Study on Expressions of 5-fluorouracil (5-FU) Related Enzymes and Acquired Resistance to 5-FU in Oral Squamous Cell Carcinoma

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INTRODUCTION

Resistance development to chemotherapy may give a major problem in the cancer treatment. Tumors, which are responsive to chemotherapy can acquire resistance during treatment by cytotoxic agents. This is clinically characterized by short period of remission and response failure to subsequent therapy. Against many drugs, a mechanism acquiring resistance is still unknown and it may depend on origin of the cells, resistance degree and methods for resistant clones used. Many investigators established sublines from murine and human tumors showing resistant to various drugs in vitro by repeated exposures for a sublethal concentration of drug to target cells[1].

5-fluorouracil (5-FU) is one of the most frequently used anti-cancer agents to various neoplasms[2] including oral squamous cell carcinomas (OSCC)[3]. It was synthesized by Heidelberger et al. in 1957[4]. The anti-tumour activities of 5-FU depends on converting ability of related cells to active metabolites. 5-FU is phosphorylated to 5-FU nucleotides through three enzymatic pathways in pyrimidine nucleotide synthesis[5-7]. When 5-FU is administered, it seems to be converted to fluorodeoxyuridine-5-monophosphate (FdUMP) by thymidine phosphate (TP) in tumor. FdUMP forms a stable and tight binding ternary complex with thymidylate synthase (TS) and 5,10-methelene-tetrahydrofolate. As a result, FdUMP prevents methylation mechanism of deoxy-uridine-5-monophosphate (dUMP) to deoxy-thymidine-5-monophosphate (dTMP) by TS, and inhibits DNA synthesis[8].

Fluorouridine-mono-phosphate (FUMP) is converted by orotate phosphoribosyl transferase (OPRT) and subsequently phosphorylated to fluorouridine-tri-phosphate (FUTP). OPRT is a key enzyme in pyrimidine de novo biosynthesis and is limiting for direct conversion of 5-FU to FUMP[9]. Several studies suggested that the mechanism of 5-FU resistance might be produced by markedly reduction of this enzyme[10,11]. FUTP is incorporated into RNA instead of uridine-tri-phosphate (UDTP) and interferes with the maturation and finally the function of RNA[12]. In the development of tumor resistance to fluoropyrimidines, importance of TS has been suggested in some studies[13].

TS plays essential role in catalizing the reductive methylation of dUMP to dTMP, which provides the intracellular de novo sources of dTMP, and moreover works at an important step in DNA biosynthesis[14]. This reaction is critical as it maintains the essential metabolic requirements for cellular proliferation and growth. As a result, TS acts as a key target of chemotherapeutic drugs[15].

As obtained from sources such as various bacteria, bacteriophage, yeast, viruses and vertebrates, TS is a dimer of identical subunits of 35Kda in each[16]. The regulation of TS expressions has been examined on cell cycle-directed events. It was shown that maximal TS activity occurred during the period of active DNA synthesis[17,18]. Thus, TS expression is directly related to cell proliferation[19,20].

Several investigators have elucidated the clinical importance of inhibition of DNA synthesis, resulting in an inhibition of TS[21-23,13]. As aforementioned, 5-FU is a standard drug converting to FdUMP and leading to TS inhibition of tumor tissues[24]. However, TS in malignancy is clinically important not only as a chemo-

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therapeutic target enzyme, but also as a prognostic index correlated with cancer activity[25]. In addition, the level of TS expression predicts response to 5-FU based chemotherapy[16,26]. Low TS expression was accompanied by a greater response rate to 5-FU containing chemotherapy and high expression of TS was associated with poor prognosis in case of gastric and colorectal carcinomas[16]. Moreover, TS levels were associated with survival in rectal and breast carcinomas[27].

Dihydropyrimidine dehydrogenase (DPD) catalyzes the first step for pyrimidine degradation; the NADPH-dependent reduction of uracil and thymine to the corresponding 5,6-dihydropyrimidines. Therefore, it is also responsible for breakdown of 5-FU to 5-flurodihydrouracil (FUH2)[28]. Approximately 85% of administered 5-FU is degraded through the catabolic pathway by this enzyme. Human liver DPD has been purified for homogeneity. A study showed that purified human liver DPD is consisted of two identical subunits with the molecular weight of 105 Kda[29]. This DPD has similar kinetic properties for the natural pyrimidine substrates, uracil and thymine, with 5-FU the preferred substrate[30]. Several studies have demonstrated significant variations in tumoral DPD amongst different human cell lines, human tumor xenograft and clinical samples. Elevated intra-tumoral DPD activity has been implicated in low anti-tumor activity of 5-FU due to increased 5-FU inactivation[31]. Etienne et al. determined tumoral/non-tumoral DPD activity ratio (normalized DPD) in biopsy specimens from the head and neck cancers before 5-FU administration for chemotherapy and reported that complete responders exhibited significantly lower normalized DPD values than partial and non-responding patients[29]. Thus, DPD activity may be a potential factor controlling 5-FU sensitivity[32].

A role of multi drug resistance-associated protein 2 (MRP2) has been identified recently. This protein acts as an ATP-driven drug efflux pump and is a member of ATP-binding cassette transporters[33]. Recent reports showed that MRP2 is expressed in human colon carcinoma cell line Caco2, and confers resistance to a number of anticancer agents such as cisplatin, camptothecin, methotrexate and 5-FU[34,35]. However, there is no report concerning in vitro establishment of 5-FU resistant OSCC cell lines, and characterization of aforementioned enzymes in 5-FU resistant mechanism. Moreover, reported data on TS and DPD expressions in OSCC are also limited and little is known about the significance of TS and DPD expressions from immunohistochemical analyses of OSCCs.

To fulfill the purpose of the study, TS and DPD proteins were investigated in OSCC. This time, we also established 5-FU resistant OSCC cell lines by repeated and step-wise increasing the concentration of 5-FU and investigated the change of proteins and mRNA level involved in it.

From above reasons, we divided our work into two experimental groups. In experimental group (I), we examined TS and DPD expressions by immunohistochemistry. Furthermore, in experimental group (II), 5-FU resistant cell lines were established, and 5-FU related aforementioned proteins and enzymes were examined.

MATERIALS AND METHODS

Experimental group (I)

I.1. Tissues tested;

Fifty tissues of OSCC were retrieved from Hiroshima University Dental Hospital. For the present analyses, biopsy or surgical specimens taken from the non-treated patients were examined.

I.2. Clinicopathologic evaluation;

Histologic examinations were carried out routinely using hematoxylin-eosin staining. Nine clinicopathologic parameters (e.g. age, gender, histologic grade, tumor size, stage, lymph node metastasis, mode of invasion, recurrence of the primary tumor and survival rate) were considered for observation. Histologic grade, lymph node metastasis and tumor size were classified according to the WHO classification. Mode of invasion was classified by Jacobsson's[36] criteria.

I.3. Immunohistochemical stainings for TS and DPD;

Immunohistochemistry of TS and DPD were examined using anti-recombinant human TS (RTSSA) and DPD (RDPDPA) polyclonal antibodies, which were generously provided by Dr. Masakazu Fukushima (Taiho Pharmaceutical Co. Ltd., Saitama, Japan). All immunohistochemical examinations were carried out using tissue sections from formalin-fixed, parrafin-

embedded specimens.

Immunohistochemical staining procedure for TS was as follows: four-μm thick sections were cut and deparaffinized in xylene and rehydrated with gradual graded alcohols. Endogenous peroxidase activity was blocked by soaking sections in 0.3% hydrogen peroxide for 20 minutes. After washing with tap water followed by distilled water, all sections were placed in appropriate 10mM citrate buffer solution (pH 6.0). For antigen retrieval, slides were heated twice at 98°C for 10 minutes in a microwave oven and then cooled for 20–30 minutes at room temperature. After washing of 2 times at 4°C in Dulbecco's phosphate buffered saline (PBS), nonspecific reactions were blocked by pre-incubation for 30 minutes at 4°Cwith normal goat serum (Vector Laboratories, Inc, CA, USA). The sections were then incubated at 4°C overnight by aforementioned primary antibody, in which the optimal concentration was 1:1000. After washing of 3 times in PBS at 4°C, slides were incubated with biotinylated anti-rabbit immunoglobulin in PBS for 30 minutes at room temperature. After washing of 3 times in PBS at 4°C, sectioned slides were incubated with Elite ABC solution (Vector Labs. Inc. CA,USA). The washing procedure was then repeated. The immunochemical reaction detected using DAB Substrate-Chromogen system (Dako Corp., CA USA) and the reaction was stopped after 3–5 minutes. After washing with tap water followed by distilled water, all sections were briefly counter stained with Mayer's hematoxylin and mounted.

Immunohistochemical staining procedure for DPD was almost similar to that of TS as following: that is four μm sections were sliced, deparrafinized in xylene and rehydrated subsequently with graded alcohols. Endogenous peroxidase activity was blocked and nonspecific reactions were blocked by pre-incubation with normal goat serum. After then the sections were incubated overnight by aforementioned primary antibody at 4°C, the optimum concentration was 1:500. After washing with PBS at 4°Cfor 60 minutes, the sections were incubated with biotinylated anti-rabbit immunoglobulin in PBS for 30 minutes. The sections were washed by PBS at 4°Cfor 30 minutes and incubated again in Elite ABC solution (Vector Laboratories, Inc, CA, USA). The washing manipulation was then carried out for 60 minutes at 4°C. The immunochemical reaction was evaluated using DAB Substrate-Chromagen system (Dako Corp., CA, USA) and the reaction was stopped after 3–5 minutes. After washing by tap water followed by distilled water, the sections were counter stained with Mayer's hematoxylin and mounted.

I.4. Evaluation of immunostainings;

TS expression was scored semi-quantitatively using the percentage of stained cells of corresponding areas of each tumor. At least, five fields were observed and the numbers of stained cells were counted. In each field, at least 200 cells were counted. TS expression could be divided in two groups based on the grade of staining; resulting in a low staining group without more than 50% cancer cells were stained, and the other is high staining group with more than 50% cells stained. Human tumor xenograft implanted into nude mice DLD-1/FdUrd, was used as the positive control of TS. The negative control was made by using non-immunized rabbit IgG instead of the primary antibody.

DPD staining expressed semi-quantitatively as stained cell percentage out of total number of tumor cells, and assigned to four gradings. Grade 1 means "<25% positive cells" in staining, Grade 2 is ">25% and <50% positive cells", Grade 3 is ">50%,but <75% positive cells" in staining and Grade 4 is ">75% positive cells" respectively. Tumor tissue from xenograft of human pancreatic cancer cell line, MIAPaCa-2 in nude mouse was used as the positive control. The negative control was made by using non-immunized rabbit IgG instead of the primary antibody.

I.5. Statistical analyses;

Obtained data were analyzed with Mann-Whitney's U test and Spearman's rank correlation test. As for TS, survival curves were estimated by Kaplan-Meire method, and their differences were evaluated by Mantel-Cox test. Here p <0.05 were considered as significant.

Experimental group (II)

II.1. Chemicals;

5-FU was generously provided by Taiho Pharmaceuticals Co. Ltd. (Japan). 3-(4,5-dimethylthiazol-2-yle)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used here were in the highest standard grade commercially available.

II.2. Cell line and cell culture;

Among nine tumor cell lines of the human head and neck, HSC-2, HSC-3, HSC-4, CA9-22, HO-1-N-1 HO-1-U-1 and more two salivary gland tumor cell lines, HSY and KSA were provided by Japanese Cancer Research Resources Bank (JCRRB). The other three human head and neck tumor cell line, Igaki, KKp and KKm were produced in our laboratory.

These cells except for KKp and KKm were cultured in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum (Whittaker, Walkersville, MD, USA), 100 U/ml penicillin, 100μg/ml streptomycin (Gibco/BRL, Grand Island, NY, USA) and were incubated at 37°Cunder 5% CO_2 in air. KKp and KKm were cultured in Keratinocyte Basal Medium (Whittaker, Walkersville, MD, USA) supplemented with Keratinocyte supplement (Sigma Aldrich, USA).

II.3. Establishment of 5-FU resistant OSCC cell lines;

HSC-3 cells were treated continuously with stepwise-increasing of 5-FU every 3 weeks up to 5μg/ ml. HSC-4 cells were treated continuously with stepwise-increasing doses of 5-FU every 3 weeks up to 5 μg/ml. Ca-9–22 cells were treated continuously with stepwise-increasing of 5-FU every 3 weeks up to 1 μg/ml. HO-1-N-1 cells were treated continuously with stepwise-increasing of 5-FU every 3 weeks up to 10 μg/ml.

II.4. Drug sensitivity assay;

Cells (1×10^4/100 μl) were seeded onto plates with 96 well. After 1 day, the cells were treated with various concentrations of 5-FU. After culture of 3 days, 20μl of MTT solution was added to each well, and its plate was then incubated at 37°Cfor 4hours.Dark blue formazan crystals were dissolved in 50 μl of dimethyle sulphoxide (DMSO) and absorbance in individual wells was calculated at 570 nm using a microplate reader (Model 450, Bio-Rad, Hercules, CA, USA). Three independent experiments were carried out for different concentrations. IC_{50} was calculated by dose effect analysis.

II.5. Protein extraction and Western blotting;

Protein extraction was performed, as previously described[37]. Concentration was determined by Bradford dye-binding protein assay (Bio-Rad, Hercules, CA, USA) with BSA (Sigma, ST. Louise, MO, USA) as the standard.

Protein samples (100μg) were dissolved in sample buffer and then subjected to 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer onto nitrocellulose filters. Immunodetection was performed using anti-TS, anti-DPD mouse monoclonal antibodies (generous gift from Dr. Masakazu Fukushima), and anti-MRP2 goat polyclonal antibody (Santa Cruz Biotechnology, Inc. CA, USA) followed by peroxidase conjugated second antibody. Immunocomplexes were visualized with enhanced chemiluminescence Western blotting detection systems (Amarsham Biosciences, UK). Anti-α-tubulin mouse monoclonal antibody (Zymed Labs. San Francisco, CA, USA) was used as the internal control.

II.6. Quantitative RT-PCR analysis;

To clarify mRNA expression levels in 5-FU resistant cell lines, we used real time flouroscence detection method. Real-time flouroscence result was detected by a laser detector of ABI Prism 7700 Sequence Detection System (Perkin-Elmer, Foster City, CA).PCR amplification was performed using a 96-wells in optical tray and caps with a 25 μl of final reaction mixture consisting of 600 nM in each primer; 200 nM probe; 5U Ampli-Taq gold; 2 μM each of dATP, dCTP and dGTP; 400μM dUTP; 5.5mM $MgCl_2$; 1 u AmpErase uracil Nglycosylase; 1 × TaqMan bufffer A containing a reference dye. The initial Taq Gold activation step was: incubation at 50°Cfor 2 minutes and at 95°Cfor 10 minutes. The thermal cycle was 40 rounds of amplification at 95°C for 15 sec and at 60°C for 1 minute. Primer and probe sequences were as follows:

OPRT:

5'-TCCTGGGCAGATCTAGTAAATGC-3'
(forward primer),

5'-TGCTCCTCAGCCATTCTAACC-3'
(reverse primer) and

5'-CTCCTTATTGCGGAAATGAGCTCCACC-3'
(probe).

β-actin:

5'-TCACCCACACTGTGCCCATCTACGA-3'
(forward primer),

5'-CAGCGGAACCGCTCATTGCCAATGG-3'
(reverse primer) and

5'-ATGCCCTCCCCCATGCCATCCTGCGT-3' (probe).

Statistical difference between mRNA expressions of original and acquired resistant cell lines were analyzed with Student's t-test. R/P ratio was calculated by mean value of mRNA expression in resistant cells divided by that in parental cells.

RESULTS

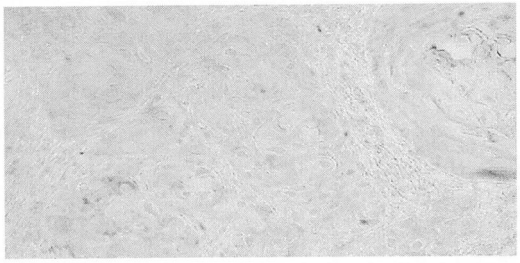
Experimental group (I)

I.1. Patients' characteristics;

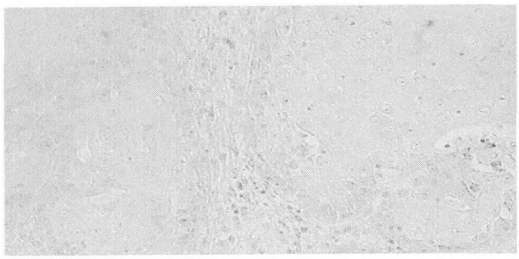
Fifty patients consisting of thirty six males and fourteen females were tested for TS in this study. As for DPD, 31 patients were examined in this study comprising of twenty two males and nine females and age ranging from 33 to 89 years (mean 62.1 years).

I.2. Immunohistochemical expressions of TS and DPD;

In cancer tissues, TS staining patterns showed variable within and among individual tumors. Immuno-

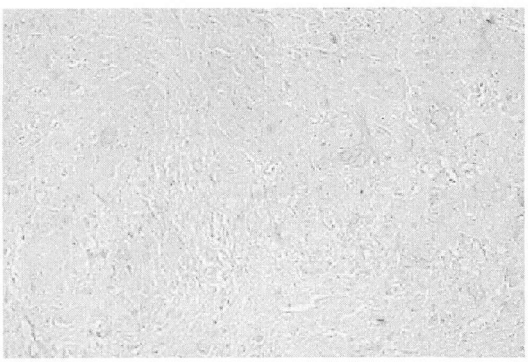


a) TS high level: Immunoreactivity (more than 50%)

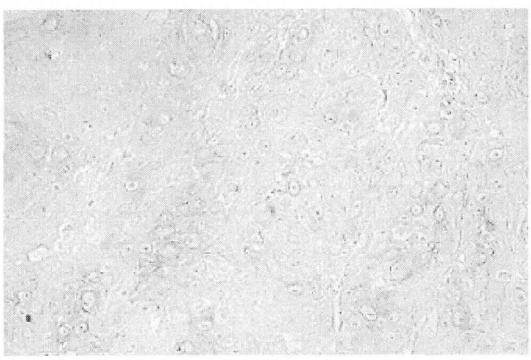


b) TS low level: Immunoreactivity (less than 50%)

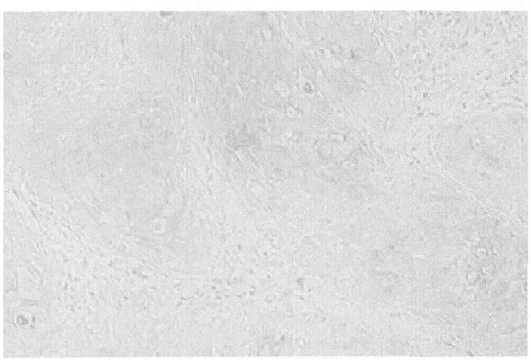
Fig. 1. TS expressions of oral squamous cell carcinoma



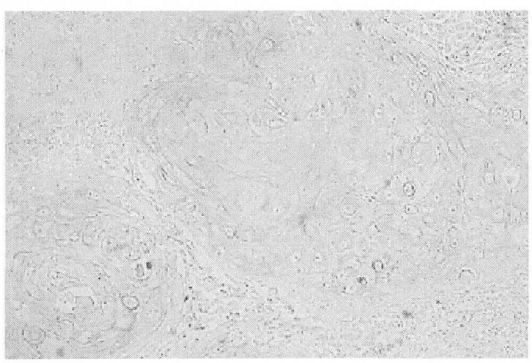
a) Grade 1: positive cells $<25\%$



b) Grade 2 : $25\% \leqq$positive cells $<50\%$



c) Grade 3 : $50\% \leqq$positive cells $<75\%$



d) Grade 4 : $75\% \leqq$positive cells

Fig. 2. DPD expressions of oral squamous cell carcinoma

positivity was observed in differentiated cells in the high staining group (Fig 1a), and undifferentiated tumor cells at the epithelial-connective tissues interface showed granular cytoplasmic staining, while terminally differentiated cells at the center of the nest did not (Fig. 1b).

DPD was diffusely distributed in the cytoplasm of tumor cells with some differences among the tumor areas (Fig 2 a, b, c, and d).

I.3. TS and DPD expressions and clinicopathologic parameters;

High and low TS groups consisted of 23 and 27 patients, respectively. Significant differences of the two groups depended on only tumor size. In TS high group advanced tumors were more than those in the low TS group ($p = 0.0023$). No significant correlation was seen between TS expression and the other clinicopathologic parameters (TableI). As for DPD, no significant correlation was found out with DPD expressions and clinicopathologic parameters (Table II).

Table I Correlation between clinicopathologic parameters and TS staining grade of OSCC

Clinicopathologic parameters	staining grade of TS		*p* value
	low (n=27)	high (n=23)	
Age (yrs)	64.3 ± 11.7	61.6 ± 11.9	N.S.[a]
Gender (M/F)	19 / 8	17 / 6	N.S.[a]
Histological grade[d]			
I	5	8	N.S.[b]
II	21	14	
III	1	1	
Tumor size[d]			
T1	7	4	0.0023[b]
T2	16	2	
T3	2	13	
T4	2	4	
Stage[c]			
I	7	4	N.S.[b]
II	9	1	
III	4	12	
IV	7	6	
Lymph node meta.[d]			
(−)	18	13	N.S.[a]
(+)	9	10	
Mode of invasion[e]			
1	3	0	N.S.[b]
2	8	7	
3	9	11	
4	7	5	
Recurred primary tumor			
(−)	23	15	N.S.[a]
(+)	4	8	

a) Mann-Whitney's U test, b) Spearman's rank correlation test.
c) Criteria of the Japan society for head and neck cancer.
d) Histologic grade, tumor size, and lymph node metastasis were classified according to the WHO classification.
e) Mode of invasion was classified by Jacobsson's criteria.

Table II Correlation between clinicopathologic parameters and DPD staining grade of OSCC

Clinicopathologic parameters	staining grade of DPD				p value
	Grade 1	Grade 2	Grade 3	Grade 4	
Age (yrs)	58.4 ± 11.8	65 ± 11.6	57.9 ± 14.7	67.7 ± 9	N.S.[a]
Gender (M/F)	7 / 3	2 / 3	5 / 1	1/2	N.S.[a]
Histologic grade[d]					
I	5	0	0 2	3	
II	11	7	7 3	0	N.S.[b]
III	0	0	0 0	0	
Tumor size[d]					
T1	4	0	0	3	
T2	6	3	4	0	N.S.[b]
T3	4	2	2	0	
T4	2	1	0	0	
Stage[c]					
I	4	0	0	3	
II	3	3	1	0	N.S.[b]
III	6	2	5	0	
IV	3	1	0	0	

a) Mann-Whitney's U test, b) Spearman's rank correlation test.
c) Criteria of the Japan society for head and neck cancer.
d) Histologic grade and tumor size were classified according to the WHO classification.

I.4. Chemo-response and TS and DPD expression;

Five patients showed no change of TS immuno-expression before and after chemotherapy, whereas 3 patients changed its expression.

Of 15 cases, 10 showed partial chemo-response, consisting of 3 patients with low TS level and 7 with high TS level. Of 5 non-responders, 3 patients showed low TS levels and 2 patients showed high TS levels, resulting seemingly in no significant correlation between TS level and chemo-response (TableIII-a and b).

Of 13, cases 9 showed partial response in which Grade 1, Grade 2, and Grade 3 for DPD were 6, 2, and 1, respectively. There were no changes in 4 patients underwent chemotherapy (Table IV).

Table III-a and b Chemo-response and TS expressions

Pre-chemotherapy → Post- chemotherapy			
High→Low	Low→High	High→Low	Low→Low
4	1	2	1

(a)

Correlation between chemo-response and TS expression		
	low	high
RR	3	7
NC	3	2

(b)

I.5. Univariate analyses of survival rate;

Five years survival rate of all the patients was 84.4%. The rates in the low and high TS group were 92.6% and 75.7%, respectively. No statistical difference was seen in survival rate between these two groups (Fig 3a).

As for tongue carcinomas, the survival rate of low TS group was significantly better than that of high TS

Table IV Chemo-response and DPD expressions

	DPD expression level			
	G.1(<25%)	G.2(25≦50%)	G.3(50≦75%)	G.4(75%≦)
RR	6	2	1	0
NC	2	1	1	0

G. = Grade

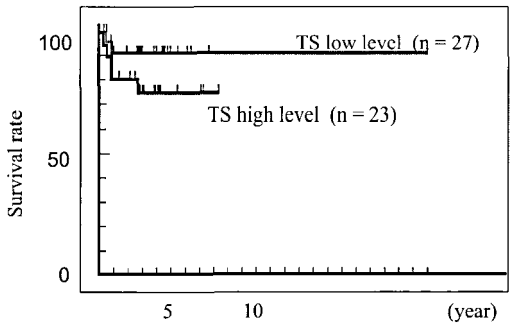


Fig. 3-a. Kaplan-Meier survival rates of OSCC patients
There was no statistical difference between TS high level and TS low level. (p = 0.0614)

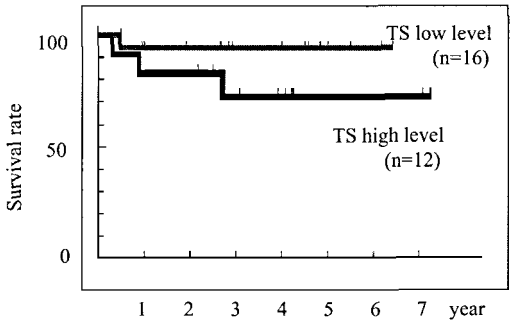


Fig. 3-b. Kaplan-Meier survival rates of tongue cancer patients
There was statistical difference between TS high level and TS low level. (p = 0.04)

group (p = 0.040) (Fig 3b). Five years survival rates in low and high TS groups were 93.8% and 72.9%, respectively.

Experimental group (II)

II.1. Expression of TS and DPD in head and neck tumor cell lines;

We examined the specificity of these antibodies by Western blotting in head and neck tumor cell lines. The bands corresponded to recombinant TS and DPD (36kDa and 108Kda). TS and DPD proteins expressed in all the cell lines (Fig. 4).

II.2. Sensitivity to 5-FU of head and neck tumor cell lines;

IC50 of 5-FU was calculated in the following cell lines i.e. 12 μg/ml in HSC-2, 8.5 μg/ml in HSC-3, 11.5 μg/ml in HSC-4, 12 μg/ml in HSY, 80 μg/ml in Ho-1-N-1, 11 μg/ml in Ho-1-U-1 (Fig. 5).

II.3. Response to 5-FU and TS and DPD expressions in tumor cell lines;

Correlation between IC50 of 5-FU and protein levels of TS and DPD of these cell lines were shown in Fig. 6. No significant correlation was observed.

II.4. Establishment of 5-FU resistant OSCC cell lines;

To obtain 5-FU resistant cells, HSC-3, HSC-4, Ca-9–22 and Ho-1-n-1 cells were treated with step-wise increasing concentrations of 5-FU. Obtained cell lines, HSC-3R, HSC-4R, Ca-9–22R and Ho-1-N-1R showed resistance of 2.7, 2, 11.2 and 2.6-fold respectively (Fig. 7a, b, c, and d).

II.5. Protein expression related to 5-FU resistance;

To determine whether or not increase of TS and DPD is related to 5-FU resistance, related proteins were semiquantified by Western blotting (Fig. 8). Table V shows that TS expression increased to 1.38-fold in HSC-4R compared to its parental cells, but did not change in the other cell lines. DPD protein had a tendency to increase more than 1.12-fold in all cell lines. These results show that these two proteins may relate to 5-FU resistance.

II.6. OPRT mRNA expression of 5-FU resistant cell lines;

The low degree of 5-FU incorporation in nucleic acid can result in down-regulation of 5-FU anabolizing gene. OPRT mRNA expression was measured by quantitative Real-Time PCR. In table VI, OPRT expression of the 5-FU resistant OSCC cell lines was significantly lower than that of original cell lines among the three cell lines. This might suggest that low incorporation of 5-FU into nucleic acid was due to partial down-regulation of OPRT.

II.7. Expression of MRP2 of 5-FU resistant cell lines;

As for MRP2 expression, this protein expressed at 190Kda as shown in Fig. 9. Table VII showed that

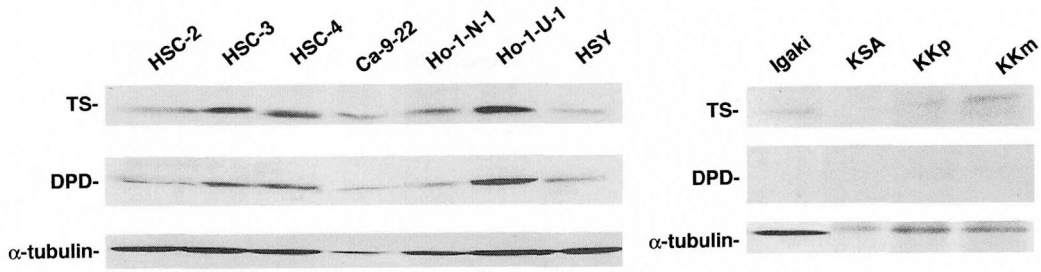
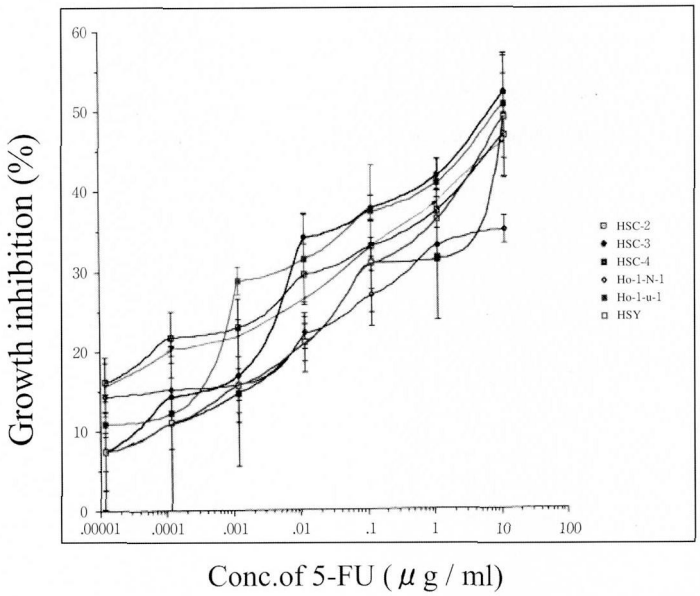


Fig. 4. TS and DPD expressions of head and neck tumor cell lines



	IC50 (μ g / ml)
HSC-2	12
HSC-3	8.5
HSC-4	11.5
HSY	12
Ho-1-N-1	80.5
Ho-1-U-1	11

Fig. 5. Growth inhibition by 5-FU in head and neck tumor cell lines

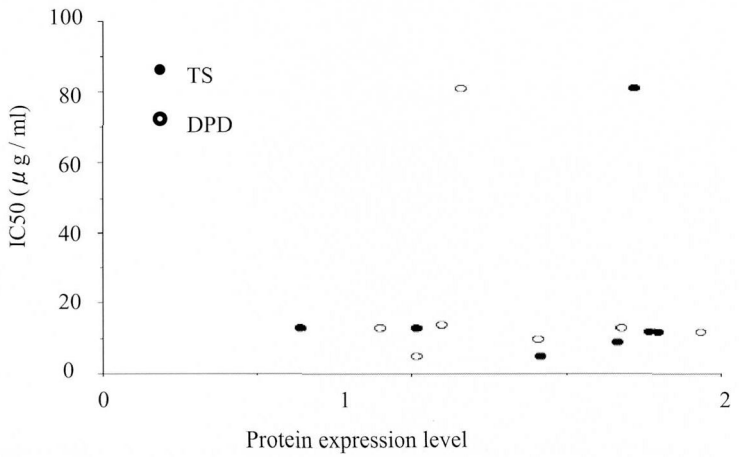


Fig. 6. Correlation between IC50 of 5-FU and protein levels of TS and DPD of cultured head and neck tumor cell lines

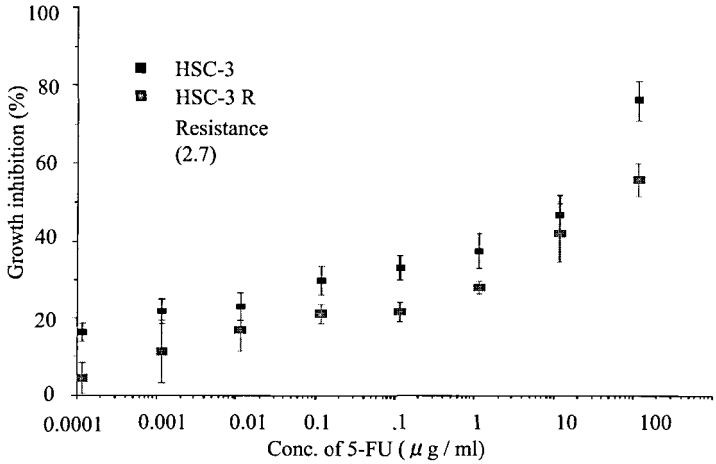


Fig. 7-a. Growth inhibition of HSC-3 cell by 5-FU

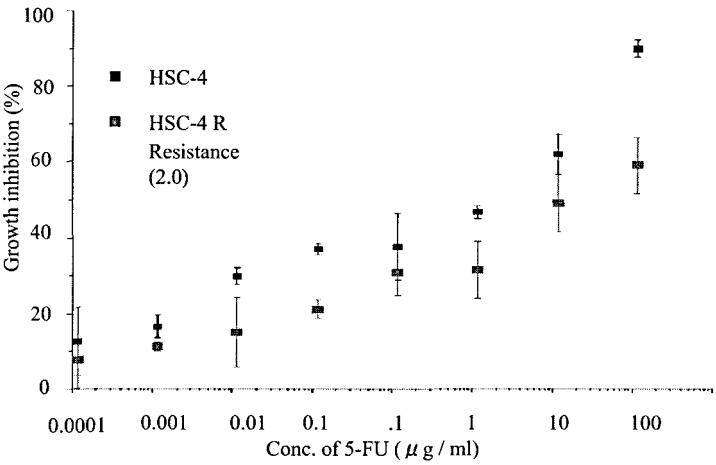


Fig. 7-b. Growth inhibition of HSC-4 cell by 5-FU

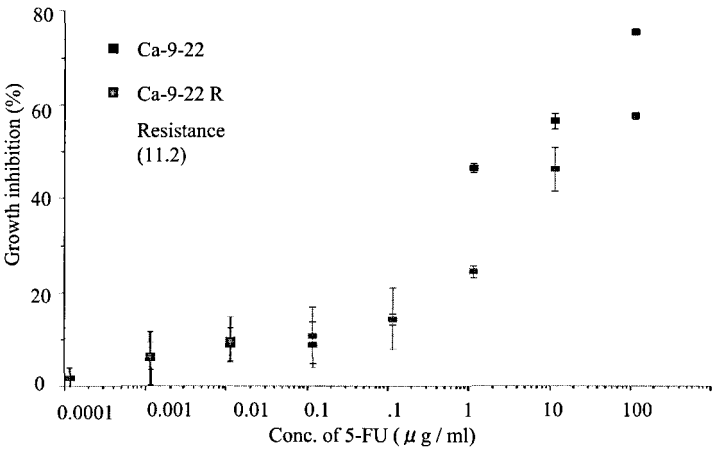


Fig. 7-c. Growth inhibition of Ca-9-22 cell by 5-FU

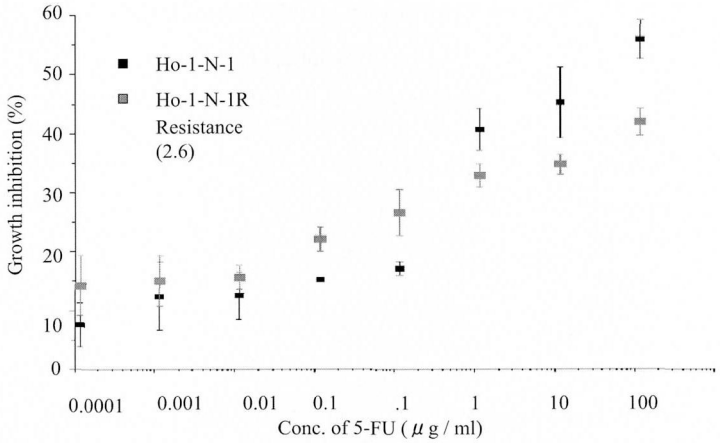


Fig. 7-d. Growth inhibition of Ho-1-N-1 cell by 5-FU

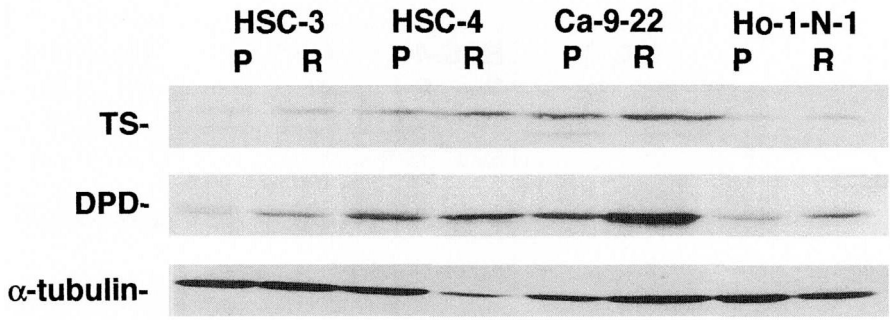


Fig. 8. TS and DPD expressions of 5-FU resistant OSCC cell lines
5-FU resistant cell (R) and corresponding parental cell (P) were assayed for protein expressions of TS and DPD by Western blot.

Table V TS and DPD expressions of 5-FU resistant OSCC cell lines

	HSC-3	HSC-4	Ca-9-22	Ho-1-N-1
	R/P ratio[a)]	R/P ratio	R/P ratio	R/P ratio
TS	1.05	1.38	0.99	1.04
DPD	1.12	1.25	1.20	1.14

a) 5-FU resistant cell (R) and corresponding parental cell (P) were assayed for protein expression of TS and DPD by Western blot. Densitometric scanning was performed on signals normalized by the internal control (α-tubulin), and the R/P ratio was calculated.

MRP2 protein increased to 2-fold in HSC-4 resistant cell line. So, it can be presumed MRP2 might be one of the causes for resistance in HSC-4 cell line.

DISCUSSION

One of the obstacles for the effective chemotherapy may depend on development of drug resistance of tumors. From these aspects, many studies have been performed to overcome the problem. The main ap-

Table VI OPRT mRNA expression of 5-FU resistant OSCC cell lines

	OPRT mRNA expression		
	Mean ± SD	R/P ratio	*P* value
HSC-3	1.0		
HSC-3R	0.73 ± 0.26	0.73[a)]	0.28[b)]
HSC-4	0.47 ± 0.20		
HSC-4R	0.79 ± 0.47	1.68	0.47
Ca-9-22	2.24 ± 2.41		
Ca-9-22R	1.49 ± 1.21	0.66	0.72
Ho-1-N-1	1.44 ± 1.30	0.55	0.62
Ho-1-N-1R	0.79 ± 0.87		

a) R/P ratio was calculated by mean value of mRNA expression in resistant cells divided by that in parental cells.
b) Statistical difference between mRNA expression of parental and resistant cells was analysed with Student's t test.

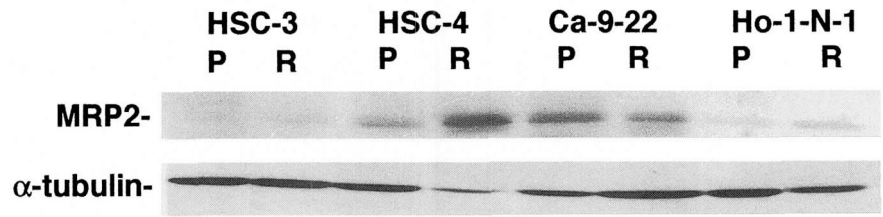


Fig. 9. MRP2 protein expression of 5-FU resistant OSCC cell lines
5-FU resistant cell (R) and corresponding parental cell (P) were assayed for protein expression of MRP2 by Western blot.

Table VII MRP2 protein expression of parental (P) and resistant (R) OSCC cell lines

	HSC-3	HSC-4	Ca-9-22	Ho-1-N-1
	R/P ratio[a)]	R/P ratio	R/P ratio	R/P ratio
MRP2	0.76	2.0	0.69	1.02

a) 5-FU resistant cell (R) and corresponding parental cell (P) were assayed for protein expression of MRP2 by Western blot. Densitometric scanning was performed on signals normalized by the internal control (α-tubulin), and the R/P ratio was calculated.

proach for identification of mechanism involved is development of drug resistant cell line. 5-FU is one of the most frequently used chemotherapeutic drugs for OSCC[37]. It acts through its active metabolites and some studies discussed on its conversion to active metabolites through different enzymatic pathway[8,12]. As 5-FU exerts two action types on tumor cells as described, it seems that either or both of them play a dominant role in the cytotoxicity depending on characteristics of pyrimidine metabolism. Aschele et al.[38] established two sub-lines from human colorectal cancer HCT-8 cells by repeated exposure to 5-FU for 4 hours or by continuous exposure for 7 days. One of the resistant sub-lines developed to 3-fold by 4-hrs exposure exhibited less incorporation of 5-FU to RNA, although the mechanism for this suppression has not been elucidated. The other sub-line developed by 7 days continuous exposure was 7-fold more resistant and ex-

pressed less folypolyglutamate synthase, thereby causing lower levels of folypolyglutamates and faster recovery of TS inhibition by 5-FU[38].

It is also shown that over expression of TS plays a major role in acquiring resistance to 5-FU treatment[8], and that inhibition of intra-tumoral DPD increased 5-FU sensitivity[39]. Recent studies elucidated that TS level was not only predictive for 5-FU response, but also has prognostic value in non-treated patients of various cancers. Johnston et al[40] investigated correlation between immunohistochemical TS expression and clinicopathologic parameters, and found that there was a significant association between the level of tumor TS expression and the degree of tumor differentiation, although only 8 cases were examined in their study.

In the experimental group (I), immunohistochemical technique was employed to examine TS and DPD expressions in OSCC patients. TS expression showed statistically significant correlation with tumor size, but not with the other parameters. Similar tendency was observed in the relation between TS expression and tumor stage classified by tumor size and nodal involvement ($p = 0.1464$). It may be due to 4 T2 cases, exhibiting low TS level were classified into stage IV as there was nodal involvement. Similar trend was seen between TS expression and recurrence of primary tumor, but there was no significant correlation ($p = 0.1029$). A large scale of study is required to conclude this issue, because the number of recurrent cases was small in the present study. It is reasonable that these three parameters showed a significant correlation or similar relationships with TS expression, which is one of indicators of cell proliferation.

Based on tumor sizes, 13 (87%) of 15 T3 cases and 4 (66%) of 6 T4 cases were observed in high TS level. T4 cases might have stopped growing or came to necrosis, because of poor nutrition due to over growth of tumor. On the other hand, low TS level was observed in 16 of 18 T2 cases (89%) and 7 of 11 T1 cases (64%), respectively. Above results may suggest that most T3 tumors are in proliferative stage, whereas most T2 tumors are in less active stage. Clinical features of these cases support this speculation.

By histology, immunopositivity was generally observed in undifferentiated cells of basal layer at the epithelial-meschenchymal interface. In cases of high TS level, immunopositivity was heterogenously observed even in differentiated cells. Of 35 cases, 21 cases (60%) showed low TS level in histological Grade II, while 8 (62%) of 13 cases showed high TS level in grade I. Grade III was only two cases. There was no statistical correlation between TS level and histologic grade.

Most of invasion front cells, mainly consisting of undifferentiated cells, seemed to be immunopositive. So, it was examined whether or not there was any relationship between TS level and mode of invasion, which might be associated with metastasis, local recurrence or prognosis (Jacobsson[36], Yamamoto[41]). But, there was no relationship.

Concerning to prognosis, there was no statistical difference in the overall survival rate between TS low level and TS high level. However, survival rate of the low TS group was significantly better than that of high TS high group in tongue cancer patients ($p = 0.040$). One of the reasons responsible for this association is tumor size relating to TS level may be one of the main prognostic factors in tongue cancers.

As for DPD, there was no such correlation between the DPD expression and clinicopathologic parameters, even though a small number of patients were examined. Furthermore, TS and DPD expressions did not correlate with chemo-response in patients.

In experimental group (II), we could established 5-FU resistant OSCC cell lines by step-wise increasing concentration of 5-FU to characterize the mechanism of resistance in oral squamous cell carcinoma.

In our study multiple parameters were involved in 5-FU resistance. These cell lines examined, such as HSC-3, HSC-4, Ca-9-22, and Ho-1-N-1, were treated for 3 weeks and acquired 2.7, 2, 11.2, and 2.6-fold resistance, respectively. But these resistances were very weak compared to those cell lines produced by Inaba et al.[42] and Y. M. Chung et al.[37]. However, we could find that the 5-FU related enzymes have an association with its resistance, if the level of resistance is low.

Though TS increased only in one cell line, it might agree with others who reported that the increase of TS expression was one of the obstacles in acquiring 5-FU resistance. We also found that in all these cell lines DPD protein showed an increasing tendency. So, DPD increase was responsible for more degradation of 5-FU in these cell lines.

Here was employed novel quantitative real time PCR technique. This can provide a more accurate and

reproducible quantification of gene expressions than the other conventional PCR method. The expression of OPRT mRNA was reduced in resistant cell lines. It may be suggested that low incorporation of 5-FU to nucleic acid was down regulated by anabolizing enzyme, OPRT.

Further investigations of MRP2 protein were performed in these resistant cell lines and found that HSC-4 cell line showed 2-fold increase of MRP2 protein compared to the parental one. So the action of MRP2 protein played an important role in this cell line, by increasing efflux of 5-FU from the cells.

In conclusion, the present study suggests that TS, but not DPD, expression level relates to tumor size in OSCC and the prognosis of the tongue cancer patients. It is also suggested that, even though not significant, TS and DPD levels increase and adversely OPRT enzyme level decreases in OSCC resistant cell lines. Furthermore, MRP2 may have a relation to 5-FU resistance. It must be necessary to elucidate acquiring mechanism of 5-FU resistance in OSCC.

SUMMARY

<Introduction>

One of the obstacles for effective chemotherapy depends on acquiring drug resistance of tumors. Many studies have been carried reported of such problems. The main approach is to know mechanism concerning development of drug resistant cell line.

5-FU is one of the most frequently used chemotherapeutic agents in OSCC. Its action mode is done through active metabolites. Several studies showed that improper or defective action of the active metabolites resulted in resistance to chemotherapy.

Thymidylate synthase (TS), a target enzyme for 5-FU, has been occasionally reported as a prognostic index correlating with malignant cancer behaviours, because of its key role in DNA biosynthesis. However, there has been little information on significance of TS in OSCC. Dihydropyrimidine dehydrogenase (DPD) is also responsible for reversible reduction of both uracil and thymine to dihydrouracil and dihydrothymine, rsepectively. This enzyme is known as catabolizing 5-FU and radiosensitizing agents 5-bromo- and 5-iodouracil. Up to date, there have been some reports on mutual correlations between DPD expression in malignant tumors and effectiveness of these therapeutic agents, but DPD has not been reported from the point of clinicopathological aspects.

To fulfil the purpose of this study, TS and DPD proteins were investigated in oral squamous cell carcinomas. 5-FU resistant OSCC cell lines were also established by repeated and continuous step-wise increasing concentration of 5-FU and examined any changed proteins or mRNA level.

<Materials and Methods>

(I) Immunohistochemical stainings were tried for 50 untreated OSCCs using anti-TS and DPD antibodies by avidin-biotin complex peroxidase method. The immuno-positive cells were classified into 2 groups (high and low levels) for TS immunostaining, and into 4 groups (Grade 1–4) for DPD immunostainings semiquantitatively. Then, the relationship between the immuno-positive grading and clinicopathologic parameters, such as age, gender, histological grade, tumor size, stage, lymph node metastasis, invasion mode, recurrence of primary tumor and survival, were examined.

(II) TS and DPD expressions in OSCC cell lines, in which 5-FU sensitivity was assayed, were investigated by Western blotting to semi-quantify their protein levels. Furthermore, relationship between 5-FU sensitivity and protein levels of TS and DPD was examined. To investigate the mechanisms of acquired resistance to 5-FU in OSCC, 5-FU resistant cell lines were tried to be established in vitro. Using these cell lines, the protein levels of TS, DPD and multi-drug resistance-associated protein (MRP)-2, a member of the ATP-binding cassette super family were semi-quantified by Western blotting. The mRNA level of orotate phosphoribosyl transferase (OPRT), an anabolizing enzyme leading to functional disorder of RNA, was semi-quantified by the real time PCR detection method.

<Results>

(I) There was statistically significant correlation between the immunohistochemical expression of TS and clinicopathologic parameters, such as tumor size ($p = 0.0023$). High TS level was mainly found in T3 and T4 cases, while low TS level was found in T1 and T2 cases. The low TS group showed significantly better prognosis than that of high TS group of tongue carcinomas ($p=0.046$). But there was no clear correlation be-

tween immunohistochemical expression of DPD and clinicopathologic parameters. No correlation was found between the TS and DPD immunoexpressions and response to cisplatin-5-FU chemoptherapy, even though a small number of cases were examined.

(II) In OSCC cell lines, no correlations were seen between TS or DPD protein level and 5-FU sensitivity. 5-FU resistant cell line showed 2-fold resistance to 5-FU in HSC-4R, 2.6-fold in Ho-1-N-1R, 2.7-fold in HSC-3R and 11.2-fold in Ca9-22R, respectively. TS protein level increased to 1.38-fold in HSC-4R, but no significant changes were observed in other three cell lines. DPD protein showed tendency to increase more than 1.12-fold in all resistant cell lines. OPRT mRNA expression was reduced in three resistant cell lines, except Ca-9-22R. MRP-2 protein increased to 2-fold in HSC-4R, although other cell lines showed slightly high or reduced expression.

<Discussion>

(I) From a basic research, it turns out TS over expression plays a major role in the resistance to 5-FU treatment and that inhibition of intra-tumoral DPD increases sensitivity. Recently it was elucidated that TS level was not only predictive for 5-FU response, but also prognostic in clinical value of non-treated cancer patients.

In the current study, immunohistochemical technique was performed in OSCC samples. This technique is easy to perform and applicable on formalin-fixed tissue. There was statistically significant correlation between TS expression and tumor size but no correlation was seen with other parameters.

In prognosis, there were no significant differences in over-all survival rate between TS high level and TS low level. However, the survival curve for low TS group was better than that of high TS group in the tongue carcinoma patients. The reason responsible for this association is due to tumor size and TS level, and it can be the main prognostic factor for tongue carcinoma.

(II) We established 5-FU resistant OSCC cell lines by step-wise increasing concentration of 5-FU to characterize the mechanism of resistance in oral squamous cell carcinoma. Here were found multiple parameters in 5-FU resistance. These cell lines tested were as follows; HSC-3, HSC-4, Ca-9-22, and Ho-1N-1 and showed resistance 2.7, 2, 11.2, and 2.6, respectively. But these resistances were very low in comparison to others. The increase of TS and DPD proteins were in agreement with other reports of two proteins contributing to 5-FU resistance.

Expression of OPRT mRNA was reduced, resulting in suggestion of that low incorporation of 5-FU to nucleic acid causing down regulation of anabolizing enzyme OPRT.

MRP2 protein increased to 2-fold in HSC-4 resistant cell line. MRP2 protein acts as an ATP driven drug efflux pump in the cell and basically found in relation to CDDP resistance. This increasing expression of MRP2 might suggest that it was also related to 5-FU resistance.

<Conclusions>

The present study showed that there was probable correlation between immunohistochemical expression of TS, and tumor size and prognosis. It was suggested that TS, DPD and OPRT might be related to 5-FU resistance of OSCC, and furthermore other factors like MRP-2 might be involved in 5-FU resistance as well.

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