Thymidylate Synthase and Dihydropyrimidine Dehydrogenase in Non-small Cell Lung Cancer: Relationship between mRNA Expression and Activity

TAKANORI MIYOSHI, KAZUYA KONDO, HARUHIKO FUJINO, YUJI TAKAHASHI, NARUHIKO SAWADA, SHOJI SAKIYAMA, MASARU TSUYUGUCHI, SUGURU KIMURA, MASAYUKI SUMITOMO and YASUMASA MONDEN

Department of Oncological and Regenerative Surgery, School of Medicine, University of Tokushima, Kuramoto-cho, Tokushima 770-8503, Japan

Abstract. Background: UFT (Tegafur + Uracil) has been reported to be effective for postoperative adjuvant chemotherapy of non-small cell lung cancer (NSCLC) in a randomized prospective study. Recently, many clinical studies have demonstrated that UFT is effective for cancer with a low activity of thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD). In the present study, we investigated TS and DPD activity in resected tumors and corresponding normal lungs and the relationship between the activity and the mRNA expression of TS and DPD in NSCLC. Materials and Methods: Seventy-seven patients underwent complete surgical resection and lymph node dissection for NSCLC. The activity of TS was determined by the FdUMP binding assay combined with gel filtration. The activity of DPD was determined by radio-enzymatic assay. Tumor tissues and their paired non-cancerous tissues were assayed. Furthermore, the mRNA expressions of TS and DPD were examined by real-time RT-PCR. Results: The mean TS and DPD activities in NSCLC were approximately 2.4-fold and 5-fold of those in normal lungs. The mean TS and DPD activities of NSCLC were 0.099 pmol/mg and 407 pmol/mg/min, respectively. Although both TS and DPD activities showed a tendency to be high for adenocarcinoma, there was no significant difference between TS and/or DPD activities and any clinical findings (age, gender, stage and histological type). The mRNA expression of DPD was correlated with DPD activity (rs=0.846, p<0.001). The mRNA expression of TS was weakly correlated with TS activity (rs=0.757, p<0.001). Conclusion: TS and DPD activities in NSCLC were higher than those in normal lungs. Assay of DPD mRNA and TS mRNA by real-time RT-PCR can be used as an indicator for the use of UFT.

Lung cancer is the most common cause of death from cancer worldwide (1). A major histological type of lung cancer is non-small cell lung cancer (NSCLC). Radical surgery is the most effective therapy for patients with localized NSCLC, although the overall prognosis remains poor. The 5-year survival rate after complete resection is reported to be 67-79% for stage I, but prognoses of patients with stage II and III are worse(2, 3). Adjuvant therapies to prevent recurrence and metastasis after surgery are needed. Cisplatin-based adjuvant chemotherapy improved survival among patients with completely resected NSCLC (4). On the other hand, UFT has been reported to be an effective postoperative adjuvant therapy for NSCLC in randomized prospective studies (5-7). Wada et al. and Kato et al. reported that UFT was used in an adjuvant setting even in patients with early stage NSCLC, and demonstrated significant efficacy (5, 8).

UFT is an anticancer drug for oral use that contains Tegafur (5-fluorouracil derivative) and Uracil (DPD inhibitor). 5-Fluorouracil (5-FU) and its derivatives are some of the most widely used chemotherapeutic drugs, especially for the treatment of gastrointestinal tumors. The anticancer effects of 5-FU are thought to relate to its two active metabolites, 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP) and 5-fluoro-uridine-5'-tri-phosphate (FUTP). FdUMP forms a ternary complex with thymidylate synthase (TS) and 5,10-methylene-tetrahydrofolate (5,10-CH2FH2), which blocks TS activity and inhibits the synthesis of thymidylate for DNA synthesis (9).
5-FU is degraded to 2-fluoro-β-alanine mainly in the liver. Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting enzyme in this process (10). Some experimental studies have shown that low DPD activity in tumor cells is related to higher 5-FU sensitivity (11-14). In some clinical studies, DPD activity in tumors was found to be correlated with the clinical response to 5-FU-based chemotherapies (15-17).

The use of biochemical assays is often not practical, particularly with the unavailability of sufficient clinical samples (e.g., biopsies). An alternative approach was performed recently using molecular methodology, quantification of DPD mRNA by reverse-transcription polymerase chain reaction (RT-PCR) (18). One of the major advantages of this method is that it can be performed with ultra-low-volume samples as compared with measurement of enzyme activities. Recently, a real-time RT-PCR method based on TaqMan fluorescence methodology was found to require fewer steps after PCR than conventional PCR methods, simplifying the procedures and making it possible to maintain quantification subsequent to PCR, while allowing a broad dynamic range of measurements (19). Quantitative measurement of genes may aid in the interpretation of results obtained by conventional techniques.

In the present study, we examined the TS and DPD activities in resected tumors and corresponding normal lungs and the relationship between the activity and the mRNA expression of TS and DPD in NSCLC. The aim of this study was to determine whether or not TS or DPD activities are related to some clinical factors, and whether they correlate with TS or DPD expression measured by real-time RT-PCR method, as a possible determinant of the response to fluoropyrimidine-based post-operative adjuvant chemotherapy.

Materials and Methods

Tumors. We used a total of 77 fresh human non-small cell lung carcinoma (NSCLC) tissue samples surgically resected between April 1993 and December 2001. These specimens were obtained at Tokushima University Hospital, Tokushima Prefectural Central Hospital, Tokushima Municipal Hospital and Tokushima Red Cross Hospital, Japan. The pathological stage was classified according to the current TNM classification revised in 1997 (2). Histological types were determined according to the WHO classification (20). Table I outlines the patient characteristics. Specimens of approximately 10 mm³ were immediately stored at -80°C pending real-time RT-PCR. All patients gave written informed consent before participation in the study.

| TS binding assay | Measurement of TS binding activity to FdUMP was based on the method of Ishikawa et al. (21, 22). Tumor tissues were homogenized in 3 volumes of 0.2 M Tris-HCl (pH 8.0) containing 20 mM 2-mercaptopoetanol and centrifuged at 10,500g for 60 min at 4°C. The supernatant obtained was incubated at 25°C for 3 h with 0.6 M NH₄HCO₃ (pH 8.0), 0.1 M 2-mercaptopoetanol, 0.1 M NaF and 15 mM 5-CMP. Furthermore, the solution was incubated at 30°C for 20 min with (6-3H)FdUMP, 2 mM tetrahydrofolate, 16 mM ascorbate, 9 mM formaldehyde, 15 mM 5-CMP, 20 mM 2-mercaptopoetanol and 100 mM NaF and centrifuged at 2000g for 5 min. The supernatant was removed and trichloroacetic acid was added to the pellet. The triitated water formed during incubation was then assayed with a liquid scintillation counter. The samples to measure total TS content were prepared by fully dissociating the ternary complex present in the cytosol to unbound TS at pH 8.0 in the preincubation period. To determine the (6-3H)FdUMP binding sites as a measure of TS content, the protein concentration was determined using a protein assay kit (Bio-Rad, Richmond, CA, USA). TS content was expressed as pmol/mg of protein.

DPD activity. Measurement of DPD activity was based on the method of Takechi et al. (23). Briefly, tumor tissue was homogenated in 3 volumes of 0.2 M potassium phosphate (pH 8.0) containing 1 mM 2-mercaptopoetanol and 1 mM EDTA, and centrifuged at 10,500g for 60 min at 4°C, and the supernatant was collected. The enzyme solution mixture (2 mM dithiothreitol (DTT), 5 mM MgCl₂, 20 μM (6-14C)5-FU (56Ci), 100 μM NADPH) was incubated at 37°C for 10 min or 30 min. After centrifugation, a 5-μl aliquot of the supernatant was applied to a thin-layer chromatography plate (silica gel 60 F254; Merck, Darmstadt, Germany). The plates were developed with a mixture of 99% ethanol and 1 M ammonium acetate (5:1, v/v). The densities of 5-FU and degradation products were calculated using an image analyzer (Bio-Rad). DPD activity was expressed as pmol/mg of protein/min.
Real-time RT-PCR. The total RNA of the sample was isolated as outlined by the manufacturer in the RNeasy mini kit (Qiagen, Inc., Chatsworth, CA, USA). The purity and amount of total RNA were estimated spectrophotometrically by measuring the absorbance of an aliquot at 260 and 280 nm. The integrity of the rRNA bands was checked by agarose gel electrophoresis in the presence of formaldehyde. Reverse transcription using up to 1 μg of total RNA was carried out in a total volume of 20 μl containing 250 pmol oligo(dT)$_{15}$, 20 units of RNasin (Promega, Madison, WI, USA) and 200 units of Molony murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD, USA) in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl$_2$, 10mM DTT and 0.5 mM deoxynucleotide triphosphate solution. Initially, the total RNA solution mixed with oligo(dT)$_{15}$ was heated at 70°C for 10 min and immediately chilled on ice. First-strand cDNAs were obtained after 60 min at 37°C and 5 min at 70°C, and these synthesized products were stored at -20°C until use.

All PCRs were performed by quantitative real-time methods using the "LightCycler Quick System 330" (Roche Molecular Biochemicals, Idaho, USA). PCR was performed in a 20-μl reaction mixture containing 16 μl of "LightCycler-FastStart DNA master SYBR Green I" (Molecular Probes, Eugene, Oregon)

Figure 1. Analysis of real-time PCR. Linear relationship between threshold and logarithm of the starting copy number of GAPDH cDNA plasmid.
USA), 10 μM of each primer, 2 μl of each 10-fold diluted cDNA sample or appropriately diluted cDNA plasmid sample, and ddH₂O in 20-μl LightCycler capillary tubes. Thermal cycling conditions comprised an initial denaturation step at 95°C for 3 min and 33 cycles at 95°C for 15 sec, 59°C for 10 sec, 72°C for 19 sec and 85°C for 1 sec. To control for variation in the number of samples, mRNA was quantified in relation to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The PCR primer sequences used were as follows: TS primers: forward primer, 5'-CCCTCTGCCAGTTCTATGTG-3' and reverse primer, 5'-AAAGGTCTGGGTTCTCGCT-3'; DPD primers: forward primer, 5'-AGGCTATACAGTTTGATCC-3' and reverse primer, 5'-AATCACCTTAACACACCG-3'; and GAPDH primers: forward primer, 5'-CAACAGCCTCAAGATCATCAGC-3' and reverse primer, 5'-TTCTAGACGGCAGGTCAGGTC-3'. They were designed according to human TS, DPD and GAPDH (24) cDNA sequences. A standard curve was constructed with 5 points representing 10-fold serial dilutions of each cDNA plasmid. Relative mRNA amounts of TS or DPD were expressed as ratios of the targeted gene to GAPDH RT-PCR products. The standard curve of GAPDH is shown in Figure 1.

Statistical analysis. Statistical analysis was performed on a personal computer with Stat View® Ver. 5.0 software (SAS Institute Inc., Cary, NC, USA). Statistical differences between the two groups were evaluated using the Mann-Whitney U-test. To evaluate the correlation between two variables, linear regression was performed and the Spearman rank correlation coefficient was calculated. A p-value of less than 0.05 was considered to be statistically significant.

Results

Enzymatic activities of TS and DPD. The mean TS content in non-cancerous lung tissues (n=48) was 0.029±0.026 pmol/mg protein, and in cancer tissues (n=48) was 0.070±0.045 pmol/mg protein. The TS content in NSCLC was approximately 2.4-fold higher than that of the normal lung (p<0.0001)(Figure 2A). The mean of DPD activity in non-cancerous tissues (n=46) was 84±60 pmol/mg protein/min, and in cancer tissues (n=46) was 414±176 pmol/mg protein/min. DPD activity in NSCLC was approximately 5-fold higher than in normal lung (p<0.0001)(Figure 2B).

The mean TS and DPD activities of NSCLC were 0.099 pmol/mg (n=77) and 407 pmol/mg/min (n=77), respectively. TS activities in adenocarcinoma (AD)(n=43) and squamous cell carcinoma (SQ)(n=28) were 0.106 and
0.090 pmol/mg, respectively. DPD activities in AD (n=43) and SQ (n=28) were 436 and 362 pmol/mg/min, respectively. There was a tendency for TS and DPD activities of AD to be higher than those of SQ, but the differences were not significant ($p=0.491$, $p=0.189$).

The relationship between TS, DPD activities and the patients’ clinicopathological factors (age, gender, stage and histological type) is shown in Table II. There was no significant difference between TS and DPD activities and any clinical findings.

**Correlation between TS and DPD activity and mRNA expression.** As shown in Figure 3, there was a statistically significant correlation between DPD activity and DPD mRNA level, with a coefficient of correlation of 0.846 ($p<0.001$). A positive correlation was also noted between TS activity and TS mRNA level (Figure 4) with a coefficient of correlation of 0.757 ($p<0.001$).

**Discussion**

TS protein is an essential enzyme for DNA synthesis and its expression has been reported to be associated with cell proliferation status. TS is a target enzyme and its expression in tumor cells is thought to be related to 5-FU sensitivity. It has been revealed in some experimental studies that high TS activity or expression in tumor cells was related to 5-FU resistance (11, 25). The role of TS in sensitivity to 5-FU, however, is still controversial. TS protein or gene expression has been reported to be strongly associated with response to 5-FU treatment and patient survival after chemotherapy (25-29), but the correlation between sensitivity to 5-FU and TS activity has been reported to be relatively poor in panels of human tumor cell lines (11, 30), and the absence of any correlation between TS activity and sensitivity to 5-FU has been documented in several recent reports (31-33).

DPD is a rate-limiting enzyme that catalyzes 5-FU degradation (10). Maintaining an intratumoral concentration of 5-FU, which may depend on intratumoral DPD activity, may be important in obtaining an effective response to 5-FU chemotherapy. Several investigators have reported that a high intratumoral DPD level is associated with low antitumor activity of 5-FU due to increased 5-FU inactivation (11, 15, 26, 31, 34).

In this study, TS and DPD activities in NSCLC tissues and their paired non-cancerous lung tissues were measured using biochemical assays. Both TS and DPD activity levels in NSCLC tissues were significantly higher than those in their paired non-cancerous lung tissues. These data are compatible with previously reported data (35-38).
NSCLC, it was not expected that a sufficient effect would be expressed by the chemotherapy of 5-FU because of the high level of DPD activity in lung cancer tissue. On the other hand, UFT is a combination drug, containing Tegafur and Uracil at a molar ratio of 1:4. Tegafur, a prodrug of 5-FU, is converted into 5-FU in vivo. Uracil inhibits 5-FU degradation by DPD. A clinical effect of UFT administration for patients with NSCLC has been reported in some prospective randomized studies (5-7). UFT had been used in an adjuvant setting even for patients in NSCLC, and demonstrated significant efficacy (5, 8). A significant survival benefit was observed with adjuvant chemotherapy, but the benefit appeared to be limited to the subgroup with T2N0 disease (8).

There were no significant correlations between any clinical factors including age, gender, stage and histological type and TS and DPD activities in NSCLC tissues. Otake et al. (36) showed a low incidence of TS-positive expression in squamous cell carcinoma of the lung in contrast to other histological types, but this study demonstrated that there were no clinical findings or markers to predict the values of TS and DPD in patients. Therefore, our results support their findings.

This is the first study to compare both expression of TS and DPD mRNA and enzymatic activity in NSCLC, in order to select the population for which UFT would be effective, so it was important to measure the TS and DPD activities of resected lung cancer tissue. Although determination of TS and DPD levels in tumor tissue is important for predicting the clinical therapeutic efficacy of 5-FU, routine measurement of enzyme activity is not technically feasible in many centers. Accordingly, we developed a quantitative real-time RT-PCR method for the determination of TS and DPD mRNA expression, which has proven extremely useful for the measurement of small specimens, such as biopsies (26), without the need for radioisotopes. In the present study, a statistically significant correlation was observed (rs=0.846, p<0.001) between tumoral DPD activity and mRNA expression. Thus, determination of DPD mRNA expression by quantitative real-time RT-PCR reflects the DPD activity of NSCLC tissue, which should in turn allow prediction of the sensitivity of the tumor to 5-FU. Also, a statistically significant correlation was found between TS mRNA level and TS content (rs=0.757, p<0.001). Ishikawa et al. (31) and Fujiwara et al. (39) used the semi-quantitative RT-PCR method and reported a good correlation between enzyme activity and TS and DPD mRNA levels in human tumor (gastric carcinoma, colon carcinoma, pancreatic carcinoma and breast carcinoma) xenografts in nude mice. Their results also showed closer correlations between enzyme activity and mRNA level of DPD (rs=0.81, 0.90) than those of TS (rs=0.62, 0.70). As the findings of human carcinoma xenografts in nude mice showed less dispersion than those of clinical specimens, their results support ours. Chu et al. (40,41) reported that TS mRNA translation was inhibited in the presence of TS protein and that there was a specific interaction of TS protein with its mRNA. This confirmed the presence of a looser correlation between TS mRNA level and TS content than between DPD mRNA level and DPD activity, and our results thus support their findings. In colorectal cancer, the sensitivity to 5-FU may be regulated by DPD mRNA (42), and high TS mRNA expression predicts non-response to raltitrexed (TS inhibitor) (43). The lack of correlation between TS mRNA...
levels below the response threshold and TS protein is disappointing, whereas this correlation was stronger between higher mRNA expression and increased protein, confirming the data from previous studies. In lung cancer, no data has yet been reported regarding the expression of TS and DPD mRNA and the response to 5-FU.

This study confirms the feasibility of treating patients with a drug regimen determined by the pattern of gene expression of their tumors. It is surprising, given the known histological heterogeneity of metastatic tumors, that such accurate predictions could be obtained from biopsy of a lesion. TS and DPD mRNA may increase the predictive value even further and allow the prospective rationalization of therapy.

In conclusion, the TS and DPD activities of NSCLC were higher than those of normal lungs, and no clinical findings could reflect TS and/or DPD activities. A statistically significant correlation was observed between tumoral DPD activity and mRNA expression, and a statistically significant correlation was observed between tumor TS activity and mRNA expression. Measurement of DPD and TS expression using real-time RT-PCR is one of the predictors for the response to fluoropyrimidine-based chemotherapy.

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References


