INTRODUCTION

In the management of HNSCC, a surgical resection often involves reconstructive or functional difficulties (loss of voice, compressive changes of the face etc.), which severely influence the patient’s quality of life. In contrast, chemoradiotherapy (CRT) with 5-fluorouracil (5-FU) may be an efficient modality of treatment, because of a relatively high radiosensitivity and both cosmetic and functional benefits. However, in some cases tumor cells may show a poor response to CRT or recur after a complete response. In these cases, additional chemotherapy may be ineffective, and radiotherapy is not applicable because of dose limitations, so a salvage operation would become an alternative treatment of choice.

5-FU is an effective and widely used...
chemotherapeutic agent for HNSCC. Sensitivity to 5-FU is influenced by two factors; thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD). TS is the key enzyme in the de novo pathway of deoxynucleotidylate (dTMP) and catalyzes the conversion of deoxyuridylate (dUMP) and 5,10-methylene tetrahydrofolate (CH2-THF) to dTMP and dihydrofolate (DHF). 5-FU is converted to fluoro-deoxyuridylate (FdUMP), which then binds TS with CH2-THF irreversibly to form a ternary complex, thus inhibiting the catalytic activity of TS.

On the other hand, DPD catalyzes the conversion of 5-FU to fluoro-β-alanine (F-BAL), and the concentration of F-dUMP is thought to be influenced by DPD activity. Since the TS activity has been shown to correlate with the number of sites bound to F-dUMP, a higher concentration of cytosolic F-dUMP is thus required to suppress the catalysis of TS in tumors with a high TS activity. Furthermore, in tumors with a high DPD activity, the percentage of 5-FU converted to F-dUMP decreases. These facts indicate that sensitivity to 5-FU tends to be lower in tumors with high TS and DPD activities.

TS/DPD expressions have been reported to be high in cancer cells treated with 5-FU in vitro. Accordingly, 5-FU exposure may induce an increase in TS/DPD expressions and lower the sensitivities to 5-FU in residual or recurrent tumors after CRT with 5-FU. However, whether TS/DPD expressions are increased or not in residual or recurrent tumors after CRT with the use of 5-FU has not yet been clarified. In order to address these issues, we obtained tissue samples of HNSCC, 1) before treatment, 2) 3-6 weeks after finishing CRT, and 3) at the time of recurrence after CRT-induced complete remission, and estimated the levels of TS/DPD expressions among these specimens through assays of TS/DPD activities and immunohistochemistry (IHC). Furthermore, the influence of CRT on the TS/DPD expressions is discussed.

**MATERIALS AND METHODS**

**Patients and samples**

This study was approved by the Ethics Committee of the University of Yamanashi and performed with the written consent of all participants. Thirty samples of HNSCC obtained from 26 patients were examined in this study. The mean age of the subjects was 64.2 ± 11.6 years. Of the cases, 23 were males and three were females.

The tumor samples were divided into three groups: Group A (n=20), which consisted of samples obtained from the subjects who were initially treated by surgery and had not previously received radiotherapy and/or chemotherapy; Group B (n=5), which contained samples that were surgically removed from residual tumors 6 weeks after CRT; and Group C (n=5), which consisted of samples obtained from recurrent tumors redeveloping after CRT. In three cases, paired normal mucosa and tumor tissues were obtained and preserved. In order to evaluate the TS/DPD expressions in all the tissue samples, the tissues were cut in half, and then one half was stained with immunohistochemistry, and the other half was used to determine the TS/DPD activities, respectively.

**Tritium release assay**

The TS activity was determined with a tritium release assay reported by Spear with modifications. Briefly, the frozen samples were homogenized in 50mM Tris-HCl buffer (pH 7.3) and centrifuged, and the cytosol fraction was separated. After determining the protein content, 0.62 µM 5,10-methylene-tetrahydrofolate (CH2-THF) and 1 µM 5-3H-dUMP were added and
incubated at 37˚C. After 10, 20, or 30 minutes, reactions were terminated by the addition of 10% activated neutral charcoal with 4% trichloroacetate. The samples were centrifuged at 14,000 × g for 5 min. The ³H-radioactivity of the separated supernatants was measured, and the time-reaction curve was determined, while the enzyme activities were also calculated.

Radioenzymatic assay

The DPD activity was determined with a radioenzymatic assay reported by Diasio 9) with modifications. Briefly, the frozen samples were homogenized and centrifuged at 105,000 × g at 4˚C for 60 min and the cytosol fraction was separated. After determining the protein contents, 6.25 mM NADPH and 125 µM 3-³H-5-FU were added and the samples were incubated at 37˚C. After 10, 20, or 30 minutes of incubation, equal volumes of hydrogen perchloride were added to the samples, and diluted two-fold with 20 mM sodium dihydrophosphate (pH 3.5) and centrifuged. The radioactivities of the supernatant fractions of 5-FU, DHFU, FUPA and FBAL were examined, the time-reaction curve was determined, and then the enzyme activities were calculated.

Immunohistochemistry

RTSSA, an anti-human TS polyclonal antibody, which was used as the primary antibody, was prepared from the serum of a rabbit sensitized with recombinant human TS, which was kindly supplied by Taiho Pharmaceutical Co., Ltd., Tokyo. We used the Envision-kit (DAKO) for the second antibody and detection system.

All samples were fixed in 4% paraformaldehyde for 24 hr and embedded in paraffin-blocks. A transplantable tumor of a human colorectal cancer, DLD-1/FdUrd, was used as positive controls. As negative controls, normal rabbit serum was applied to every specimen instead of the primary antibody. The blocks were sliced into 4 µm sections and mounted on silan-coated glass slides. The sections were deparaffinized in xylene and then were rehydrated in decreasing ethanol concentrations and then autoclaved. After cooling to room temperature (RT), the sections were incubated in 0.3% hydrogen-peroxide in methanol for 20 min and washed in deionized water (dH₂O) and Tris-buffered saline with 0.1% Tween 20 (TBS-T). Protein block solution (DAKO) was applied to the sections and incubated for 5 min at RT. After removing the excess protein solution, 100-times diluted primary antibody was added and incubated for 60 min at RT. After washing, the second antibody was added and incubated for 30 minutes at RT. After washing, DAB-solution was applied and incubated for 4 min at RT. After washing, the sections were counterstained with hematoxylin, dehydrated and mounted with malinol (Muto-kagaku, Tokyo). Three high power fields were examined by two observers who did not know the patients’ backgrounds and immunoreactivity was evaluated and classified using a visual grading system based on the cytoplasm staining as negative(-), weak(+), moderate(++) and strong(+++)10).

Statistical analysis

The regularity of distribution was analyzed with using the Chi-square test. Any correlations between TS and DPD were analyzed with Pearson’s parametric correlation test. Differentiation between TS/DPD in every group was analyzed with Student’s t-test (non-paired) when comparing two groups and with one-way analysis of variance when comparing in three groups. P values of less than 0.05 were considered to be significant.
RESULTS

Enzymatic activity of TS/DPD

A weak but significant correlation was observed between the TS and DPD activities of the head and neck tumors ($r=0.457$, $p=0.04$, Fig. 1).

The mean TS activities of the tumors and normal mucosa were $10.786 \pm 9.517$ (n.s.-19.9) and $1.833 \pm 0.850$ (1.2-2.8) pmol/min/mg protein, respectively, whereas the mean DPD activities of the tumors and normal mucosa were $108.1 \pm 59.0$ (n.s.-249) and $66.3 \pm 24.0$ (79-171) pmol/min/mg protein, respectively. The activities of both TS and DPD in the tumor samples tended to be higher than those of the normal mucosa, but not by a significant amount (Fig 2-A, B, $p=0.06$, $p=0.12$). In addition, in paired samples of tumors and normal mucosa obtained from the same subjects, the tumor DPD activity was significantly higher in comparison to the normal mucosa ($p=0.033$). The tumor TS activity was higher than the normal mucosa but the difference was not significant ($p=0.12$).

The TS activities of the tumors before treatment (Group A), the residual tumors after CRT (Group B) and the recurrent tumors obtained following a complete response after CRT (Group C) were $12.2 \pm 10.4$, $0.7 \pm 1.0$, and $9.06 \pm 5.58$ pmol/min/mg protein, respectively, whereas the DPD activities of these groups were $111.8 \pm 51.5$, $56 \pm 55.3$, and $127.8 \pm 96.3$ pmol/min/mg protein, respectively. Both the TS and DPD activities of Group B were significantly lower in comparison to those of Group C.

Fig. 1. Correlation between the TS and DPD activities of the head and neck cancer cells. A weak but significant correlation ($r=0.457$, $p=0.04$) was observed between the TS and DPD activities in the head and neck cancer cells.

Fig. 2. The mean TS and DPD activities of the cancer cells and normal mucosa. The mean TS (A) and DPD (B) activities of the cancer cells tended to be higher in comparison to the normal mucosa; however, not by a significant amount. ns = not significant.
Group A (Fig. 3, p=0.005, p=0.022). In addition, the TS activity of Group B was significantly lower than that of Group C (p=0.012), however, the DPD activity of Group B tended to be lower than that of Group C but the difference was not significant (p=0.100). In contrast, no significant differences were observed between the TS and DPD activities of Groups A and C (p=0.245, p=0.314).

In addition, we were able to examine the tumor tissues before and after various treatment modalities from each of three subjects. In case 1, the tumor was surgically removed without postoperative CRT following the recurrence. The TS and DPD activities of the recurrent tumor were likely to be higher than those of the primary tumor. In case 2, the patient was initially treated with surgery followed by CRT and a recurrence developed. The recurrent tumor showed a lower DPD activity, although the TS activity was unchanged in comparison to the primary tumor. In case 3, the patient was treated with preoperative CRT followed by surgery, and 12 months after the recurrence a salvage operation was performed. The TS and DPD activities of the recurrent tumor were remarkably higher in comparison to the tumor tissue obtained after CRT.

**Immunohistochemistry**

TS and DPD staining was observed in the cytosol and the results were consistent with previous reports (Fig. 4). The grades of immunoreactivity in the tumor tissues were stronger in comparison to the normal mucosa, although weak staining in the superficial or intermediate layers of the normal mucosa were observed with these antibodies (data not shown). Serial sections revealed that both TS and DPD were co-expressed in the tumor cells of all groups (Fig. 5).

Secondly, the relationship between the TS/DPD activities and grades of immunostaining was examined. No significant difference was observed in the TS and DPD activities between the stronger stained and weaker stained specimens (data not shown).
Fig. 4. Immunohistochemical staining of TS (A) and DPD (B) in head and neck cancer cells. Both TS (A-1) and DPD (B-1) staining were observed in the cytosol of the cancer cells. A-2, B-2 = negative controls. Magnification $\times 100$.

Fig. 5. Colocalization of TS and DPD immunoreactivity using a serial section. H-E staining (A), TS immunoreactivity (B), and DPD immunoreactivity (C). Magnification $\times 40$. 
DISCUSSION

In this study, we found that the TS/DPD activities of the residual tumors just after CRT were significantly lower in comparison to the primary tumors before treatment. The TS activities of the residual tumors just after CRT were also significantly lower than those of the recurrent tumors. However, their DPD activity tended to be lower in comparison to the recurrent tumors after CRT. We had expected higher expressions of TS/DPD after CRT; however, the reverse was the case.

Peters et al\(^6\) has shown that the sensitivity to 5-FU decreases when tumor cells are cultured with 5-FU and the TS activity of these cells increases. It has also been demonstrated that the TS expression is regulated at a translational level, and TS mRNA has a binding site to which TS binds to inhibit TS translation\(^11\). When F-dUMP forms a ternary complex with TS and then irreversibly inhibits TS activity, free TS concentrations decrease. This decrease in turn reoperates TS mRNA translation, thus inducing the upregulation of TS expression\(^11-13\). Furthermore, the tumor DPD activity has been shown to increase after treatment with Tegafur and Uracil (UFT) in urinary tract cancer patients\(^7\). These reports suggest that the low sensitivity to 5-FU of tumors treated with 5-FU may be due to the elevated expressions of TS/DPD. The reason for the discrepancy between our results may be that there seems to be a balance between the TS/DPD expressions triggered with either chemotherapy (inductive effects) or radiotherapy (inhibitory effects). Therefore, when the inhibitory effects of radiotherapy are stronger than the inductive effects of 5-FU, TS/DPD expressions may decrease. Our results suggest that radiotherapy might show stronger effects than chemotherapy in terms of TS/DPD expressions in the case of concurrent chemoradiotherapy with 5-FU. Further studies may be necessary to address these issues.

We have also demonstrated that the TS/DPD activities of the recurrent tumors were not significantly different from those of the untreated primary tumors. Clinically, chemotherapy is sometimes not effective for recurrent tumors, which seems to be due to the higher expressions of TS/DPD activities in recurrent tumors. However, no significant differences in these values have been found between untreated primary and recurrent tumors. These results indicate that recurrent tumors’ resistance to 5-FU may be due to other mechanisms besides higher TS/DPD expressions. In order to confirm these facts, more data will have to be collected and examined.

The problem with this study is whether or not the tumor cells were viable in the tissues obtained from the samples after CRT. When tumor cells become necrotic after chemoradiotherapy, the percentage of viable tumor cells may not be high enough to correctly estimate the TS/DPD enzymatic activity. In order to carefully evaluate the TS/DPD expressions in the tissue samples after CRT, the tissues were cut in half, and then one half was stained with immunohistochemistry to confirm if enough viable tumors cells were present. These results demonstrated that more than 60% viable tumor cells were observed in every specimen of Groups A, B, and C and no difference was found among the samples.

Studies from three cases imply that primary surgery had no effect on the TS/DPD activities in the tumor cells at all. However, the decrease in those values observed after CRT seemed to recover or increase when a tumor recurred. These results suggest that CRT inhibits the TS/DPD expressions in HNSCC to the levels of
normal mucosa at first, but these enzymatic activities can reactivate afterward during tumor regrowth. Further studies, which compare the values of TS/DPD between before and after CRT in the same subjects, have to be necessary in order to show the direct evidence of inhibitory effect of CRT on TS/DPD activities.

A salvage operation is the preferred treatment when tumors remain after CRT completion. However, this study suggests that sensitivity to 5-FU may increase just after CRT due to the suppressed TS/DPD activities. It is thus conceivable that 5-FU chemotherapy immediately after CRT may be an alternative treatment to surgery for residual tumors. This is the first report to examine the ST/DPD activities in tumors both after CRT and at the time of recurrence. These findings may help create a novel therapeutic approach for the treatment of residual tumors after CRT in HNSCC.

REFERENCES


