Expression of Thymidylate Synthase and Dihydropyrimidine Dehydrogenase in Primary Oral Squamous Cell Carcinoma and Corresponding Metastases in Cervical Lymph Nodes: Association with the Metastasis Suppressor CD82

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Abstract. *Thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) are 5-fluorouracil (5-FU) metabolizing enzymes and are involved in the sensitivity of carcinoma patients to 5-FU. Although 5-FU is often used for the treatment of oral carcinoma, there has not been any investigation into the expression of these enzymes in metastatic lymph nodes or of their roles in the effectiveness of 5-FU in treating lymph node-metastatic cancer. Oral squamous cell carcinoma (OSCC) often metastasizes to the lymph nodes, and these enzymes may be significant in the survival of patients with this disease. This study investigated the expression of TS and DPD in cervical lymph node metastases and its relationship with primary OSCC, as well as the interaction between these enzymes and Kangai 1(KAI1/CD82) which is a metastasis suppressor protein. Surgical specimens from 20 cases of OSCC with lymph node metastasis, 20 cases of OSCC without lymph node metastasis, and 10 cases of normal mucosa were examined by immunohistochemistry. The relationship between TS and DPD expression and clinicopathological data was analyzed. TS and DPD proteins were overexpressed in primary OSCC compared to that in normal mucosa. TS expression of the primary oral cancer cells in the group with lymph node metastasis was higher than that of those without. DPD expression did not significantly correlate with the occurrence of lymph node metastasis, nor was it different between primary oral cancer cells and cervical metastases. CD82 expression was significantly reduced in lymph node metastases. These findings indicate that TS and CD82 may be of great value in assessing lymph node metastasis of OSCC, and could be taken as new targets for therapy of metastatic OSCC.*

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The anticancer effect of systemic 5-fluorouracil (5-FU) administration is caused by three mechanisms: inhibition of DNA synthesis, dysfunction of DNA and dysfunction of RNA (1, 2). Thymidylate synthase (TS), which is a rate-limiting enzyme in *de novo* DNA biosynthesis, catalyzes the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine (dTMP), an essential step in DNA biosynthesis (3, 4). In the presence of 5,10-methylene-tetrahydrofolate, 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), which is a metabolic product of 5-FU, forms a slowly reversible covalent complex with TS and thereby blocks the DNA synthetic process (3, 5). Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting enzyme in the catabolism of 5-FU, and is the enzyme primarily responsible for 5-FU resistance in carcinoma cells (1, 2, 6). Recently, increased interest has been focused on the biological roles of TS and DPD as independent prognostic factors, as well as determinants of response to 5-FU-based therapy for cancer patients (7, 8).

Although some reports have indicated that TS and/or DPD expression is related to the sensitivity to and cytotoxicity of 5-FU (9), its clinical meaning is not necessarily clear. In our previous study, we also suggested that the effect of 5-FU on primary oral cancer was significantly correlated with DPD expression in those cells (10). However, we had not determined if the metastatic cells were present in cervical lymph nodes, and there has not been any research into the biological roles of metastatic cancer cells in cervical lymph nodes.

In oral squamous cell carcinoma (OSCC), cervical lymph node metastasis is a most important prognostic factor, and the incidence of occult cervical lymph node metastasis is 20-50% (11-14). Most patients with OSCC who have either suspected or proven metastasis in regional lymph nodes are candidates for composite resection in which the lesion, surrounding tissues, and lymph nodes of the neck are all removed (15). Despite its clinical importance, there are relatively few methods of identifying the biology of metastatic oral cancer cells in the lymph nodes. Although chemotherapy is thought to have limited efficacy for cervical lymph node metastases, adjuvant chemotherapy does have the potential to improve the survival of such cancer patients. Therefore, it is important to investigate the biological mechanism of cancer cell metastasis to cervical lymph nodes.

5-FU is commonly administered to treat oral carcinoma (16). In this study, we investigated the expressions of TS and DPD in OSCC patients with and without lymph node metastasis, and analyzed the clinical and pathological findings in each case. CD82, also known as KAI1, has an important role to play in the invasiveness and metastasis of cancer cells (17). Altered expression levels of CD82 in different types of human cancer have been implicated as having prognostic value and as being linked to the long-term survival of the patients (17). In addition, we examined expression of CD82 in OSCC and determined the correlation of TS and DPD expression and the expression of CD82 as a marker of cancer metastasis.

Patients and Methods

Patients. OSCC specimens from 20 patients with cervical lymph node metastasis treated at the Department of Oral and Maxillofacial Surgery, Nagasaki University Hospital, Japan, between 2000 and 2005 were collected, and 20 OSCC patients without metastasis in the same period were randomly selected (Table I). Ten specimens of normal oral mucosa were used as controls. Overall, the average patient age was 64.5 years (range, 43-91 years), and the gender distribution of the patients was 26 males and 14 females. The primary sites of the OSCCs were the gingiva (n=20), the tongue (n=13), the floor of the mouth (n=5), and the buccal mucosa (n=2). All tumors were staged by the UICC–TNM staging system (18), and pathological differentiation was graded by the WHO classification system (19) (Table I).

Immunohistochemical examination. Specimens used for immunohistochemistry were fixed in 4% buffered formalin and embedded in paraffin. To avoid a reduction in immunoreactivity, the fixation time did not exceed 48 hours. Four-micrometer-thick tissue sections were cut and prepared for histologic examination, which were carried out routinely using hematoxylin and eosin stain. The expression of TS and DPD was studied immunohistochemically using polyclonal antibodies, which were generously donated by 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. Antibody to CD82 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Table I. *Patient characteristics (n=40).*

	Metastasis group	No metastasis group
Gender		
Male	12	14
Female	8	6
TNM categories		
T1	2	8
T2	8	6
T3	0	0
T4	10	6
N0	0	20
N1	13	0
N2	7	0
M0	20	20
M1	0	0
Stage		
I	0	8
II	0	6
III	8	0
IV	12	6
Histology		
Well-differentiated	11	16
Moderately differentiated	6	4
Poorly differentiated	3	0

The immunohistochemical staining procedure was as follows: 4-μm sections were cut from the paraffin-embedded specimens. The sections were dewaxed with xylene and rehydrated gradually with graded alcohols. Endogeneous 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 the aforementioned primary antibodies to TS (dilution, 1:1000), DPD (dilution, 1:500) and CD82 (dilution, 1: 200) at 4˚C overnight. After being washed 5 times in PBS, the sections were incubated with ENVISION+ (DAKO, Glostrup, Denmark) for 30 minutes. The immunochemical reaction was demonstrated with a solution of 3,3'-diaminobenzidine tetrahydrochloride in 50 mM Tris buffer (pH 7.6) containing 10 μL of 30% H_2O_2. 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.

Evaluation of immunohistochemical staining. All staining was evaluated as described previously. The immunostaining score for TS, DPD, and CD82 were calculated by counting the positive cells among more than 500 epithelial cells in randomly selected fields. Averages of 0-10% were deemed negative (–), whereas those averaging between 10% and 50% were deemed positive (+) and those 50% or higher were deemed highly positive (++). The two observers who assessed all staining results were blinded to the clinical outcome of the patients.

Statistical analysis. The association between immunohistochemical expressions and clinicopathological features was evaluated using contingency table analysis (Fisher's exact test). Differences were considered significant at $p<0.05$.

Table II. *Thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD) and CD82 expressions of the group with metastasis (M+) and without metastasis (M–) of OSCC.*

	TS expression			DPD expression			CD82 expression		
	Positive	Negative		Positive	Negative		Positive	Negative	
M+	20/20	0/20		14/20	6/20		11/20	9/20	
M–	15/20	5/20	p=0.024	17/20	3/20	p=0.225	18/20	2/20	p=0.015

Results

Expression of TS and DPD in primary oral cancer cells. We investigated the distributions of TS and DPD in surgically obtained OSCC specimens using immunohistochemical staining. TS and DPD expressions were confirmed by the presence of brown-stained cytoplasm in the cells in the specimens (Figure 1), and were demonstrated in the carcinoma cell nest. In controls, TS expression was negative in all 10 cases, while DPD was expressed in 3 out of 10 cases. TS and DPD expression in cancer cells was significantly higher than that in controls. Of the 20 OSCC cases with cervical lymph node metastasis, 20 were positive for TS, and 14 were positive for DPD in the corresponding primary tumor (Table II). In the group without cervical lymph node metastasis, 15 out of 20 cases were TS-positive, and 17 of 20 were DPD-positive (Table II). TS expression in primary sites of the group with metastasis was significantly higher than that of the group without.

Expression of TS and DPD in the cervical lymph nodes. In the cervical lymph node metastases, 20 of 20 were TS-positive, and 15 out of 20 cases were DPD-positive (75%) (Figure 2, Table III). TS expression in primary sites and in the lymph node metastases showed significant correlation. However, there was no significant correlation between DPD expression in primary sites and in lymph node metastases. Expression of TS and DPD did not show any significant correlation in relation to any other clinical factors.

Expression of CD82 and association with TS and DPD. In 11 out of 20 patients, CD82 was expressed in the primary oral tumor (Figure 3). CD82 was expressed in cervical lymph node metastases in only 4 out of 20 patients. In these 4 patients, CD82 expression was positive in both the primary site and lymph nodes, and in 7 out of 11 patients, CD82 expression was positive in the primary site but negative in the cervical lymph node. In 9 patients, CD82 expression was negative in both the primary site and lymph nodes (Table III). Eighteen out of 20 CD82-positive patients were without lymph node metastasis. CD82 expression in the primary sites of the group with lymph node metastasis was significantly lower than that of the group without. There was no significant association between expression of TS and DPD and expression of CD82.

Table III. *Comparison of thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD) and CD82 expression in primary and cervical lymph node tumor.*

Patient no.	TS		DPD		CD82	
	Primary	Lymph node	Primary	Lymph node	Primary	Lymph node
1	+	++	+	+	+	+
2	++	–	–	–	+	–
3	++	+	++	+	–	–
4	++	+	+	–	–	–
5	+	+	++	++	+	–
6	+	++	+	+	–	–
7	+	+	–	+	–	–
8	++	+	–	+	+	–
9	++	++	+	++	+	–
10	++	++	++	+	–	–
11	+	++	+	+	+	+
12	+	+	+	+	–	–
13	++	++	++	++	–	–
14	++	+	++	++	–	–
15	+	+	+	+	+	–
16	+	–	–	–	+	–
17	+	+	–	–	–	–
18	++	+	++	–	+	–
19	+	–	–	+	+	+
20	++	+	+	+	+	+

Evaluation of immunohistochemical staining: –, negative, +, positive, ++, highly positive.

Discussion

Although there are many reports about the relationship of tumor TS and DPD expression with response to 5-FU (20-22), to our knowledge, there are few reports concerning TS and DPD expression in metastatic tumors of lymph nodes and their relationship with primary tumors.

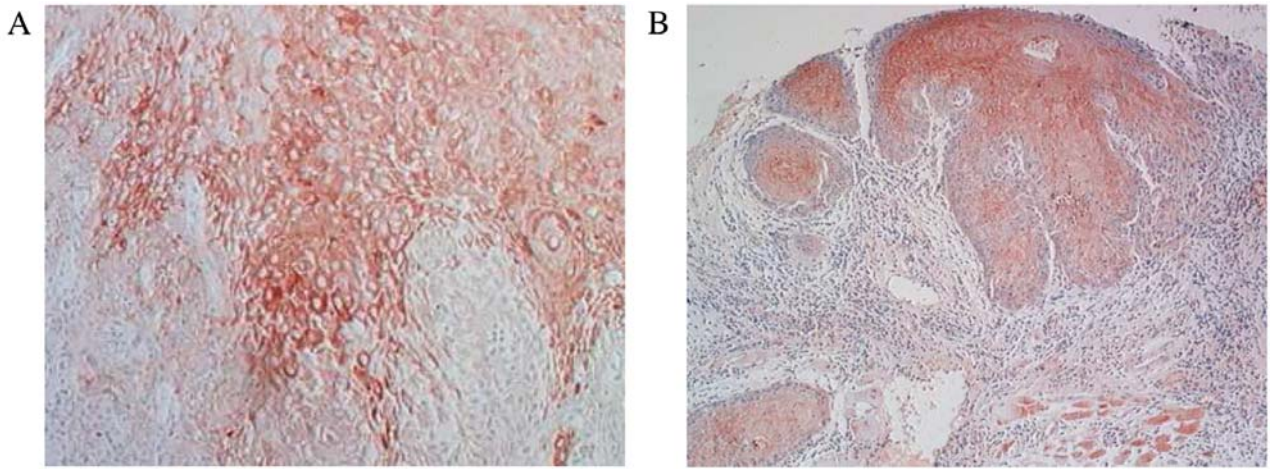

Figure 1. *Immunohistochemical staining for thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) in oral squamous cell carcinoma. Brown staining for TS and DPD were observed in the cytoplasm of tumor cells. A: TS, B: DPD.*

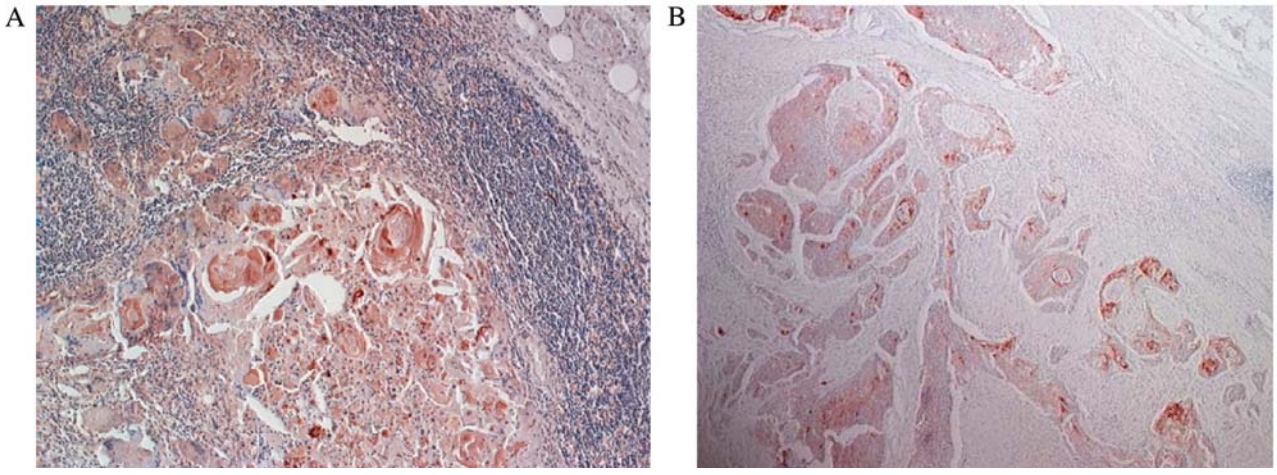

Figure 2. *Immunohistochemical staining for TS and DPD in cervical lymph node metastasis. A: TS, B: DPD.*

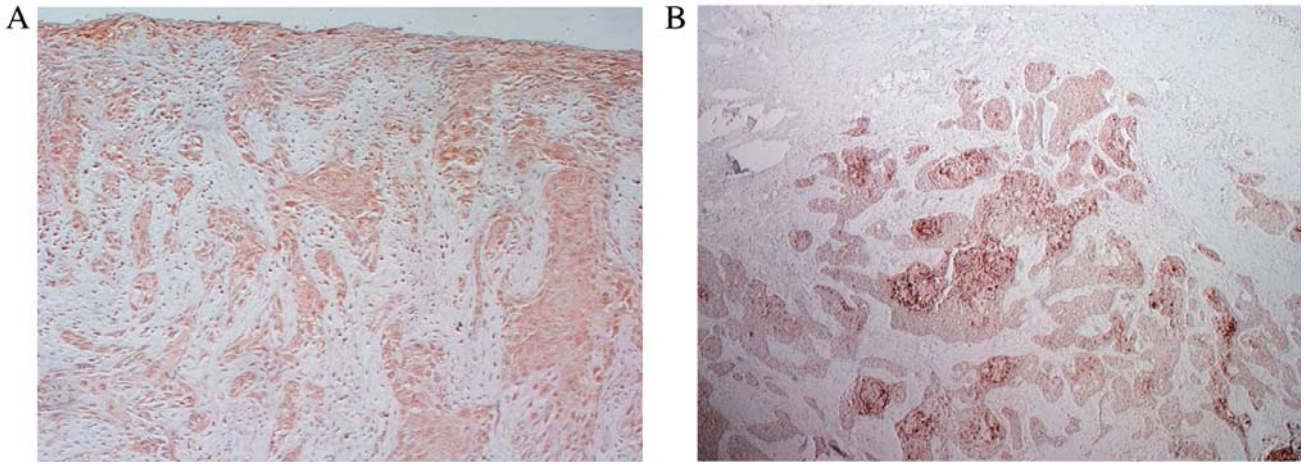

Figure 3. *Immunohistochemical staining for CD82 in oral squamous cell carcinoma. A: Primary site, B: cervical lymph node.*

Clinicopathologic factors associated with the development of cervical lymph node metastases have been well studied, in particular concerning tumor size and depth, differentiation, mode of invasion, microvascular invasion, and histologic grade of malignancy (23). The presence or absence of lymph node metastasis is one major prognostic factor for survival in patients with negative cervical lymph nodes (23). Although 5-FU is one of the most useful chemotherapy drugs for head and neck carcinoma, there is some controversy about its efficacy (8), in particularly for lymph node metastases (10).

In the current study, we found that TS expression levels showed a significant correlation between primary oral tumors and cervical lymph node metastases, but DPD levels showed no interaction between the two sites. Both TS activity and mRNA levels are high in cancerous tissue, reflecting more highly active DNA synthesis compared with that in normal mucosa (16). TS activity is higher during DNA replication but decreases when cells are not dividing and is therefore associated with proliferation (16). In our previous study of squamous cell carcinoma tissue, we also reported that expression of TS and Ki-67, which is a proliferation marker, showed a significant correlation (10). Aggressive tumors tend to metastasize, so we suggest that TS-positive cells have greater potential for metastasis to lymph nodes.

Metastatic cancer cells of lymph nodes are resistant to chemotherapy. DPD is present mainly in the liver and more than 80% of administered 5-FU is catabolized by DPD (24). The expression level of DPD influences selective cytotoxity and is important in predicting chemosensitivity to 5-FU. Many reports have discussed the relationship between DPD expression in the tumor and the efficacy of 5-FU-based chemotherapy (4, 25, 26). In our previous study, we reported that TS was not related to drug resistance, but DPD was related to the 5-FU response of the primary site in OSCC (10). In the current study, DPD expression in cervical lymph nodes was lower than that in primary sites. This finding suggests that DPD expression is not related to the lack of efficacy of 5-FU for lymph node metastases.

There is some controversy about DPD as a prognostic factor. Several studies suggested that intratumoral DPD expression was related to cell proliferation and differentiation (1, 27). However, Li *et al*. (28) reported that DPD was not a prognostic factor in breast cancer. Yasumatsu *et al*. (8) showed that DPD was not related to either the malignancy of tongue carcinoma or patient survival. In the current study, we did not find any relationship between DPD expression and prognosis either.

CD82 structurally belongs to the tetrapsin family, while being categorized as a metastasis-suppressor gene on functional grounds. CD82 plays an important role in the invasiveness and metastasis of cancer cells (17). Marked reduction of CD82 protein has been observed in highly metastatic cancer cells. Loss or reduced expression of CD82 in primary tumors of penile squamous cells was related to positive lymph node metastasis and poor prognosis as compared to negative or positive CD82 expression in the lymph nodes (29). In our findings, CD82 expression showed a significant difference between the group with metastasis and that without. Furthermore, in the group with metastasis, there was a significant correlation between expression in primary sites and lymph node metastatic cells. However, there was no significant difference between CD82 expression and TS expression.

CD82 has an ectopic effect on adhesion by strengthening the interactions between E-cadherin and β-catenin. In inducing this effect, CD82 may reduce the likelihood of cellular dissemination from the primary tumor (30). Furthermore, CD82 indirectly regulates the function of matrix metalloproteinase by up regulating their tissue inhibititors (31). Metastasis is a complex cascade process that involves a number of orchestrated events by cancer cells in order for them to break away from the primary tumor, break down tissue barriers and invade a new organ (secondary site) to form new tumors (17). CD82 is obviously involved in a number of cellular events that are somewhat mirrored in clinical studies.

In conclusion, TS and CD82 play important roles in lymph node metastasis, and we suggest that there are somewhat different metastatic mechanisms underlying the effect of TS and CD82 in oral carcinoma.

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