Predictive Value of Thymidylate Synthase and Dihydropyrimidine Dehydrogenase Expression in Tumor Tissue, Regarding the Efficacy of Postoperatively Administered UFT (Tegafur+Uracil) in Patients with Non-small Cell Lung Cancer

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Abstract. *Background: UFT (tegafur + uracil) has been reported to be effective as an adjuvant in postoperative chemotherapy for non-small cell lung cancer (NSCLC) in a randomized prospective study. Thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) expression were investigated in resected tumors and the relationship between their expression and clinical factors in NSCLC patients was examined. Patients and Methods: Fifty-four NSCLC patients had undergone complete surgical resection and lymph node dissection, and had been administered UFT post-surgery. The TS and DPD expression in the tumor tissues was evaluated by immunohistochemical staining. The relationship between TS and/or DPD expression and clinicopathological factors was examined. Results: There were 38 TS-negative and 16 TS-positive cases, and 22 DPD-negative and 32 DPD-positive cases. There was no significant difference between the patients with TS or DPD and those without TS or DPD in age, gender, histological type or p-stage. The 5-year survival rates of patients positive and negative for TS were 50.0 and 89.5%, while 10-year survival rates were 23.3 and 79.7%, respectively ($p<0.001$). The 5-year survival rates of TS-positive and TS-negative patients in p-stage I were 54.6 and 95.5%, while 10-year survival rates were 22.7 and 95.5%, respectively ($p<0.001$). There was no significant difference between DPD-positive and DPD-negative patients in prognosis. Conclusion: The oral administration of UFT after surgery might improve the survival of NSCLC patients when TS levels in tumor tissues are low. Immunohistochemical evaluation of TS and DPD expression may be useful for predicting the efficacy of UFT after complete resection in NSCLC.*

Lung cancer is the most common cause of death from cancer worldwide (1) and non-small cell lung cancer (NSCLC) is a major histological type. 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 has been reported as 67-79% for stage I, but the prognosis for patients with stage II or III disease is poor (2, 3). Adjuvant therapies to prevent recurrence and metastasis after surgery are needed. 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 metabolities, 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-CH_2FH_2), which blocks TS activity and inhibits the *de novo* synthesis of thymidylate for DNA synthesis (4).

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 (5). Some experimental studies have shown that weak DPD activity in tumor cells is related to greater sensitivity to 5-FU (6-9). In some clinical studies, DPD activity in tumors has been found to be correlated with the clinical response to 5-FU-based chemotherapies (10-12).

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Key Words: Thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), non-small cell lung cancer.

UFT is an anticancer drug for oral use, developed in Japan, where it has been widely used mainly for treating malignant tumors originating from the digestive system. It contains tegafur (a 5-FU derivative) and uracil (a DPD inhibitor) at a molar ratio of 1:4. Tegafur is converted into 5-FU *in vivo*. UFT has been reported to be an effective postoperative adjuvant for NSCLC in randomized prospective studies (13-15). Wada *et al.* and Kato *et al.* have reported that UFT used in an adjuvant setting for patients with early stage NSCLC demonstrated significant efficacy (13, 16).

In the present study, TS and DPD expression in resected tumor tissues was investigated using immunohistochemical staining, and the relationship between the efficacy of UFT as an adjuvant for chemotherapy and the expression of TS and DPD in NSCLC was analyzed retrospectively.

Table I. *Clinical factors in patients with lung cancer.*

Factor	Number
Patients	54
Age, mean (range)	61.7±9.6 (38-80)
Gender (M/F)	43/11
Histological types	
adenocarcinoma	32
squamous cell ca.	15
large cell ca.	6
ad-sq cell ca.	1
p-Stage	
I (IA/IB)	33 (19/14)
II (IIA/IIB)	6 (0/6)
III (IIIA/IIIB)	14 (11/3)
IV	1

Patients and Methods

Tumors. The study group comprised 54 patients with NSCLC who had undergone complete surgical resection and lymph node dissection and had been administered UFT post-surgery during the period from 1988 to 1997 at the University of Tokushima hospital. The administration of UFT (260 mg/m^2 per day or 400 mg per body per day) was started within one month after surgery and continued for more than six months. The pathological stage of the disease was determined according to the current TNM classification revised in 1997 (2). The histological types were determined according to the WHO classification (17). Table I outlines the patient characteristics. All patients gave written informed consent to participate in the study.

Immunohistochemical staining. Surgical specimens were fixed in 10% formalin, embedded in paraffin, and cut into 4-μm sections. TS and DPD expression in tumor tissues was evaluated by immunohistochemical staining (IHC) using the rhTS polyclonal antibody RTSSA (18, 19) and DPD polyclonal antibody RDPDPA (20, 21) by the labeled streptavidin biotin (LSAB) method. The relationship between TS and/or DPD expression and clinicopathological factors was examined.

The peroxidase blocking reagent was obtained from DAKO Co. Ltd. (Santa Barbara, CA, USA). Normal goat serum, biotinylated goat anti-rabbit IgG, and streptavidin-biotynylated-peroxidase complex were obtained from DAKO (LSAB kit). Diaminobenzidine tetrahydrochloride (DAB) was purchased from Vector Co. Ltd. (Burlingame, CA, USA). All other chemicals were commercial products of analytical grade.

The staining procedure was as follows. After tissue sections were deparaffinized and rehydrated, endogenous peroxidase activity and nonspecific binding were blocked. The sections were then incubated with anti-TS antibody (1:1000) or anti-DPD antibody (1:400) as a primary antibody at 4˚C overnight. Biotinylated goat anti-rabbit IgG antibody was applied for 30 minutes at room temperature. Peroxidase activity was visualized with the DAB solution for 3 minutes at room temperature. Counterstaining was performed with hematoxylin for 1 minute at room temperature.

Sections of human colonic cancer tissue obtained from nude mice implanted with the cell line DLD-1/FdUrd and positive for TS were used as positive controls in each staining. Sections of human pancreatic cancer tissue obtained from the implanted cell line MIAPaCa-2 in nude mice that is positive for DPD were used as positive controls in each staining. The negative control was prepared by omitting the primary antibody for each section.

Evaluation of staining. Each section was evaluated separately by two of us (T.M. and K.K.) without knowledge of the clinical data. The TS and DPD expression was semiquantitated using a visual grading system in which the intensity of staining was categorized as "negative" (less than 10% positively-stained cancer cells) and "positive" (more than 10% positively-stained cancer cells) (Figures 1, 2).

Statistical analysis. Statistical analysis was performed with Stat ViewTM Ver. 5.0 software (SAS Institute Inc., Cary, NC, USA). A comparison of clinicopathological features between two groups was performed with the Chi-square test or Student's *t*-test. Survival after surgery was analyzed by the Kaplan-Meier method and an evaluation of the difference was conducted with the log-rank test. A *p*-value of less than 0.05 was considered to be statistically significant.

Results

Expression of TS. The relationship between TS expression and clinicopathological factors is summarized in Table II. TS expression was positive in 16 patients (29.6%) and negative in 38 (70.4%). The TS-positive group seemed to be older than the TS-negative group (66.1 *versus* 59.9 years), but the difference was not significant. There were no significant differences between the TS-positive and TS-negative patients in gender, histological type or stage of disease.

Expression of DPD. The relationship between DPD expression and clinicopathological factors is summarized in Table III. DPD expression was positive in 32 patients (59.3%) and negative in 22 (40.7%). There was no significant difference between the DPD-positive and DPD-negative patients in age, gender, histological type or tumor stage.

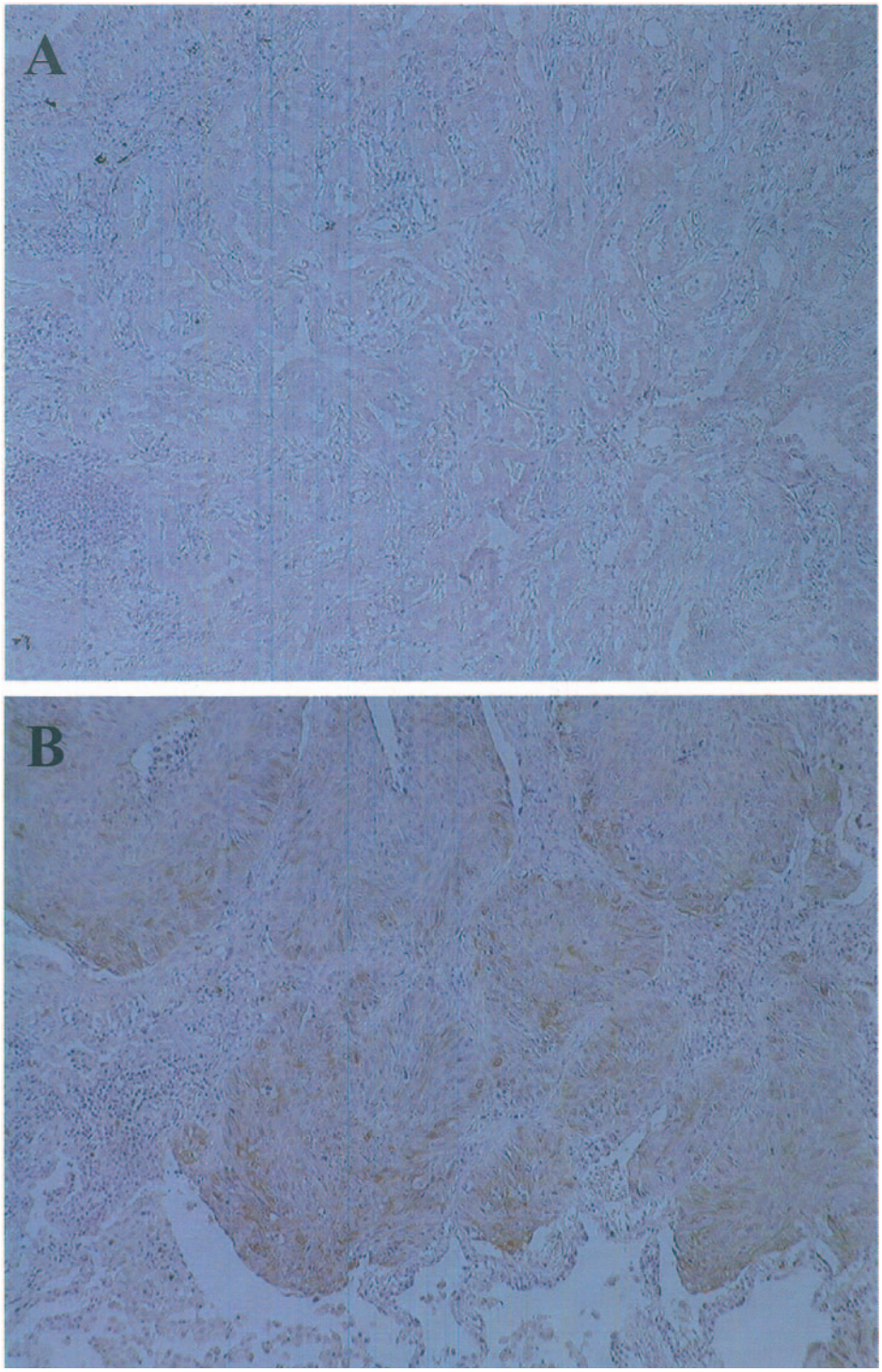

Figure 1. *Immunohistochemical staining for TS in tumor tissues. The intensity of the staining was evaluated using a visual grading system as "Negative" (A) or "Positive" (B). TS expression was identified as granular cytoplasmic staining in tumor cells (H&E stain x100).*

Survival. A comparison of survival rates after surgery according to the TS and DPD staining is shown in Figures 3-5. The 5-year survival rates of patients positive and negative for TS were 50.0 and 89.5%, while the 10-year survival rates were 23.3 and 79.7%, respectively, with significant differences in both cases ($p<0.001$). Furthermore, the 5-year survival rates

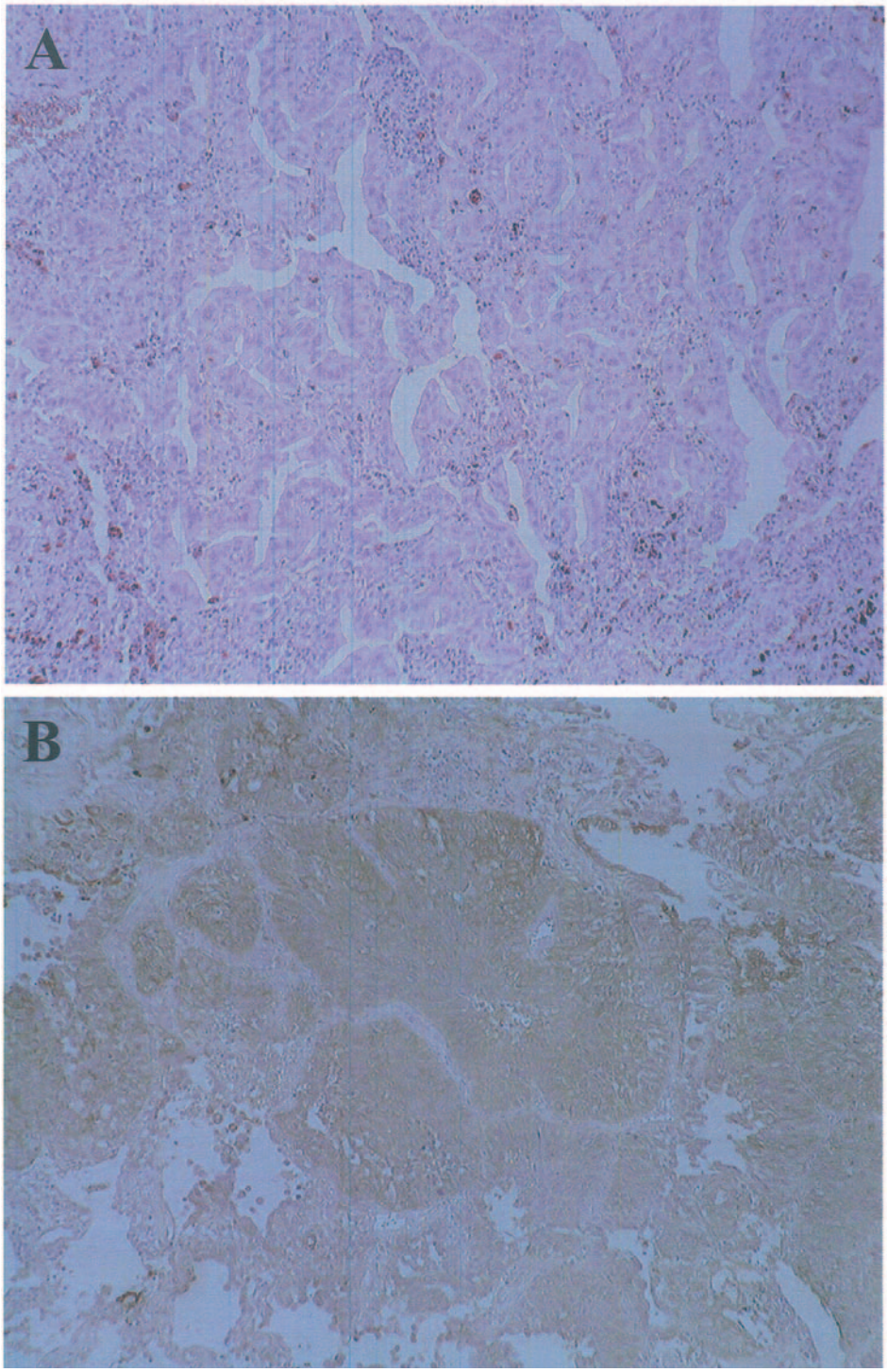

Figure 2. *Immunohistochemical staining for DPD in tumor tissues. The intensity of the staining was evaluated using a visual grading system as "Negative" (A) or "Positive" (B). DPD expression was identified as granular cytoplasmic staining in tumor cells (H&E stain x100).*

of stage I patients positive and negative for TS were 54.6 and 95.5%, while the 10-year survival rates were 22.7 and 95.5%, respectively, with a significant difference between these groups ($p<0.001$) (Figure 4). The 5- and 10-year survival rates of TS-positive patients seemed to be lower than those of TS-negative patients (40.0% *versus* 80.0%,

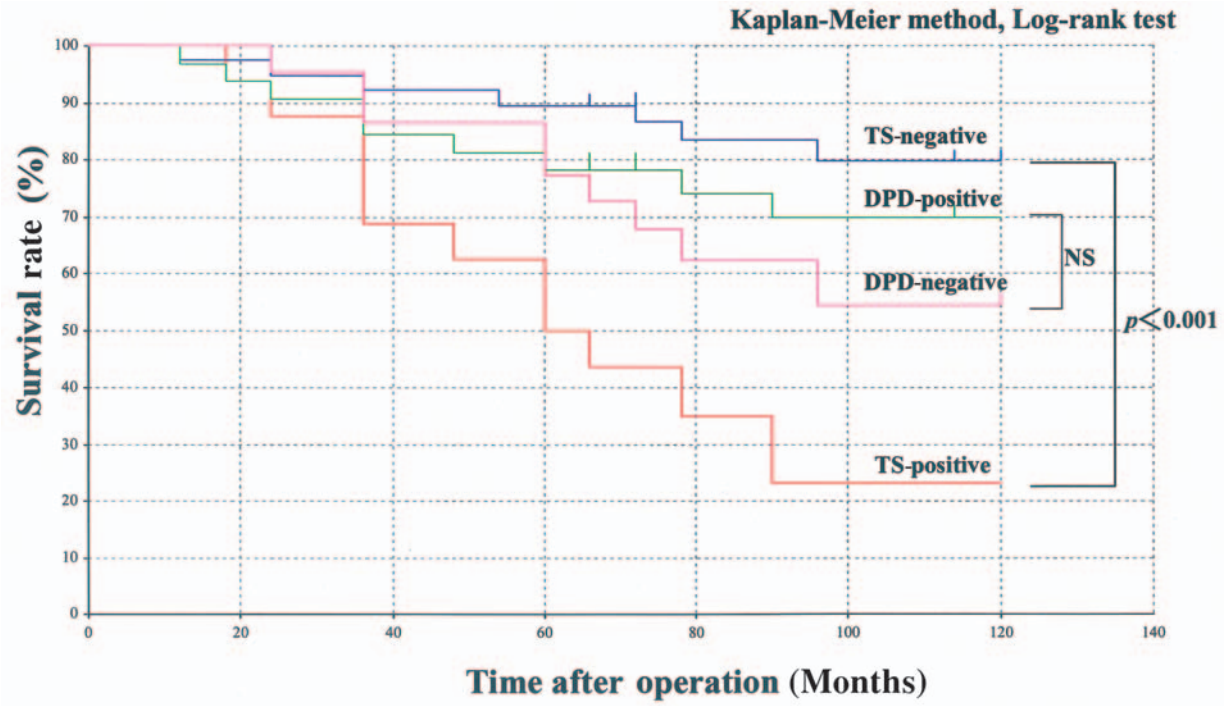

Figure 3. *Comparison of 10-year survival curves of all patients. The TS-positive group had a significantly worse prognosis than the TS-negative group (p<0.001). There was no significant difference between the DPD-negative and DPD-positive groups (p=0.390).*

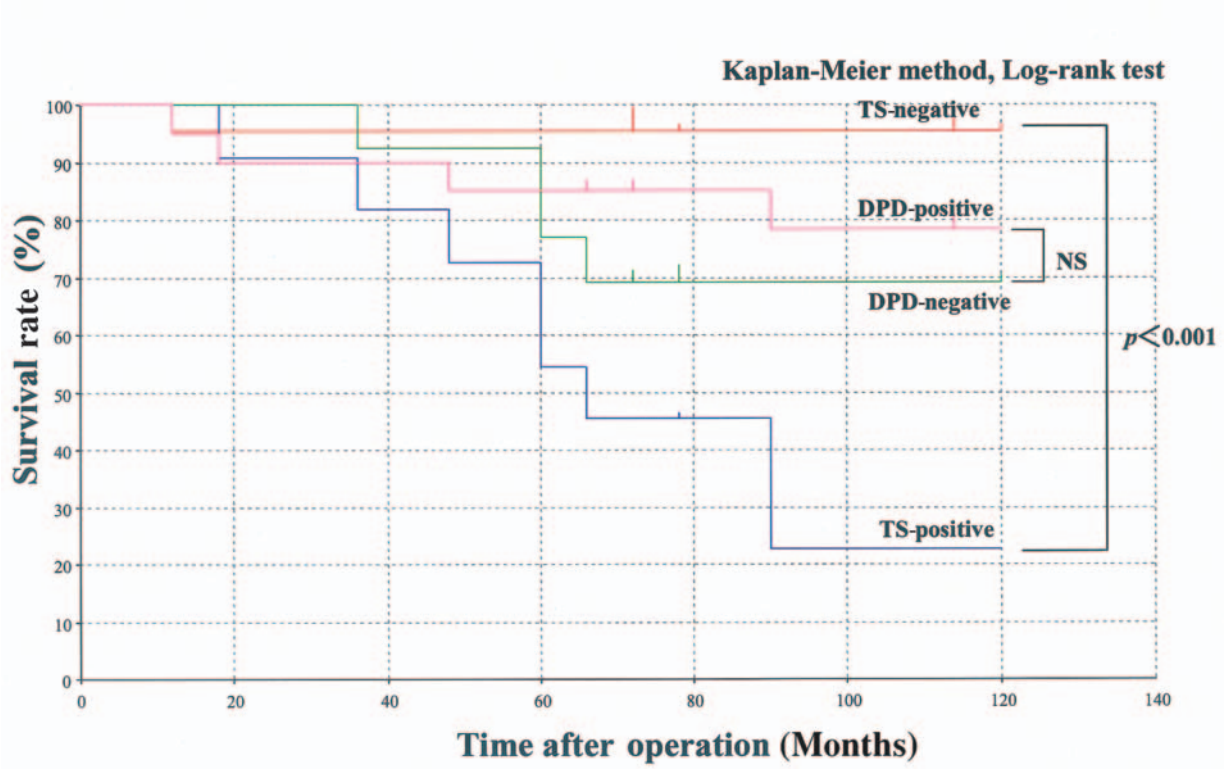

Figure 4. *Comparison of 10-year survival curves of p-stage I patients. The TS-positive group had a significantly worse prognosis than the TS-negative group (p<0.001). There was no significant difference between the DPD-negative and DPD-positive groups (p=0.486).*

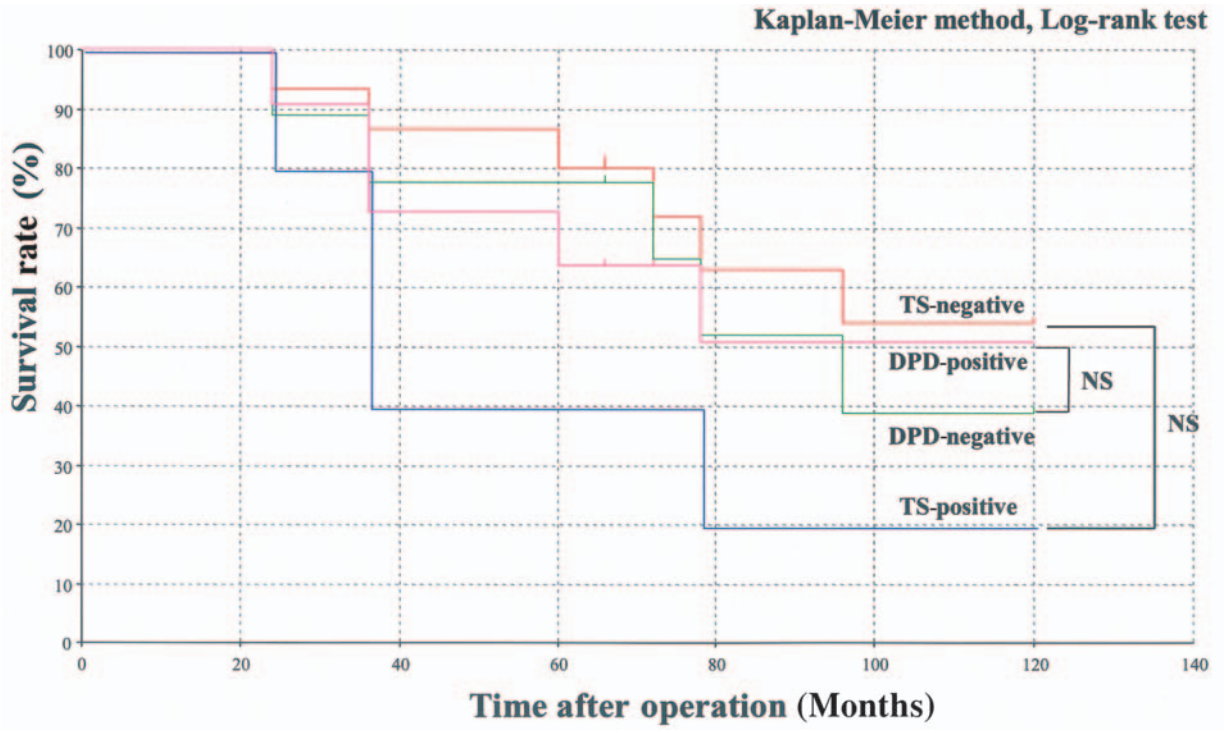

Figure 5. *Comparison of 10-year survival curves of p-stage II, III and IV patients. There was no significant difference between the TS- or DPD-negative group and TS- or DPD-positive group (p=0.097, 0.844).*

Table II. *Relationship between clinical factors and TS.*

Factor	TS-negative n=38	TS-positive n=16	*P*-value
Age (years)	59.9±8.9	66.1±10.2	0.058
Gender			
Male	30	13	0.848
Female	8	3	
Histological types			
Adenocarcinoma	23	9	
Squamous cell ca.	10	5	0.934
Other	5	2	
p-Stage			
I	22	11	
II	6	0	0.241
III, IV	10	5	

Table III. *Relationship between clinical factors and DPD.*

Factor	DPD-negative n=22	DPD-positive n=32	*P*-value
Age (years)	63.5±9.0	60.5±10.0	0.264
Gender			
Male	17	26	0.721
Female	5	6	
Histological types			
Adenocarcinoma	15	17	
Squamous cell ca.	5	10	0.530
Other	2	5	
p-Stage			
I	13	20	
II	2	4	0.827
III, IV	7	8	

20.0% *versus* 54.0%) among the stage II, III and IV cases, but the difference was not significant (Figure 5).

The 5-year survival rates of patients positive and negative for DPD were 78.1 and 77.3%, while the 10-year survival rates were 69.9 and 54.4%, respectively, with no significant difference in either case. Similarly, the 5-year survival rates of DPD-positive and DPD-negative stage I patients were 85.0 and 76.9%, while the 10-year survival rates were 78.5 and 69.2%, respectively, with no significant difference between these groups (Figure 4). The 5-year survival rates of the DPD-positive and DPD-negative patients were 63.6 and 77.8%, while the 10-year survival rates were 50.9 and 38.9%, respectively, with no significant difference among the stage II, III and IV cases (Figure 5).

Discussion

The results of the present study showed that the patients with TS-negative tumors treated with surgery and UFT had significantly better survival than the patients with TS-positive tumors ($p<0.001$) and in the patients with p-stage I tumors the difference was particulary remarkable. A significant survival benefit using UFT as an adjuvant for NSCLC apparently limited to the subgroup with T2N0 disease has also been demonstrated by Kato *et al.* (16). This finding suggests that the expression of TS in tumors serves as a possible predictor of the efficacy of administering UFT after complete resection for NSCLC. In other words, the evaluation of TS expression in p-stage I NSCLC patients might lead to individualized treatment. However, high-level p-stage (II, III or IV) or high-level TS expression in p-stage I patients might need an adjuvant other than UFT.

In our previous study, the levels of TS and DPD activity were higher in cases of NSCLC than in normal lungs, and no clinical findings reflected the TS and/or DPD activities. The measurements of TS and DPD expression using real-time reverse transcription-polymerase chain reaction (RT-PCR) were predictive of the response to fluoropyrimidine-based chemotherapy (22).

TS is an essential enzyme for DNA synthesis and its expression has been reported to be associated with cell proliferation status (23). Its expression in tumor cells is thought to be related to sensitivity to 5-FU. The role of TS in sensitivity to 5-FU, however, is still controversial. While TS protein or gene expression has been reported to be strongly associated with the response to 5-FU treatment and patient survival after chemotherapy (24-28), the correlation between sensitivity to 5-FU and TS activity has been reported to be relatively poor in panels of human tumor cell lines (6, 29), and the absence of any correlation between TS activity and sensitivity to 5-FU has been documented in several recent reports (30-32). Fujiwara *et al.* reported that the role of TS in sensitivity to 5-FU might be more accurately demonstrated by real-time RT-PCR than by enzyme activity because it provides a very broad dynamic range, and TS mRNA levels are associated with the response to 5-FU treatment and patient survival after 5-FU-based chemotherapy (33).

In contrast to TS, in the present study, the expression of DPD did not influence the prognosis in cases of NSCLC. Nakagawa *et al.* have reported that the oral administration of UFT after surgery might improve the survival of patients with p-stage I NSCLC when the level of DPD expression in tumor tissue is low (21). No difference in prognosis between high-DPD tumors and low-DPD tumors in the UFT-administered group was reported. Therefore, the uracil of UFT might be able to inhibit DPD activity, and there is a possibility that it could promote the effect of 5-FU.

In conclusion, the present study suggested that oral administration of UFT after surgery might improve the survival of NSCLC patients when the TS expression level in the tumor tissue is low. The expression of DPD did not influence the prognosis in NSCLC. The uracil of UFT might be able to inhibit DPD activity, and there is a possibility that it could promote the effect of 5-FU. However, as this study was performed retrospectively, a prospective randomized study will be needed to fully elucidate the role of TS expression in NSCLC as a predictor of UFT efficacy.

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