Thymidylate synthase and dihydropyrimidine dehydrogenase expression in oral squamous cell carcinoma: An immunohistochemical and clinicopathologic study

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Objective. Thymidylate synthase (TS) is the target enzyme for 5-fluorouracil (5-FU), and dihydropyrimidine dehydrogenase (DPD) is the first enzyme that metabolizes 5-fluorouracil. Until now, only the enzyme activities of TS and DPD have been investigated; however, there are few reports about the immunohistochemistry of TS and DPD and none regarding oral carcinoma. The purpose of this article was to investigate the expression of TS and DPD in oral squamous cell carcinoma.

Study design. In this study, 109 oral squamous cell carcinomas were investigated for the immunohistochemical expression of TS and DPD proteins.

Results. The expressions of TS in carcinoma cases was significantly higher than in controls (P < .05, t test). DPD was expressed both in carcinomas and in areas adjacent to the carcinomas. There was no correlation between the clinical factors and the TS labeling index or between the clinical factors and the DPD labeling index (DPD-LI). Pathologically, DPD-LI was significantly different in both the World Health Organization classification and Anneroth's classification. The TS labeling index was significantly correlated with the Ki-67 LI (P < .05, Pearson's correlation coefficient). Although TS showed no correlation between tegafur-uracil response and TS labeling index, there was a significant correlation between the tegafur-uracil response and DPD-LI.

Conclusions. TS may reveal tumor cell proliferation, but DPD-LI may correlate with a response to anticancer drug treatment.


5-Fluorouracil (5-FU) is one of the most widely used chemotherapeutic agents for the treatment of oral cancer. The main mode of action of 5-FU is thought to be through its active metabolite, 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP). FdUMP suppresses thymidylate synthase (TS). The mechanism of 5-fluorouracil (5-FU) resistance involves modifications related to the intracellular anabolism and alterations of TS. TS is a rate-limiting enzyme in de novo DNA biosynthesis, catalyzing the methylation of deoxuryridine monophosphate (dUMP) to deoxythymidine (dTMP) for the folate cofactor 5,10-methylene tetrahydrofolate. However, with the administration of 5-FU, the metabolic product FdUMP forms a tight-binding covalent complex with TS and thereby blocks the DNA synthetic process. Several reports have indicated that tumoral
Table 1. Correlation between clinical factors and expressions of TS and DPD

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>TS-LI (%)</th>
<th>DPD-LI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoma cases</td>
<td>109</td>
<td>21.3 ± 14.6</td>
</tr>
<tr>
<td>Controls</td>
<td>10</td>
<td>6.3 ± 4.2</td>
</tr>
<tr>
<td>Age (range 34-91)</td>
<td>(mean ± SD)</td>
<td>(mean ± SD)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>67</td>
<td>24.2 ± 14.6</td>
</tr>
<tr>
<td>Female</td>
<td>42</td>
<td>19.4 ± 14.4</td>
</tr>
<tr>
<td>T categories</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>28</td>
<td>21.6 ± 15.3</td>
</tr>
<tr>
<td>T2</td>
<td>40</td>
<td>20.3 ± 15.4</td>
</tr>
<tr>
<td>T3</td>
<td>8</td>
<td>17.7 ± 16.1</td>
</tr>
<tr>
<td>T4</td>
<td>33</td>
<td>23.0 ± 12.9</td>
</tr>
<tr>
<td>N categories</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>75</td>
<td>20.5 ± 14.6</td>
</tr>
<tr>
<td>N1</td>
<td>16</td>
<td>22.0 ± 13.8</td>
</tr>
<tr>
<td>N2</td>
<td>18</td>
<td>23.6 ± 14.1</td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>25</td>
<td>21.3 ± 14.9</td>
</tr>
<tr>
<td>II</td>
<td>31</td>
<td>20.4 ± 16.7</td>
</tr>
<tr>
<td>III</td>
<td>12</td>
<td>22.5 ± 15.9</td>
</tr>
<tr>
<td>IV</td>
<td>41</td>
<td>21.5 ± 12.6</td>
</tr>
</tbody>
</table>

TS-LI. Thymidylate synthase labeling index; DPD-LI, dihydropyrimidase dehydrogenase labeling index.

*P < .05, t test.

TS expression is significantly related to the response to 5-FU-based chemotherapy and to patient survival in gastric and colorectal cancers.6-10 Other reports have shown that tumoral TS expression and TS immunohistochemical staining grade could be used as independent prognostic factors for patients with colorectal and gastric carcinoma.11,12 TS is thought to be important both as an indicator of DNA synthesis/tumor growth and as a target enzyme of 5-FU. 5-FU is catabolized to 2-fluoro-β-alanine, mainly in the liver, by 3 enzymes: dihydropyrimidase dehydrogenase (DPD, the first and rate-limiting enzyme), followed by dihydropyrimidase and β-ureidopropionase. DPD exists not only in liver cells, but also in human tumor cells. Several studies have reported3,13-14 that DPD activities are associated with drug efficacy and prognosis. In head and neck cancer, Etienne et al15 determined tumoral/nontumoral DPD activity ratios (normalized DPD) in tumor biopsy specimens from patients before 5-FU treatment and reported that complete responders exhibited significantly lower normalized DPD values than partial and nonresponding patients.

Almost all studies concerning TS and DPD in cancers have determined enzyme activity by biochemical and molecular techniques.7-10 These assays may be sensitive but may also present special technological difficulties. Immunohistochemical evaluation of TS and DPD is simple and easy to perform. There are few immunohistochemical analyses of TS,11,12 especially in oral cancer.

In this study, we examined the expression of TS and DPD immunohistochemically by using polyclonal antibodies; we also investigated the relationship between the immunohistochemical score and the clinicopathologic factors. Furthermore, we examined the effectiveness of tegafur-uracil (UFT) on oral squamous cell carcinoma by an analysis of TS and DPD.

**PATIENTS AND METHODS**

One hundred nine oral carcinoma patients who underwent treatment between 1986 and 1999 at the First Department of Oral and Maxillofacial Surgery, Nagasaki University School of Dentistry, Japan, were enrolled in this study (Table I). There were 67 men and 42 women. The mean age was 64 years (range, 34-91 years). Oral carcinoma biopsies were fixed in 4% buffered formalin and embedded in paraffin. To prevent a reduction in immunoreactivity, the fixation time did not exceed 48 hours. Tissue sections 4-μm thick were cut and prepared for histologic examination. Histologic examination was carried out with hematoxylin and eosin staining. All tumors were staged following the International Union against Cancer staging system.16 Furthermore, each case was analyzed by Anneroth’s classification17 and each was evaluated for histologic malignancy grade by using Anneroth’s score. As controls, normal oral mucosa (n = 10) were collected and analyzed.

The expression of TS and DPD were studied immunohistochemically with polyclonal antibodies, which were generous gifts from Taiho Pharmachemical Co Ltd, Saitama, Japan. These polyclonal antibodies have been demonstrated by Western blot analysis and immunohistochemistry to react specifically with intracellular TS and DPD. For tumor cell proliferating factor evaluation, MIB-1 (Immunotech, Marseille, France) was used as the monoclonal antibody for Ki-67.

For the immunohistochemical staining procedure, 4-μm-thick sections were cut from the paraffin-embedded cancer tissue. The sections were dewaxed with xylene and rehydrated gradually with graded alcohols. Endogenous peroxidase activity was blocked by soaking the sections in 3% hydrogen peroxidase for 30 minutes. After being washed in Dulbecco’s phosphate-buffered saline (PBS), the sections were incubated with primary antibodies of TS (dilution, 1:1000) and DPD (dilution, 1:500) at 4°C overnight. After being washed 5 times in PBS, the sections were incubated with EN-VISION+ (Dako Corp, Carpenteria, Calif) for 30 minutes. Regarding Ki-67 staining, tissue sections of 4-μm thickness were placed on albumin-coated glass slides and prewarmed overnight, followed by an antigen retrieval procedure of 121°C autoclaving treatment for 5 minutes in 0.01 mol citrate buffer solution (pH...
6.0). The slides were treated with 3% H₂O₂ in methanol to deactivate peroxidase and incubated with 10% rabbit serum for 30 minutes to block nonspecific binding. Next, they were incubated with the primary antibody antihuman Ki-67 (MIB-1; Immunotech), then diluted at 1:50 overnight in a humidified chamber. Slides were washed in PBS, and the sections were incubated with ENVISION+ (Dako) for 30 minutes. The immunohistochemical reaction was revealed with a solution of 3,3'-diaminobenzidine tetrahydrochloride in 50 μmol Tris buffer (pH 7.6) containing 10 μL of 30% H₂O₂. The reaction was stopped after 10 minutes by the addition of tap water. The sections were then briefly counterstained with Mayer’s hematoxylin and mounted.

Labeling indices (LI) for immunostaining TS, DPD, and Ki-67 were calculated by counting the positive cells among more than 500 epithelial cells in 5 randomly selected fields.

The percentage of immunoreactive positive cells (LI) was summarized as mean ± SD (%). Significance was assessed by means of the Student t test and Pearson’s correlation coefficient (statistical significance when $P < .05$).

RESULTS
Expression of TS and DPD in oral cancer
TS was expressed in the cytoplasm in tumor cells and was typically seen in the central portion of tumor cell nests (Fig 1, 2). Cases that revealed TS positivity at the peripheries were few. Although TS was seen the control oral mucosal epithelial cells, there was a significant difference ($P < .05$, t test) in TS-LI between controls and carcinoma cases. TS in controls was expressed in the granular and spinosum layer.

TS-LI ranged from 0 to 50.8%, and the mean TS-LI was 21.3%. Twelve of all cases were TS-negative. The mean TS-LI of the control cases was 6.3%. There was a significant difference between TS-LI for the control group and carcinoma group.

DPD was expressed in the cytoplasm in the tumor cells, and DPD expression was seen diffusely in the cell nests (Fig 3). DPD was also expressed in all control cases (Fig 4). Although DPD was expressed in the adjacent area to the carcinoma lesion, there was no correlation ($P < .05$, t test) between DPD-LI of carcinoma cells and mucosa adjacent to the carcinoma. DPD-LI ranged from 0 to 44.2%, and the mean DPD-LI was 13.8%. The mean DPD-LI of the control group was 10.2%. There was no correlation ($P > .05$, t test) between LI of the control group and the carcinoma group.
There was no correlation between TS-LI and DPD-LI of oral carcinoma cells \( (P > .05, \text{Pearson's correlation coefficient}) \).

**Correlation between TS and DPD expressions and clinical and pathologic factors**

Clinical factors had no correlation \( (P < .05, t \text{ test}) \) with either TS-LI or DPD-LI (Table I). With respect to the World Health Organization histologic grade, there was a significant difference between DPD-LI for the well-differentiated group and DPD-LI for the moderately and poorly differentiated groups (Table II). We divided the cases into 3 groups by Anneroth's classification: low-malignancy group, medium-malignancy group, and high-malignancy group. DPD-LI showed a significant difference \( (P < .05, t \text{ test}) \) between the high-grade tumor group and the low-grade tumor group. TS-LI showed no significant difference \( (P < .05, t \text{ test}) \) in any pathologic factors.

**Correlation between TS and Ki-67**

There was a significant correlation \( (P < .05, \text{Pearson's correlation coefficient}) \) between TS-LI and Ki-67 LI. Although expression of Ki-67 could be seen in the peripheral regions of the tumor cell nests, expression of TS could be seen in the central portions of the tumor cell nests (Fig 5). There was no correlation \( (r = 0.237, \text{Pearson's correlation coefficient}) \) between Ki-67-LI and DPD-LI and no correlation of localization between Ki-67 protein and DPD protein.

**Analysis of TS and DPD according to clinical response to UFT**

Cases treated either with UFT before surgery or by UFT alone totaled 27 of 109 (Table III). UFT dosage was 300 mg/d from 2 weeks to 6 weeks. Response to UFT was observed in 8 of 27 patients, with UFT nonresponse seen in 19 of 27 patients. There was no correlation \( (P > .05, t \text{ test}) \) between TS-LI in the UFT response group and TS-LI in the UFT nonresponse group. DPD-LI in the UFT response group was lower than that in the UFT nonresponse group. The DPD tumoral/nontumoral ratio of the UFT response group was significantly lower \( (P < .05, t \text{ test}) \) than that of the UFT nonresponse group.

**DISCUSSION**

Recently, an immunohistochemical study of TS was reported for cancer of digestive organs. Concerning the relationship between TS and clinicopathologic factors, Yamachika et al.\(^{11}\) reported that TS in colorectal cancer showed significant differences with respect to clinical stage and survival. Kuniyasu et al.\(^{12}\) also reported that in gastric carcinoma the TS staining grade was significantly correlated with 3 clinicopathologic variables: depth of invasion, peritoneal metastasis, and stage. Furthermore, they reported that TS expression was significantly correlated with survival in gastric carcinoma patients. Patients with high TS staining had significantly worse prognoses than those with low TS staining. These results suggest that immunohistochemical expression of TS could be used as an indicator of tumor progression and prognosis in gastric carcinoma. They reported that these results were similar to those previously reported in which TS was investigated by other methods.

TS activity and mRNA levels were high in cancerous tissue, reflecting more active DNA synthesis compared with normal gastric mucosa.\(^{18}\) TS activity is higher during DNA replication and decreases when cells are nondividing\(^{19}\) and is associated with proliferation.\(^{20}\) The activity of TS increases markedly as the cell passes from late G1-phase to early S-phase in the cell cycle and acts as a catalyzing enzyme in the final step in dTMP synthesis.\(^{21}\) Ki-67 is a proliferation cell nuclear antigen located on chromosome 10 and is correlated with the cell cycle and cell growth.\(^{22}\) Ki-67 increases in late S phase and more in G1-G2 and in mitosis. It is not detectable in G0 because it is absent in resting or nonproliferating cells. In this study, there was a significant correlation between TS and Ki-67.

Yamachika et al.\(^{11}\) divided colorectal cancer patients into 2 groups: adjuvant chemotherapy–treated group and nonadjuvant chemotherapy group. In the nonadjuvant chemotherapy group, TS-positive cases had a significantly lower 10-year survival rate than TS-negative cases. In patients with adjuvant chemotherapy, there was no difference in the survival rates of the TS-positive group and TS-negative group. Recent studies
Table II. Correlation between pathologic factors and expressions of TS and DPD

<table>
<thead>
<tr>
<th></th>
<th>No. of patients</th>
<th>TS-LI (%) mean ± SD (%)</th>
<th>DPD-LI (%) mean ± SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHO classification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-differentiated</td>
<td>84</td>
<td>21.2 ± 13.7</td>
<td>16.6 ± 10.4</td>
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<tr>
<td>Moderately differentiated</td>
<td>20</td>
<td>19.3 ± 18.2</td>
<td>5.5 ± 6.5</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>5</td>
<td>29.7 ± 12.2</td>
<td>0.5 ± 1.1</td>
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<tr>
<td>Anneroth’s classification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low malignancy</td>
<td>12</td>
<td>16.0 ± 11.8</td>
<td>23.9 ± 16.3</td>
</tr>
<tr>
<td>Medium malignancy</td>
<td>83</td>
<td>21.2 ± 14.6</td>
<td>14.2 ± 9.0</td>
</tr>
<tr>
<td>High malignancy</td>
<td>14</td>
<td>28.0 ± 16.5</td>
<td>6.0 ± 5.9</td>
</tr>
<tr>
<td>Pattern of invasion (Anneroth’s classification)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>27.7 ± 9.2</td>
<td>10.1 ± 7.9</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>17.0 ± 12.8</td>
<td>16.4 ± 10.7</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>24.8 ± 16.1</td>
<td>13.3 ± 11.2</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>20.1 ± 14.6</td>
<td>12.7 ± 11.2</td>
</tr>
</tbody>
</table>

WHO, World Health Organization.
*P < 0.05, t-test

that used TS binding assays have demonstrated that the
prognoses of patients with cancer and their responses to
5-FU were significantly related to TS expression.12
However, on the basis of immunostaining studies of
tumor specimens, Findlay et al13 have suggested that
TS protein expression in primary colorectal tumors
does not correlate with the clinical response to 5-FU.
Peters et al14 also reported no significant relationship
between 5-FU sensitivity and TS activity in vitro, sug-
gest that sensitivity was mainly related to a balance in
the activities of anabolic and catabolic enzymes.

Little is known about the DPD activity in human
tumors, and only a few studies have been reported.25,26
DPD was detectable by chemical activity and immuno-
histochemistry in normal tissue, as well as in tumor
tissue. An analysis of DPD activity in colorectal cancer
of 63 patients25 who underwent biopsy showed that
DPD activity was highly variable and was higher in
normal tissue than in tumors. The tumor-normal activity
ratio ranged from 0.19 to 3.32 (median, 0.76). Tumor
DPD activity was a median of 76% of that found
in adjacent normal tissue. This difference may contrib-
ute to the favorable differential between antitumor ac-
tivity and systemic toxicity from 5-FU. A higher degree
of 5-FU degradation would occur in normal tissues than
in colorectal tumors. DPD activity has also been mea-
sured in surgical specimens from 56 head and neck

cancer patients before administration of 5-FU–based
therapy.26 Tumor DPD activity ranged from 13-193
pmol per minute per milligram of protein, and the
median ratio of tumor-to-adjacent normal tissue was
1.04. The tumor-to-nontumor ratio was higher in the
nonresponding patients than in those achieving a partial
or complete response, suggesting that increased cata-
obolism influences tumor response to 5-FU therapy by
decreasing the amount of drug available to form cyto-
toxic nucleotides. The experimental data on 19 human
cancer cell lines exhibiting spontaneous sensitivity to
5-FU showed that the lower the DPD activity, the
greater the 5-FU sensitivity.15 Immunohistochemical
Table III. Analysis of TS and DPD according to clinical response of tegafur/uracil (UFT)

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>TS (%) (mean ± SD)</th>
<th>DPD (%) (mean ± SD)</th>
<th>Tumoral/nontumor DPD activity ratio (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response</td>
<td>8</td>
<td>19.2 ± 14.2</td>
<td>6.2 ± 3.3</td>
</tr>
<tr>
<td>No response</td>
<td>19</td>
<td>20.3 ± 16.1</td>
<td>12.2 ± 5.6</td>
</tr>
</tbody>
</table>

Patients treated by UFT only before surgery or by UFT only for chemotherapy totaled were 27. UFT response rates were 8 patients and UFT no-response rates were 19 patients.

*P < .05, t test.

studies of DPD are very few, but Takenoue et al. reported that the intensity of immunohistochemistry was significantly correlated with protein expression. They suggested that the immunohistochemical approach may be used as a method of predicting the sensitivity of 5-FU in colorectal carcinoma.

High TS activity, high TS mRNA levels, and low DPD mRNA levels in cancerous tissue imply selective cytotoxicity of 5-FU for gastric cancer tissue as opposed to normal gastric mucosa. Ishikawa et al. concluded that high tumoral TS levels and low tumoral DPD mRNA may indicate a selective cytotoxicity of 5-FU with gastric cancer. Kirihara et al. used gastrointestinal cancer cells, and reported that TS gene expression increased remarkably in response to 5-FU. Enhanced expression was greater in 5-FU-resistant cells than in 5-FU-sensitive cells. DPD gene expression and activity correlated well with cell growth inhibition by 5-FU in gastrointestinal cancer cells. Gene expression was enhanced by 5-FU selectively in 5-FU-resistant cells. In addition, inhibition of DPD caused an increase in 5-FU activity. These results indicate that DPD in cancer cells acts on 5-FU degradation, thus relating to 5-FU-resistance. Although the detailed mechanism remains unclear, 5-FU directs an increase in TS and DPD expression in 5-FU-resistant cells. It was suggested that DPD acts on 5-FU degradation, and TS induction leads to a decrease in 5-FU activity.

It is important to investigate the expression of TS and DPD for 5-FU response prediction. In this study, there was no correlation between TS and DPD expression. If TS and DPD were coregulated, there would be little or no additional benefit for response prediction from immunohistochemical study of both enzymes. If the expression is independently regulated, some proportion of the low TS patients might be identified as nonresponders by their high DPD expression. Salonga et al. showed that there was no correlation between DPD gene and TS gene expression; these findings were similar to our results with immunohistochemistry. Because the response of colorectal tumors to chemotherapy has been associated with survival, measuring TS and DPD gene expression not only predicts response to 5-FU but also identifies patients with improved survival.

Although UFT response showed no correlation with TS-LI in this study, UFT response had a low DPD-LI and low tumoral-to-nontumoral DPD-LI ratio. UFT is an oral antineoplastic agent that combines the 5-FU prodrug tegafur (1-[2-tetrahydrofuryl]-5-fluorouracil) and uracil (molar ratio 1:4). Tegafur is hydroxylated and converted to 5-FU by hepatic microsomal enzymes and may sustain the levels of 5-FU in tumors. Uracil is also known to competitively inhibit the activity of hepatic DPD, thus blocking the catabolism of 5-FU, resulting in increased plasma and tumor concentrations. UFT has a longer half-life than 5-FU and has proved to be effective in a variety of tumors in animals and humans. UFT is a very useful drug for oral carcinoma, and chemotherapy including UFT is a worthwhile treatment. Hence, it is important to investigate TS, DPD, and other factors by immunohistochemistry before treatment.

Our results suggest that DPD has greater clinical importance than TS for the treatment of oral carcinoma with 5-FU.

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