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Thymidylate Synthase, Thymidine Phosphorylase and Orotate Phosphoribosyl Transferase Levels as Predictive Factors of Chemotherapy in Oral Squamous Cell Carcinoma

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We conducted a clinicopathologic study on protein and mRNA levels of thymidylate synthase (TS), thymidine phosphorylase (TP) and orotate phosphoribosyl transferase (OPRT) using biopsy tissue specimens before treatment. The mRNA levels have been measured in tumor cells microdissected from paraffin-embedded specimens (Danenberg Tumor Profile method: DTP method). We studied the mRNA and protein expression as effect predictive factors in chemotherapy. The subjects consisted of 20 cases of untreated oral squamous cell carcinoma who had undergone chemotherapy with TS-1 (16 males and 4 females, tongue in 8 cases, upper gingiva in 3 cases, lower gingiva in 3 cases, buccal mucosa in 5 cases and floor of the mouth in 1 case). TS gene expressions of the responders were lower than those for the nonresponders. Furthermore, regarding males who were less than 70 years of age, stage I and II, well differentiated type and tongue, TS mRNA expression of the responders were lower than that for the nonresponders. The mRNA expression of OPRT for the male responders was lower than that for the nonresponders. No remarkable difference was observed by immunohistochemistry. In this study, the measurement of the TS levels using the DTP method may potentially act as a predictive factor of antitumor effectiveness.

Key words: thymidylate synthase, thymidine phosphorylase, orotate phosphoribosyl transferase, oral cancer

I. Introduction

In anticancer drugs, the therapeutically effective blood concentration and the toxicity-development blood concentration are closely related. A therapeutic plan that considers the prognostic factors and the effective predictive factors is required in the selection of patients in whom a good response can be expected before implementing effective and safe cancer therapy. In advanced oral cancer, radiation and chemotherapy are used concomitantly with surgical therapy, and as part of chemotherapy, 5-fluorouracil (5-FU) is one of the anticancer drugs that is most widely used for head and neck cancer. Thymidylate synthase (TS), thymidine phosphorylase (TP), orotate phosphoribosyl transferase (OPRT) are referred to as 5-FU-related enzymes, and it has been indicated that the enzyme activity and enzyme content may be correlated with the antitumor effects of 5-FU-based drugs.

Recently, the relationship between the expression of the metabolic enzymes of 5-FU in tumors and anticancer efficacy has been studied [6, 8, 13, 18, 19]; however, the mRNA of metabolic enzymes that have been measured in specimens includes not only the mRNA expression level of tumor cells but also the entire tumor tissue, including the interstitium, and the number of specimens is also limited. There have been relatively few reports in which the level of

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expression of the mRNA in TS, TP, and OPRT has been measured in tumor tissue using specimens of formalin-fixed paraffin-embedded slices of biopsy tissue obtained using laser capture microdissection in oral cancer as samples (Danenberg Tumor Profile method: DTP method [5, 16]).

This report describes the findings of a clinicopathological study while also investigating the mRNA expression with laser capture microdissection and protein expression of TS, TP and OPRT.

II. Patients and Methods

The subjects consisted of 20 cases of untreated oral squamous cell carcinoma who had undergone chemotherapy and surgery at Oral and Maxillofacial Surgery Department, Tokyo Women's Medical University Hospital from January 1998 to September 2006, including 16 males and 4 females. Their ages ranged from 52 to 80, with an average of 69.6. The primary site included the tongue in 8 cases, upper gingiva in 3 cases, lower gingiva in 3 cases, buccal mucosa in 5 cases, and the floor of the mouth. As for the stages of progression, 4 cases were stage I, 14 cases were stage II, 1 case was stage III, and 1 case was stage IV. As for the degree of differentiation, 15 cases were of the well differentiated type and 5 cases were of the moderately differentiated type.

Formalin-fixed paraffin-embedded slice specimens were prepared from biopsy tissue specimens before treatment of the oral squamous cell cancer, and 4 μm sliced strips were used for immunostaining and 10 μm sliced strips for the DTP method. We conducted a clinicopathologic study of the mRNA expression and the protein expression of TS, TP and OPRT, while also evaluating the relationship regarding the therapeutic effects of chemotherapy.

TS-1 (Taiho Pharmaceutical Co, Ltd. Tokyo Japan) was given for 14 days as neoadjuvant chemotherapy [17]. The dosage of TS-1 was selected as follows: in a patient with body surface area (BSA) (<1.25 m^2, 80 mg/day; 1.25 m^2≤ BSA <1.5 m^2, 100 mg/day; 1.5 m^2≥ BSA, 120 mg/day). The effect on the tumors was determined 7 days after the administration of TS-1 was completed and the evaluation method was implemented while observing the therapeutic effect evaluation standard according to the General Rules for Clinical Studies on Head and Neck Cancer [1]. There were 2 complete responses, 8 partial responses and 10 cases with no change. Both the complete responses and the partial responses were considered to be responders.

This study was approved by the Institutional Review Board of the Tokyo Women's Medical University.

Danenberg Tumor Profile

A representative formalin-fixed, paraffin-embedded tumor specimen obtained from primary tumors was selected by a pathologist after examining the haematoxylin-eosin-stained slides. Ten μm thick sections were stained with nuclear fast red to enable the visualization of histology for laser capture microdissection (PALM Microlaser Technologies AG, Munich, Germany) (Fig. 1), which was performed to ensure that only tumor cells were studied. Microdissected samples were collected into a microcentrifuge tube. Six hundred μl of xylene was added to each tube. After centrifugation for 7 min at 14000 rpm, the supernatant was discarded, and the washing step was repeated three times. The deparaffinised materials were rehydrated in xylene:ethanol:water at the following ratios (95:95:5, 95:90:10, 95:80:20, 95:75:25, and 95:70:30). After each step, the rehydration medium was removed after centrifugation for 7 min at 14000 rpm. After discarding the last supernatant, the pelleted sections were resolved in 70% ethanol. Four hundred μl of buffer (4 M guanidine isothiocyanate solution including 0.5% sarcosine and 8 μl 1 M DTT) were then added to the dried tissue and homogenised mechanically. For RNA demodification, homogenates were heated at 95°C for 30 min. RNA was extracted from homogenates by addition of 50 μl of 2 M sodium acetate (pH 4.0), 500 μl of water-saturated phenol, and 100 μl of chloroform-isoamyl mixture (49:1). RNA was recovered from the water phase by isopropanol pre-

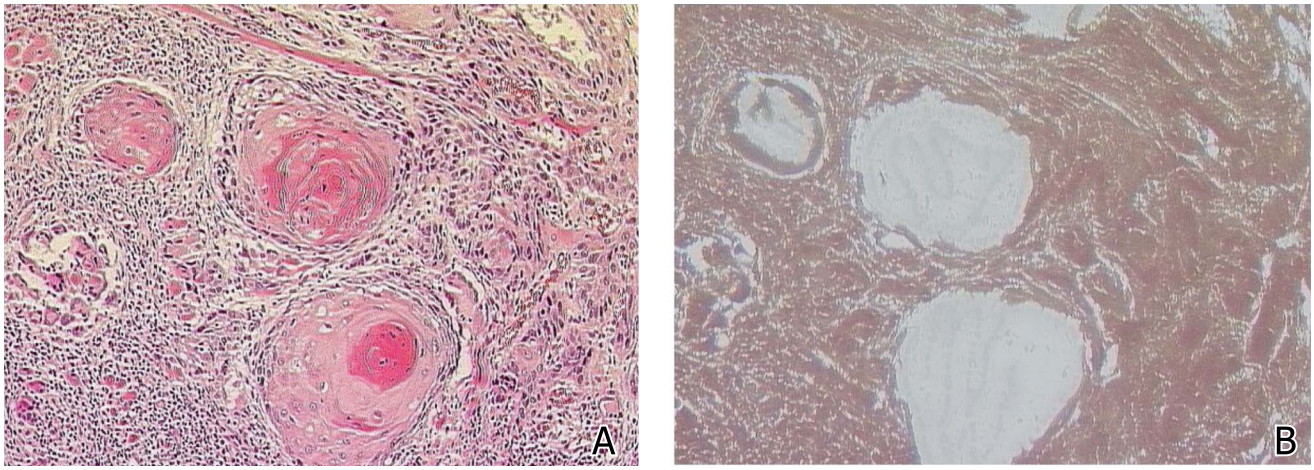

Fig. 1. Laser captured microdissection of cancer cell, Hematoxylin-eosin stain (**A**) After laser captured microdissection of cancer cell (**B**).

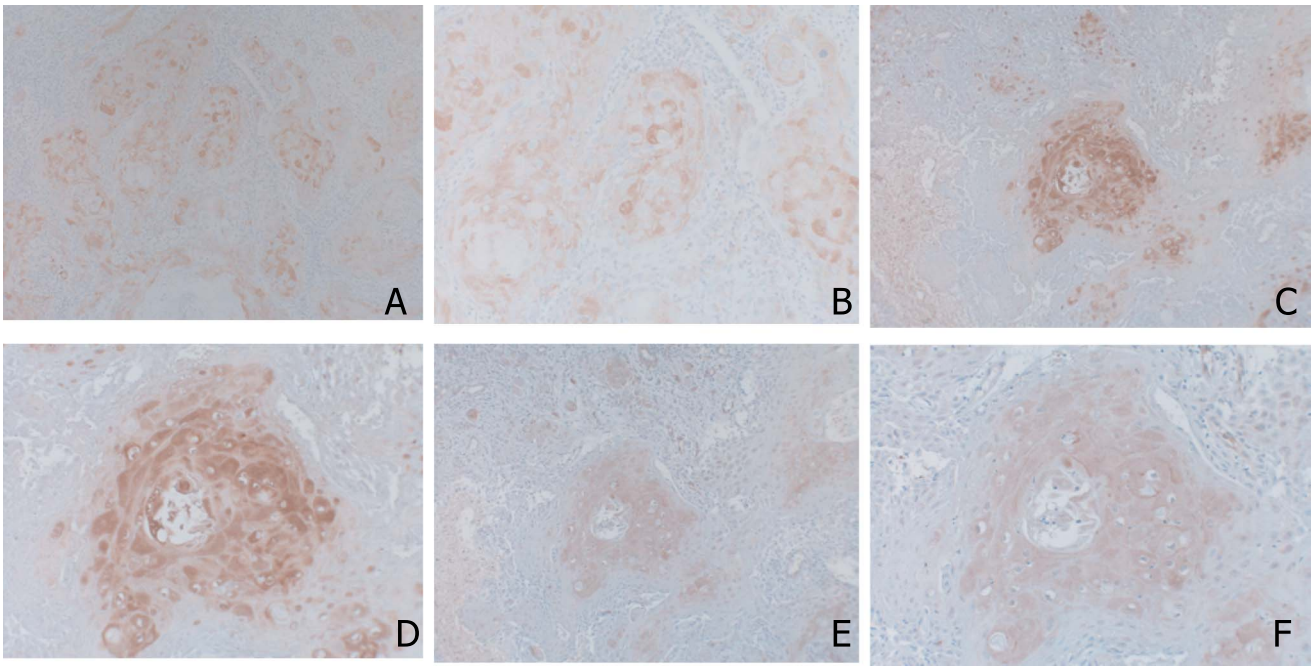

Fig. 2. Immunohistochemical staining for TS×100 (**A**), TS×200 (**B**), TP×100 (**C**), TP×200 (**D**) and OPRT×100 (**E**), OPRT×200 (**F**). Staining is observed in the cytoplasm of cancer cells.

cipitation and transferred to a new tube and precipitated with 10 µl glycogen and 400 µl isopropanol for 30 min at –20°C. After centrifuging for 7 min at 14000 rpm, the pellet was washed with 500 µl 75% ethanol. After drying, the pellet was dissolved in 50 µl 5 mM Tris-HCL (pH 8.0). Reverse transcription was carried at 39°C for 45 min using 400U of MMLV reverse transcriptase, 1×first strand buffer, 0.04 µl random hexamers, 10 mM DTT, and 1 mM deoxynucleoside triphosphate. The target cDNA sequences were amplified by quantitative PCR using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System (Taqman), Applied Biosystems, Foster City, CA, USA). Polymerase chain reaction was carried for each gene of interest, and β-actin was used as an internal reference gene. The 25 µl PCR reaction mixture contained 600 nmol/l of each primer, 200 nmol/l each of dATP, dCTP, and dGTP, 400 µmol/l dUTP, 5.5 mmol/l $MgCl_2$, and 1×TaqMan buffer A containing a reference dye. The primers and probe sequences used were as follows: TS primers: GCCTCGGTGTGCCTTTCA and CCCGTGATGTGCGCAAT, probe 6FAM-TCGCCAGCTACGCCCTGCTCA; TP primers: CCTGCGGACGGAATCCT and GCTGTGATGAGTGGCAGGCT, probe 6FAM-CAGCCAGAGATGTGACAGCCACCGT; OPRT primers TAGTGTTTTGGAAACTGTTGAG GTT and CTTGCCTCCCTGCTCTCTGT, probe 6FAM-TGGCATCAGTGACCTTCAAGCCCTCCT; β-actin primers: TGAGCGCGGCTACAGCTT and TCCTTAATGTCACGCACGATTT probe 6FAM-ACCACCACGGCCGAGCGG.

The PCR conditions were 50°C for 10 sec and 95°C for 10 min, followed by 42 cycles at 95°C for 15 sec and 60°C for 1 min. The relative gene expression of TS, TP and OPRT was determined based on the threshold cycles of each gene in relation to the threshold cycle of the corresponding internal standard β-actin. Gene expressions were quantified as the ratio between two measurements (gene of interest/β-actin).

Immunohistochemistry

Multiple 4 µm-thick sections of the paraffin-embedded tissue were examined for immunohistochemistry. The primary antibodies utilized in immunohistochemistry were rabbit polyclonal antibodies specific for TS (diluted 1:500), OPRT (diluted 1:500) a mouse monoclonal antibodies specific for TP (diluted 1:500). Prior to immunostaining, sections (TS, OPRT) were processed for microwaving for 23 min in 10 mM citrate buffer (pH 6.0). Sections were deparaffinized, rehydrated, quenched for 20 min at 4°C with 3% hydrogen peroxide for inhibiting endogenous peroxidase activity, rinsed in 150 mM phosphate-buffered saline (pH 7.6), incubated overnight at 4°C with the primary antibodies. Immunoreaction product deposits with the primary antibodies were visualized using an Envision kit (Dako). 3,3'-Diaminobenzidine tetrahydrochloride (Dojin, Kumamoto, Japan) solution was used as the chromogen, and hematoxylin was used as the counterstain. The sections from which the primary antibodies were omitted served as negative reaction controls. The labeling indices for TS, TP and OPRT proteins were determined by calculating the percentage of immunoreactive cells in more than 500 cancer cells (Fig. 2).

Statistical analysis

Significance was assessed by Mann-Whitney's U-test. P values of less than 0.05 were considered to be significant.

III. Results

The characteristics of the patients are summarized in Table 1. In all 20 cases, laser capture microdissection of cancer cells was possible and the mRNA of TS, TP and OPRT could be measured. The median value of the mRNA expression was 3.9 for TS, 51.8 for TP and 1.4 for OPRT, which was selected for a cutoff value to separate high and low levels of expression.

TS, TP, and OPRT protein were observed in the cytoplasm by immunostaining, and the median value of the protein expression was 12.4% for TS, 16.9% for TP, and 17.2% for OPRT, which was selected for a cutoff value to separate high and low levels of expression. The mRNA expression of TS in the response group was low in 9 of 10 cases, and the protein expression was low in 5 of 10 cases. The mRNA expression of TP in the response group was low in 6 of 10 cases, and the protein expression was low in 5 of 10 cases. The mRNA expression of OPRT in the response group was low in 9 of 10 cases, and the protein expression was low in 8 of 10 cases.

Regarding the gender, the age, the clinical stage, the degree of differentiation and the site, TS, TP and OPRT mRNA and protein expression are shown in Table 2. The TP mRNA expression in patients who were less than 70 years of age was lower than that in patients older than 71 years of age ($p<0.05$). There was no remarkable difference for TS and OPRT.

mRNA expression of TS, TP and OPRT according to response is shown in Figure 3. The average values of TS mRNA expression were 2.6 and 5.3 for responders and non-responders, respectively, and TS was therefore lower in the responders ($p<0.05$). The average values of TP mRNA expression were 53.9 and 49.6 for responders and non-responders, respectively. The average values of OPRT mRNA expression were 0.9 and 1.9 for responders and nonresponders, respectively. There were no remarkable differences for TP and OPRT.

Protein expression of TS, TP and OPRT according to response is shown in Figure 4. The average values of TS protein expression were 10.9% and 14% for responders and nonresponders, respectively. The average values of TP protein expression were 22.4% and 11.5% for responders and nonresponders, respectively. The average values of OPRT protein expression were 7.8% and 26.5% for responders and nonresponders, respectively. There were no remarkable differences for TS, TP and OPRT.

Furthermore, response and nonresponse findings were studied after the patients were categorized by gender, age, clinical stage, degree of differentiation and site (Table 3).

Table 1. *Patient clinical characteristics and mRNA and protein expression of TS, TP and OPRT*

Case	Gender	Age	Site	Stage	Differentiation	TS		TP		OPRT		response
						mRNA/ β-actin	Protein (%)	mRNA/ β-actin	Protein (%)	mRNA/ β-actin	Protein (%)	
1	M	70	Tongue	II	Well	1.2	14	42.2	55	0.8	1	CR
2	F	66	Buccal mucosa	I	Well	3.3	17	51.6	18	0.9	1	CR
3	M	56	Tongue	II	Well	1.7	19	34.3	8	1	1	PR
4	M	66	Tongue	II	Well	2	1	47	8	1.1	1	PR
5	M	71	Buccal mucosa	II	Moderately	4.3	14	61.6	40	0.7	17	PR
6	M	65	Buccal mucosa	II	Well	1.2	22	22.7	3	1.2	26	PR
7	M	76	Buccal mucosa	II	Well	2.5	2	53.1	4	0.5	2	PR
8	M	76	Buccal mucosa	II	Well	3.1	3	53.2	16	0.7	4	PR
9	F	84	Upper gingiva	II	Well	3.5	12	123	44	0.9	24	PR
10	F	77	Upper gingiva	II	Well	3.2	5	51.3	28	1.8	1	PR
11	M	52	Tongue	I	Well	3.5	29	131	20	2.6	90	NC
12	F	75	Tongue	II	Well	2.3	7	42.8	1	0.4	1	NC
13	M	66	Tongue	I	Moderately	7.7	13	31.2	8	2.1	21	NC
14	M	66	Tongue	I	Moderately	2.1	1	24.9	1	0.75	5	NC
15	M	71	Tongue	II	Well	10.8	15	25.8	1	2.7	1	NC
16	M	64	Upper gingiva	II	Moderately	8.8	13	23	11	2.49	41	NC
17	M	75	Lower gingiva	IV	Well	3.7	31	84.9	50	0.9	90	NC
18	M	67	Lower gingiva	III	Well	6.4	10	26.5	3	1.43	3	NC
19	M	80	Lower gingiva	II	Well	3	1	79.5	18	1.04	1	NC
20	M	69	Floor of mouth	II	Moderately	4.8	20	27.3	2	5.1	12	NC

TS: thymidylate synthase, TP: thymidine phosphorylase, OPRT: orotate phosphoribosyl transferase.
CR: complete response, PR: partial response, NC: no change

mRNA expression of TS and OPRT for the male responders was significantly lower than that for the nonresponders. TS mRNA expression with the responders who were less than 70 years of age were significantly lower than that for the nonresponders. TS mRNA expression of stage I, II with the responders were significantly lower than that for the nonresponders. TS mRNA expression of responders with a well differentiated type was significantly lower than that for the nonresponders. TS mRNA expression of the responders in the tongue was significantly lower than that for the nonresponders. There was no remarkable difference in the protein levels of TS, TP and OPRT by immunostaining.

Table 2. *Patient and tumor characteristics according to level of mRNA and protein expression of TS, TP and OPRT*

	Number of patients	TS		TP		OPRT	
		mRNA/β-actin	Protein (%)	mRNA/β-actin	Protein (%)	mRNA/β-actin	Protein (%)
Gender							
Male	16	4.1	13	48	15.3	1.5	19.8
Female	4	3.1	10.3	67.1	22.8	1	6.75
Age (years)							
≤70	11	3.8	14.6	41.9*	12.5	1.7	20.1
>70	9	4	10	63.8	23	1	15.7
Clinical stage							
I=4	18	3.8	11.5	51.4	16	1.4	13.9
II=14							
III=1	2	5	20.5	55.6	26.5	1.1	46.5
IV=1							
WHO classification							
Well	15	3.4	12.5	57.9	18.5	1.2	16.5
Moderately	5	5.5	12	33.6	12.4	2.2	19.2
Site							
Tongue	8	3.9	12.3	47.4	12.8	1.4	15.1
Upper gingiva	3	5.1	10	65.6	27.7	1.7	22
Lower gingiva	3	4.3	14	63.5	23.6	1.1	31.3
Buccal mucosa	5	2.9	11.6	48.4	16.2	0.8	10
Floor of mouth	1	4.8	20	27.3	2	5.1	12

Mann-Whitney's U-test *p<0.05, TS: thymidylate synthase, TP: thymidine phosphorylase, OPRT: orotate phosphoribosyl transferase.

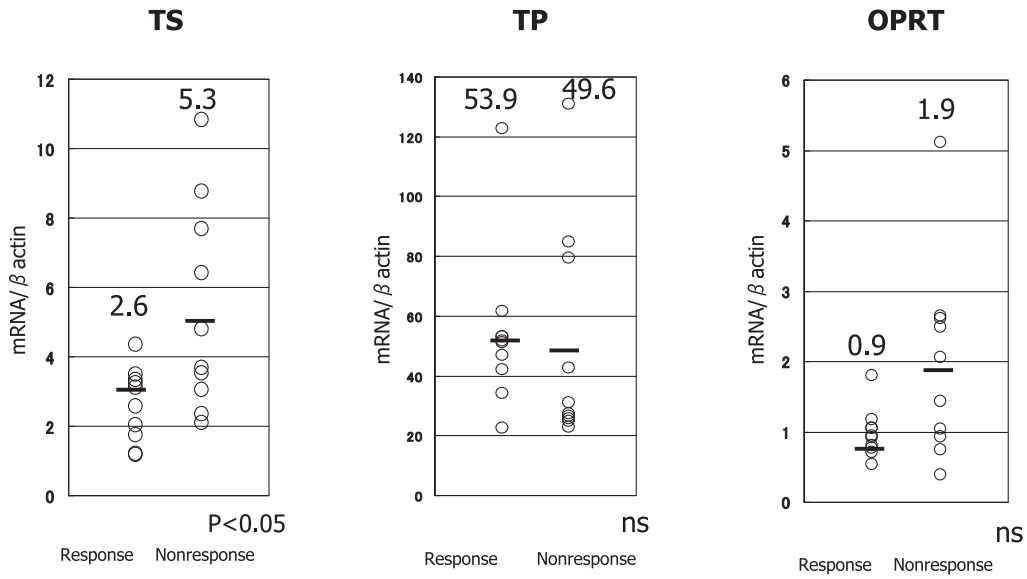

Fig. 3. Expression of mRNA of TS, TP, and OPRT according to response, TS was significantly lower in the response group (Mann-Whitney U test).

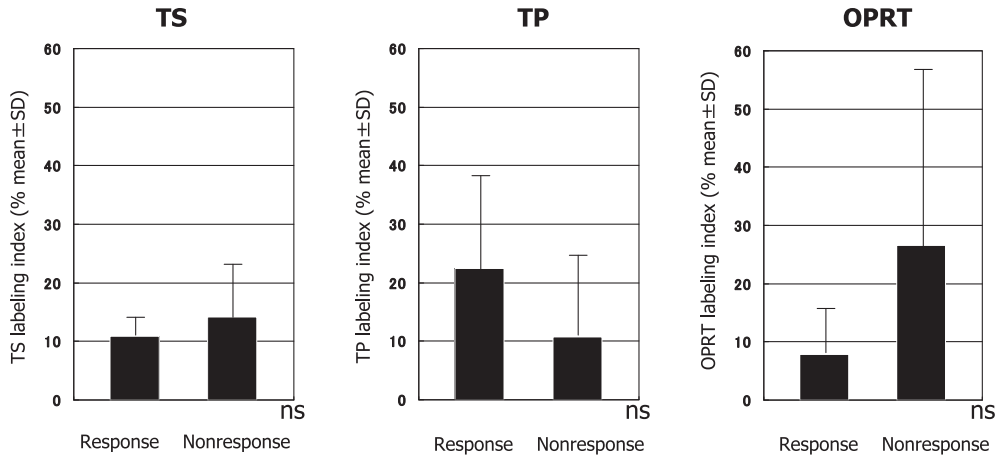

Fig. 4. The labeling indices for TS, TP and OPRT proteins were determined by calculating the percentage of immunoreactive cells in cancer cells according to response. The differences were not significant (Mann-Whitney U test).

Table 3. *mRNA expression of TS, TP and OPRT accoding to response*

	Number of patients			TS	TP	OPRT
Gender						
Male	16	Res	7	2.3 **	44.8	0.8 *
		Nonres	9	5.5	50.4	2.1
Female	4	Res	3	3.3	75.2	1.2
		Nonres	1	2.3	42.8	0.3
Age (years)						
≤70	11	Res	5	1.9 *	39.5	1
		Nonres	6	5.5	43.9	2.4
>70	9	Res	5	3.3	68.3	0.9
		Nonres	4	4.9	58.2	1.2
Clinical stage						
I=4	18	Res	10	2.6 *	53.9	0.9
II=14		Nonres	8	5.3	48.1	2.1
III=1	2	Res	0			
IV=1		Nonres	2	5	55.6	1.1
WHO classification						
Well	15	Res	9	2.4 *	53.1	1
		Nonres	6	4.9	65	1.5
Moderately	5	Res	1	4.3	61.5	0.7
		Nonres	4	5.8	26.6	2.6
Site						
Tongue	8	Res	3	1.6 *	41.1	0.9
		Nonres	5	5.2	51.1	1.6
Upper gingiva	3	Res	2	3.3	87	1.3
		Nonres	1	8.7	22.9	2.4
Lower gingiva	3	Res	0			
		Nonres	3	4.3	63.5	1.1
Buccal mucosa	5	Res	5	2.9	48.4	0.8
		Nonres	0			
Floor of mouth	1	Res	0			
		Nonres	1	4.8	27.3	5.1

Mann-Whitney's U-test *p<0.05 **p<0.01, TS: thymidylate synthase, TP: thymidine phosphorylase, OPRT: orotate phosphoribosyl transferase.
Res: responders, Nonres: nonresponders.

IV. Discussion

It is generally believed that there are three phosphorylation pathways for 5-FU. One is the 5-FU → FUMP pathway by OPRT, another is the 5-FU → 5-fluorouridine (FUrd) → FUMP pathway via uridine phosphorylase and uridine kinase, and yet another is the 5-FU → 5-fluorodeoxyuridine (FdUrd) → FdUMP pathway via thymidine phosphorylase and thymidine kinase.

TS is an enzyme that catalyses conversion of 2'-deoxyuridine-5'-monophosphate to 2'-deoxythymidine-5'-monophosphate. An active metabolite of fluorouracil, 5'-fluorodexyuridinylate, forms stable ternary complexes with TS and folate cofactor, thereby preventing DNA.

Cases with a low TS protein expression level tended to have a high antitumor efficacy of S-1 in oral squamous cell carcinoma [12], and no significant difference has been reported when the differentiated type, gender and stage were studied with immunostaining [7]. Other reports have also demonstrated no significant difference in the differentiated type and stage when TS was measured with the DTP method [15] while also showing cases with low TS values to have a high antitumor efficacy [4]. It has been reported that if the TS enzyme content in tumor cells is low, then the antitumor effects of 5-FU are high in gastric cancer and colorectal cancer [3, 9, 10]. In this study, the result was similar, and cases with low TS gene expression were responders in 9 of 10 cases. In addition the average values of TS gene expression with the responders were lower than the expression with the nonresponders. Regarding males who were less than 70 years of age, stage I and II, well differentiated type and tongue, the TS mRNA expression of the responders were significantly lower than for the nonresponders. As a result, the TS gene expression is thus considered to be a useful predictive factor.

It is believed that TP converts 5-FU into FdUMP, forming a complex with TS, while also inhibiting TS enzymes from causing DNA damage. For TP, in comparison to TS

and OPRT, studies on the effect on tumors are relatively new, and such reports are very few. No difference in the TP mRNA expression level due to the response in oral squamous cell carcinoma has been reported [4]. While it is reported that cases with high mRNA expression level of TP in tumors have low effects of 5-FU therapy in metastatic colon cancer [11], it is also reported that cases with a high TP expression have a better prognosis in subjects for postoperative adjuvant chemotherapy [14]. In this study, the TP mRNA expression in patients who were less than 70 years of age were significantly lower than the expression in those older than 71 years of age. The protein expression showed a similar tendency. This indicated that TP demonstrates differential expression, depending on age. There was no remarkable difference in the mRNA and protein expression of TP due to the response.

OPRT is an enzyme that converts fluorinated pyrimidine series anticancer drugs into active nucleotides, which are regarded as a main enzyme in the first step involved in DNA and RNA inhibition. Therefore, if the OPRT activity value is low, the metabolism of FdUMP does not progress, and thus the antitumor effects of 5-FU are assumed to decrease.

In this study, mRNA and protein expression tended to be low in the response groups. OPRT mRNA of the male responders was also significantly lower than for the non-responders. Those findings were different from previous expectations. This cannot be compared, because of the small number of cases, but buccal mucosa cancer is included in 5 of 10 cases in the response group (low in all 5 cases), and well-differentiated cases are included in 9 of 10 cases in the response group (low in 8 of 9 cases), and therefore it can be assumed that OPRT expression of buccal mucosa cancer or well-differentiated cases show less OPRT expression. It has been reported that no significant difference was observed when OPRT mRNA was measured and the response was compared for oral squamous cell carcinoma [4]. In colon cancer, there are reports that responding tumors had a statistically higher OPRT gene expression than non-responding tumors [2].

We expected to find differences in the numbers, but no remarkable differences were found in Tables 2 and 3. This is because there are only a small number of cases, hence it would be desirable to study a greater number of cases in the future.

In this study, the mRNA expression and protein expression showed similar trends but had slight differences in the measured values. This may have been caused by differences in the methods of quantitative and qualitative measurement. Immunohistochemical staining, which is a simple procedure that is inexpensive to perform, can visualize the expression status of each enzyme, thereby distinguishing between interstitial cells and tumor cells, but it is less quantitative. In addition, the conventional measurement of the level of mRNA expression with reverse transcript-polymerase chain reactions constitutes not only the level of expression of mRNA in tumor cells but also the entire tumor tissue, including tumor stroma, and the number of specimens was limited. On the other hand, a method that selects tumor cells only in the quantitative measurement of mRNA with DTP using the specimens of formalin-fixed paraffin-embedded slices of biopsy tissue as samples is a useful method. So far there have only been a few studies published on the mRNA expression with the DTP method regarding oral cancer, and therefore further study is still required. In this study, the measurement of the TS levels using the DTP method may potentially act as a predictive factor of antitumor effectiveness. It is assumed that studies on the prognostic factors in oral cancer and the predictive factors of the effect of 5-FU-based drugs would make chemotherapy more effective against oral cancer and therefore would have significance in establishing a therapy that is suitable for individual oral cancer patients.

V. Acknowledgements

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VI. References

1. Fisher, R. I., Gaynor, E. R., Dahlberg, S., Oken, M. M., Grogan, T. M., Mize, E. M., Glick, J. H., Coltman, C. A. Jr. and Miller, T. P. (1993) Comparison of a standard regimen (CHOP) with three intensive chemotherapy regimens for advanced non-Hodgkin's lymphoma. *N. Engl. J. Med.* 328; 1002–1006.
2. Harada, K., Kawashima, Y., Yoshida, H. and Sato, M. (2006) Thymidylate synthase expression in oral squamous cell carcinoma predicts response to S-1. *Oncol. Rep.* 15; 1417–1423.
3. Ichikawa, W., Uetake, H., Shirota, Y., Yamada, H., Takahashi, T., Nihei, Z., Sugihara, K., Sasaki, Y. and Hirayama, R. (2003) Both gene expression for orotate phosphoribosyl transferase and ratio to dihydropyrimidine dehydrogenase influence outcome following fluoropyrimidine-based chemotherapy for metastatic colorectal cancer. *Br. J. Cancer* 89; 1486–1492.
4. Ichikawa, W., Takahashi, T., Suto, K., Nihei, Z., Shirota, Y., Shimizu, M., Sasaki, Y. and Hirayama, R. (2004) Thymidylate synthase and dihydropyrimidine dehydrogenase gene expression in relation to differentiation of gastric cancer. *Int. J. Cancer* 112; 967–973.
5. Ichikawa, W., Takahashi, T., Suto, K., Yamashita, T., Nihei, Z., Shiota, Y., Shimizu, M., Sasaki, Y. and Hirayama, R. (2004) Thymidylate synthase predictive power is overcome by irinotecan combination therapy with S-1 for gastric cancer. *Br. J. Cancer* 91; 1245–1250.
6. Kawano, K., Yanagisawa, S., Kusukawa, J., Sunagawa, H., Shiba, R., Goto, M., Shinohara, M., Fukuda, J., Oishi, M., Ikemura, K., Takahashi, T., Sugihara, K., Inokuchi, T., Mimura, T. and Goto, H. (2006) Intratumoural expression of thymidylate synthase is an independent predictor of prognosis in patients with squamous cell carcinoma of the tongue: results from a retrospective study. *Int. J. Oral Maxillofac. Surg.* 35; 265–269.
7. Kawasaki, G., Yoshitomi, I., Yanamoto, S. and Mizuno, A. (2002) Thymidylate synthase and dihydropyrimidine dehydrogenase expression in oral squamous cell carcinoma: An immunohistochemical and clinicopathologic study. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 94; 717–723.

8. Kornmann, M., Schwabe, W., Sander, S., Kron, M., Strater, J., Polat, S., Kettner, E., Weiser, H. F., Baumann, W., Schramm, H., Hausler, P., Ott, K., Behnke, D., Staib, L., Beger, H. G. and Link, K. H. (2003) Thymidylate synthase and dihydropyrimidine dehydrogenase mRNA expression levels: predictors for survival in colorectal cancer patients receiving adjuvant 5-fluorouracil. *Clin. Cancer Res.* 9; 4116–4124.
9. Leichman, C. G., Lenz, H. J. and Danenberg, P. V. (1997) Quantitation of intratumoral thymidylate synthase expression predicts for disseminated colorectal cancer response and resistance to protracted-infusion fluorouracil and weekly leucovorin. *J. Clin. Oncol.* 15; 3223–3229.
10. Lenz, H. J., Leichman, C. G. and Leichman, L. (1996) Thymidylate synthase mRNA level in adenocarcinoma of the stomach: a predictor for primary tumor response and overall survival. *J. Clin. Oncol.* 14; 176–182.
11. Metzger, R., Danenberg, K., Leichman, C. G., Salonga, D., Schwartz, E. L., Wadler, S., Lenz, H. J., Groshen, S., Leichman, L. and Danenberg, P. V. (1998) High basal level gene expression of thymidine phosphorylase in colorectal tumors is associated with nonresponse to 5-fluorouracil. *Clin. Cancer Res.* 4; 2371–2376.
12. Obara, S., Yamamoto, K., Hosogai, N. and Yoshimura, Y. (2005) Evaluation of TS-1 based treatment and expression of thymidylate synthase and dihydropyrimidine dehydrogenase on oral squamous cell carcinoma. *Oral Oncol.* 41; 276–282.
13. Ochiai, T., Sugitani, M., Nishimura, K., Noguchi, H., Watanabe, T., Sengoku, H., Kihara, A. and Okano, M. (2001) Expression and pathophysiologic features of orotate phosphoribosyl transferase activity in gastric carcinoma. *Cancer Chemother.* 28; 345–350.
14. Saito, S., Tsuno, N., Nagawa, H., Sunami, E., Zhengxi, J., Osada, T., Kitayama, J., Shibata, Y., Tsuruo, T. and Muto, T. (2000) Expression of platelet-derived endothelial cell growth factor correlates with good prognosis in patients with colorectal carcinoma. *Cancer* 88; 42–49.
15. Sakakura, K., Chikamatu, K. and Shino, M. (2006) Expression of thymidylate synthase and dihydropyrimidine dehydrogenase in oral squamous cell carcinoma: possible markers as predictors of clinical outcome. *Acta Otolaryngol.* 126; 1295–1302.
16. Schneider, S., Uchida, K., Brabender, J., Baldus, S. E., Yochim, J., Danenberg, K. D., Salonga, D., Chen, P., Tsao-Wei, D., Groshen, S., Hoelscher, A. H., Schneider, P. M. and Danenberg, P. V. (2005) Downregulation of TS, DPD, ERCC1, GST-Pi, EGFR and HER2 gene expression after neoadjuvant three-modality treatment in patients with esophageal cancer. *J. Am. Coll. Surg.* 200; 336–344.
17. Tsukuda, M., Kida, A., Fujii, M., Kono, N., Yoshihara, T., Hasegawa, Y. and Sugita, M. (2005) Randomized scheduling feasibility study of S-1 for adjuvant chemotherapy in advanced head and neck cancer. *Br. J. Cancer* 93; 884–889.
18. Yasuno, M., Mori, T., Koike, M., Takahashi, K., Toi, M., Takizaki, T., Shimizu, S., Yamaguchi, T. and Matsumoto, H. (2005) Importance of thymidine phosphorylase expression in tumor stroma as a prognostic factor in patients with advanced colorectal carcinoma. *Oncol. Res.* 13; 405–412.
19. Yoshitomi, I., Kawasaki, G., Yanamoto, S. and Mizuno, A. (2006) Orotate phosphoribosyl transferase mRNA expression in oral squamous cell carcinoma and its relationship with dihydropyrimidine dehydrogenase expression and the clinical effect of 5-fluorouracil. *Oral Oncol.* 42; 880–887.