage I (n = 16), stage II (n = 2), stage III (n = 1), stage IV (n = 3), and G1 (n = 3), G2 (n = 17), G3 (n = 2), respectively. Briefly, cell suspensions were prepared by treating finely minced cancer tissues with collagenase (3 mg/ml; Sigma Chemical Co., St. Louis, MO). After washing in RPMI 1640, the cell suspensions were layered on discontinuous gradients consisting of 2 ml of 100%, 2 ml of 80%, and 2 ml of 50% Ficoll-Hypaque in 15-ml plastic tubes, and were centrifuged at 400 × g for 30 min. Lymphocyte-rich mononuclear cells were collected from the 100% interface, and cancer cells and mesothelial cells from the 80% interface. Cell suspensions enriched with cancer cells were sometimes contaminated by monocyte-macrophages, mesothelial cells, or lymphocytes. To eliminate additional contamination of host cells, we layered the cell suspensions on discontinuous gradients of 2 ml each of 25, 15, and 10% Percoll in complete medium in 15-ml plastic tubes and centrifuged them for 7 min at 25 × g at room temperature. Cancer cells depleted of lymphoid cells were collected from the bottom, washed, and suspended in complete medium. Cancer cells were >93% viable on average according to the trypan blue dye-exclusion test. The cancer cells were maintained in monolayers on plastic dishes in complete medium. The cancer cells of primary culture were used as target cells for lysis of 5-FU in the MTT assay.

Measurement of TS Activity in RCC and Normal Kidney. The activity of TS was determined by the dUMP binding assay combined with gel filtration as described previously (7, 20). RCC and normal kidney were sonicated in the homogenate buffer [50 mM Tris-HCl, 1 mM EDTA, and 5 mM MgCl2 (pH 7.4)] at maximum output (Sonifier cell disruptor 350; SmithKline), and centrifuged at 105,000 × g at 4°C for 60 min in a Beckman ultra-centrifuge (model TL-100). The super-

![Image](https://example.com/image.png)
natants from each sample were divided into several tubes and frozen at −80°C until use.

The test supernatant was incubated with [3H]FdUMP and 5,10-CH2-FH4 at 30°C for 20 min, then the mixture was gel-filtered using a PD-10 column (Pharmacia Biotech, Uppsala, Sweden) to separate TS-bound from free [3H]FdUMP. The sample was eluted with PBS (−), and the total radioactivity of the fractions containing protein was measured. Protein content of the supernatant was measured using the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL). Internal standards were used to compare assays. We analyzed all of the samples at the same time. This method made it possible to estimate TS activity 1 fmol/mg protein. Repeated measurements yielded the same results.

TS activity greater than the median value was regarded as high activity, and TS activity less than the median value was regarded as low activity.

Measurement of DPD Activity in RCC and Normal Kidney. RCC and normal kidney were homogenized in 4 volumes of 50 mM Tris-HCl (pH 8.0) containing 5 mM 2-mercaptoethanol, 25 mM K, and 5 mM MgCl2. The homogenate was centrifuged at 105,000 × g for 1 h at 4°C, and the supernatant fluid was used for the measurement of DPD activity as described before (7, 16). Briefly, the assay mixture, in a final volume of 250 µl, consisted of 50 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 25 mM NaF, 50 mM nicotinamide, 5 mM ATP, 1 mM NADPH, [6-3H]5-FU (0.2 µCi, 20 mM), and the enzyme extract (100 µl). The mixture was incubated for 30 min at 37°C, and the reaction was stopped by heating at 100°C in a water bath. After centrifugation at 3,000 rpm, the supernatant (100 µl) was treated with 10 µl of 2 M KOH for 30 min at room temperature. Then, the mixture was treated with 5 µl of 2 m perchloric acid and centrifuged. An aliquot (20 µl) of the supernatant was spotted onto a TLC plate (Merck silica gel 60F254 precoated plate; 2.5 × 10 cm, thickness 0.25 mm; Merck, Whitehouse Station, NJ), and developed with a mixture of chloroform, methanol, and acetic acid (17:3:1, v/v/v). The spots of 2-fluoro-α-alanine and 2-fluoro-α-ureidopropionic acid, 5-FU degradation products, were scraped into vials and mixed with 10 ml of ACS-II scintillation fluid (Amersham, Buckinghamshire, United Kingdom). The radioactivity was measured in a Wallac 1410 liquid scintillation counter (Pharmacia Biotech). Internal standards were used to compare assays. This method made it possible to estimate DPD activity 0.4 pmol/mg protein/min. We analyzed all of the samples at the same time. Repeated measurements yielded the same results.

DPD activity greater than the median value was regarded as high activity, and DPD activity less than the median value was regarded as low activity.

Cytotoxicity Assay. MTT assay was used to determine tumor cell lysis as described previously (21, 22). Briefly, 100 µl of target cell suspension (2 × 10⁵ cells) were added to each well of 96-well flat-bottomed microtiter plates (Corning Glass Works, Corning, NY), and each plate was incubated for 24 h at 37°C in a humidified 5% CO2 atmosphere. After incubation, the supernatant was aspirated, and tumor cells were washed three
times with RPMI 1640, and 200 μl of drug solution or complete medium for control were distributed in the 96-well plates. Each plate was incubated for 24 h at 37°C. After incubation, 20 μl of MTT working solution (5 mg/ml; Sigma Chemical Co.) was added to each culture well, and the cultures were incubated for 4 h at 37°C in a humidified 5% CO₂ atmosphere. The culture medium was removed from the wells and replaced with 100 μl of isopropanol (Sigma Chemical Co.) supplemented with 0.05 N HCl. The absorbance of each well was measured with a microculture plate reader (Immunoreader; Japan Intermed Co. Ltd., Tokyo, Japan) at 540 nm. The percentage of cytotoxicity was calculated by the following formula: percentage cytotoxicity = (absorbance of experimental wells/absorbance of control wells) × 100.

**Statistical Analysis.** All of the determinations were made in triplicate. For statistical analysis, Student’s t test and Pearson’s correlation test were used. Postoperative disease-specific survival was determined by the Kaplan-Meier method. The Cox-Mantel test was used to establish the statistical difference in recurrence between the patients with high and low levels of TS activities. Factors related to disease-specific survival in patients with RCC were also analyzed by multivariate analysis. A P of 0.05 or less was considered significant.

**RESULTS**

Activity of TS in RCC and Normal Kidney. The values of TS activity in RCC and normal kidney in patients with RCC were summarized in Fig. 1. The mean TS activity in RCC was ∼5-fold higher than that in normal kidney. The median levels of TS activity in normal kidney and RCC were 3.1 and 10.5 fmol/mg protein, respectively. The level of TS activity in normal kidney in patients with RCC was similar to that in patients with renal pelvic cancer or ureteral cancer (data not shown). These findings demonstrated that TS activity in RCC was significantly higher than that in normal kidney.

The Level of TS Activity in RCC. We then examined TS activity in RCC as a function of histological stage and grade of the disease. The activity of TS was 2.5-fold higher in T₁ RCC than that in T₀ RCC (Fig. 2). Furthermore, TS activity in T₃/₄ RCC was ∼2.5-fold higher than that in T₁/₂ RCC. The level of TS activity in M₁ RCC was significantly (2.5-fold) higher than that in M₀ RCC (Fig. 3). TS activity in stage III RCC was 3-fold and 3.5-fold higher than those in stage I and II RCC, respectively (Fig. 4). TS activity in stage IV RCC was 3-fold and 4-fold higher than those in stage I and II RCC, respectively. In addition, TS activity in stage III/IV RCC was 3.2-fold higher than that in stage I/II RCC.

The level of TS activity in grade 3 RCC was 3-fold and 2-fold higher than that in grade 1 and 2 cancer, respectively (Fig. 5). TS activity in clear cell RCC was 3.5-fold higher than that in papillary RCC (Fig. 6).

These findings showed that TS activity was in parallel with the stage progression and the increase of the histological grade of RCC.

Correlation between the Level of TS Activity and Postoperative Disease-specific Survival in Patients with RCC. RCC patients undergoing radical nephrectomy were evaluated for the postoperative clinical course. The postoperative disease-specific survival was estimated by Kaplan-Meier analysis. On the basis of the analysis, patients with RCC were divided into two groups, namely, those with high TS activity (greater than the median value) and those with low activity (less than the median value). Patients with low TS activity had a longer disease-specific survival as compared with those with high activity in the 5-year follow-up (Fig. 7). However, multivariate analysis showed that TS activity was not an independent prognostic factor in patients with RCC.

Relationship between TS and DPD Activities in RCC. DPD is the initial and rate-limiting enzyme in the three-step pathway of pyrimidine nucleotides catabolism (9, 10). In contrast, TS is an important enzyme in pyrimidine nucleotides synthesis. We then examined the association between TS and DPD activities in RCC. There was no correlation between the levels of TS and DPD activities in RCC (Fig. 8).

Correlation between the Level of TS Activity in RCC Cells and Their Sensitivity to 5-FU. We examined the association between TS activity level in RCC cells and their sensitivity to 5-FU. Twenty-two primary cultures derived from surgical specimens were used as targets. The findings in Fig. 9 demonstrate that there was a positive correlation between the level of TS activity in RCC cells and their sensitivity to 5-FU. Similar findings were observed with different doses of 5-FU (data not shown).
Correlation between the Levels of TS and DPD Activities in RCC Cells, and Their Sensitivity to 5-FU. Our previous studies have demonstrated that DPD activity in bladder cancers and RCCs inversely correlates with their sensitivity to 5-FU (11, 16). Furthermore, this study has shown that TS activity in RCC correlated positively with their sensitivity to 5-FU. We then examined the association between TS/DPD activities in RCC and their sensitivity to 5-FU. TS and DPD activities greater than the median value were regarded as high activities, and TS and DPD activities less than the median value were regarded as low activities. RCC cells with both high TS activity and low DPD activity were more sensitive to 5-FU than those with either low TS activity or high DPD activity (Table 1). These findings suggest that the levels of TS and DPD activities in RCC may be a significant predictive parameter for 5-FU efficacy.

DISCUSSION

In the present study, we demonstrated that TS activity was up-regulated in RCC, compared with normal kidney, and that the level of TS activity correlated with both the progression of the stage and the increase of the grade of RCC. Furthermore, clear cell RCC had higher TS activity compared with papillary RCC. The prognosis in patients with papillary RCC has been reported to be better than that in patients with clear cell RCC (23, 24). Because TS activity increases in parallel with cancer growth rate and DNA synthesis (1), RCC with high TS activity may be a highly growing phenotype. In addition, patients with low TS activity had a longer disease-specific survival as compared with those with high activity in the 5-year follow-up. Although we report a few patients during a short-term follow-up, our findings suggest that TS may play an important role in regulating the malignant potential of RCC, and that low TS activity may be considered a good prognostic sign. However, TS activity was not an independent prognostic parameter related to natural history after radical nephrectomy.

The present study is the first to show that the activity of TS in RCC was significantly higher than that in normal kidney, and that the level of TS activity was correlated with the increase of the stage and the grade of RCC. Our current findings are consistent with those of others, which demonstrated that TS activity in cancer tissues was higher than that in normal tissues (17, 25). The association of TS activity with stage/grade status in RCC may be a reflection of the rates of cancer cell proliferation. It has been reported that the level of TS activity increases 20-fold when the cells enter the S phase from the G0 phase in synchronized cells (26). Because TS binds to the c-myc mRNA as a part of a ribonucleoprotein complex (27), it is likely that TS may be involved in the coordinate regulation of a lot of other genes. Thus, these findings suggest that TS may be necessary for carcinogenesis as well as cell proliferation in RCC. However, additional studies are needed to determine the biological interaction between TS and growth modulation of RCC.
The overall response rate of immunotherapy and/or chemotherapy against RCC has improved. However, metastasis and recurrence of RCC remain major problems. Therefore, new therapeutic approaches are required for the patients. The up-regulation of TS activity in RCC compared with normal kidney, especially in high stage and high grade RCC, identifies TS as a therapeutic target. Accordingly, inhibition of TS activity may provide a therapeutic means of preventing growth of RCC. Because TS is the target enzyme of 5-FU (4, 5), our observation that elevated TS activity in primary cultured RCC cell lines was associated with high 5-FU sensitivity may be of potential clinical importance in the management of patients with RCC. Chemoinmunotherapy including 5-FU may be effective against RCC with high TS activity. However, the mechanisms responsible for 5-FU resistance in cancer cells are multifactorial. Furthermore, 5-FU-containing chemoinmunotherapy is not always effective against RCC (28, 29). In addition, the lower the TS activity is, the greater the response rate to 5-FU-containing chemotheraphy is achieved (30). These findings suggest that overcoming 5-FU resistance of RCC or patient selection may be also necessary in the treatment of RCC with 5-FU.

TS activity in RCC was 5-fold higher than that in normal kidney. The activity of TS in primary cultured RCC cell lines was positively correlated with their sensitivity to 5-FU. Therefore, the high ratio of cancer:normal TS activity may contribute to the favorable differential between anticancer effect and adverse effect of 5-FU. Thus, a higher degree of 5-FU sensitivity may occur in cancer tissues compared with that in normal tissues.

Several studies and our observation suggested that high TS activity may be related to a favorable response to 5-FU (6–8). In contrast, other studies have demonstrated that either overexpression of TS protein or high TS activity is associated with 5-FU resistance (28, 29). These findings suggest that TS may be a critical enzyme in DNA synthetic process in RCC with high TS activity, high response of primary cultured RCC cells with high TS activity to 5-FU was observed in this study. Because only 2 RCC patients in this study were treated with 5-FU, additional studies are needed to clarify TS activity in RCC as a predictive marker related to treatment response in 5-FU-treated patients.

Most of the administered 5-FU is degraded through the catabolic pathway with DPD (12, 13). DPD activity is highly associated with 5-FU pharmacokinetics (14, 15). Our previous report has demonstrated that DPD activity in RCC is inversely correlated with their sensitivity to 5-FU (11). Fur-
thermore, this study showed that TS activity in primary cultured RCC cells was positively correlated with their sensitivity to 5-FU. In addition, primary cultured RCC cells with both high TS activity and low DPD activity were more sensitive to 5-FU than those with either low TS activity or high DPD activity. These findings suggest that the levels of TS and DPD activities in RCC may be important predictive indicators for 5-FU efficacy. Moreover, the measurement of TS activity as well as DPD activity may be necessary for the evaluation of efficacy of 5-FU-containing chemotherapy.

In conclusion, the current study demonstrated that the activity level of TS in RCC was correlated with the increase of histological stage and grade, and that elevated level of TS activity in primary cultured RCC cell lines was associated with high response to 5-FU. These findings suggest that the assessment of TS activity may be useful both in the management and in the treatment of RCC. Because the level of TS activity could be used as a prognostic parameter in patients with RCC and a predictive indicator for 5-FU efficacy against RCC, the accurate prediction of prognosis and 5-FU efficacy may help select patients for more intensive surgical or immunchemotherapeutic approaches including 5-FU. However, additional studies are needed to determine the regula-
Table 1  

<table>
<thead>
<tr>
<th>Level of TS and DPD activities in RCC</th>
<th>% Cytotoxicity (mean ± SD)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Both high TS activity and low DPD activity ($n = 7$)</td>
<td>39.7 ± 6.2$^b$</td>
</tr>
<tr>
<td>Either low TS activity or high DPD activity ($n = 15$)</td>
<td>30.8 ± 6.7</td>
</tr>
</tbody>
</table>

$^a$ The direct cytotoxic effect of 5-FU (100 μM) on primary cultured RCC cells was assessed by a 1-day MTT assay. The RCC cells were derived from 22 patients with RCC.

$^b$ P < 0.05 versus RCC with either low TS activity or high DPD activity.

REFERENCES


Significance of Thymidylate Synthase Activity in Renal Cell Carcinoma

Yoichi Mizutani, Hiromi Wada, Osamu Yoshida, et al.


**Updated version**
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/9/4/1453

**Cited articles**
This article cites 28 articles, 18 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/9/4/1453.full.html#ref-list-1

**Citing articles**
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
/content/9/4/1453.full.html#related-urls

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.