SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 2: 297-301, 2011

Thymidylate synthetase and dihydropyrimidine dehydrogenase mRNA levels in esophageal cancer

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Received September 1, 2010; Accepted November 19, 2010

DOI: 10.3892/ol.2010.227

Abstract. This study investigated the mRNA levels of thymidylate synthetase (*TYMS*) and dihydropyrimidine dehydrogenase (*DPYD*) in esophageal squamous cell carcinoma (ESCC). *TYMS* and *DPYD* gene expression was quantified using real-time RT-PCR in 56 patients with ESCC, co-amplified with glyceraldehyde-3-phosphate dehydrogenase as an internal standard. The results were analyzed with reference to the clinicopathological characteristics and the prognosis of the ESCC patients. The *TYMS* and *DPYD* expression levels in patients positive with lymphatic invasion were significantly higher compared to those in patients who exhibited negative lymphatic invasion (*TYMS* P=0.0127, *DPYD* P=0.0127). Patients were classified into the groups high *TYMS*/*DPYD*, high *TYMS* but low *DPYD*, low *TYMS* but high *DPYD* and low *TYMS*/*DPYD*. The highest survival rate was found in the group with low *TYMS*/*DPYD* and the lowest survival rate in the group with high *TYMS*/*DPYD* (P=0.017). It was concluded that, on the basis of the multivariate analysis, *TYMS* mRNA expression is a candidate that serves as an independent prognostic factor for ESCC patients.

Introduction

The prognosis of patients with esophageal cancer remains poor, prompting the search for novel treatment strategies. Given the high malignant potential of this type of cancer, many patients developed local recurrence of the tumor or distant metastasis within a short period of time. Molecular biological studies have shown that esophageal squamous cell carcinoma (ESCC) is caused by the accumulation of multiple genetic changes in oncogenes and tumor suppressor genes (1,2). Thymidylate synthetase (*TYMS*) plays a role in catalyzing the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), a crucial synthetic step in nucleotide metabolism. Dihydropyrimidine dehydrogenase (*DPYD*) is also a key enzyme in the metabolic pathway involved in the degradation of the pyrimidine bases uracil and thymine.

TYMS and *DPYD* are significant enzymes in *de novo* DNA synthesis and the salvage pathway in cancer cells, respectively. This study investigated the *TYMS* and *DPYD* mRNA expression in ESCC by real-time RT-PCR using a LightCycler system. The results were analyzed with reference to the clinicopathological characteristics and prognosis of the ESCC patients.

Materials and methods

Tissue samples. Tissue samples were obtained from 56 patients with primary ESCC who underwent radical esophagectomy at the Department of Surgery, Nagoya City University Medical School, between 1996 and 2000. The study design was approved by the Institutional Review Board of the university hospital and written consent was obtained from each patient. The tumors were classified according to the Guidelines for the Clinical and Pathological Studies on Carcinoma of the Esophagus (3). The patient population comprised 44 males and 12 females (mean age 63.2±8.4 years; range 46-80). The samples were immediately frozen in liquid nitrogen and stored at -80˚C until use. None of the patients received chemotherapy or radiation therapy prior to or following surgery.

RT-PCR assays for thymidylate synthetase and dihydropyrimidine dehydrogenase. The RNA concentration was determined using a spectrophotometer and adjusted to a concentration of 200 ng/ml. RNA (1 μg) was reverse transcribed by the Superscript II enzyme (Gibco BRL, Gaitherburg, MD, USA) with 0.5 mg oligo(dT) (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The reaction mixture was incubated at 42˚C for 50 min followed by incubation at 72˚C for 15 min. To ensure the quality of mRNA extraction and reverse transcription, the samples were subjected to PCR amplification with oligonucleotide primers specific for the constitutively

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Key words: thymidylate synthetase, dihydropyrimidine dehydrogenase, esophageal cancer, LightCycler

Table I. Correlation of *TYMS* mRNA expression in esophageal cancer with clinicopathological characteristics.

Characteristics	No. of patients (n=56)	*TYMS* expression relative to *GAPDH*	P-value
Age at surgery			0.9638
≤65 years	33	1.542	
>65 years	23	1.568	
Gender			0.7396
Male	44	1.637	
Female	12	1.896	
Pathological subtype			0.3309
Well	16	1.98	
Non-well	40	1.382	
Tumor status			0.6742
T1/2	21	1.402	
T3/4	35	1.644	
Lymph node status			0.5005
n^+	41	1.666	
n^-	15	1.243	
Pathological stage			0.4452
0/I/II	20	1.268	
III/IV	36	1.711	
Lymphatic invasion			0.0126
Negative	11	0.775	
Positive	42	1.746	
Blood vessel invasion			0.9554
Negative	19	1.566	
Positive	34	1.532	

TYMS, thymidylate synthetase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

expressed gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and normalized to it. PCR was performed using LightCycler-Fast start DNA Master SYBR-Green I kit (Roche Molecular Biochemicals, Mannheim, Germany). The primer sequences for the *TYMS* gene were: forward primer, 5'-TTACCTGAATCACATCGAGC-3' and reverse primer, 5'-ATATCCTTCGAGCTCCTTTG-3'. The cycling conditions were: initial denaturation at 95˚C for 10 min, followed by 60 cycles at 94˚C for 15 sec, 55˚C for 5 sec and 72˚C for 8 sec. The primer sequences for the *DPYD* gene were: forward primer, 5'-GTTCTGGCTACCAGGCTAT-3' and reverse primer, 5'-CATAAGGTGTTGTCCTGGAA-3'. The cycling conditions were: initial denaturation at 95˚C for 10 min, followed by 60 cycles at 94˚C for 15 sec, 56˚C for 5 sec and 72˚C for 6 sec. Amplified cDNAs were separated on 1% agarose gels and the bands were visualized by ethidium bromide.

Statistical methods. Data are expressed as the means ± standard deviation (SD). Statistical analysis was performed using the Stat-View software package (Abacus Concepts, Berkeley, CA, USA). The Mann-Whitney U test was used to evaluate the significance of the expression in paired groups. The survival of patients with ESCC was examined using the Kaplan-Meier method, and the survival times were compared using the log-rank test. Survival was measured from the day of surgery. Multivariate analysis was performed using Cox's regression model and the logistic multivariate regression model. $P<0.05$ was considered to be statistically significant.

Results

Analysis of thymidylate synthetase and dihydropyrimidine dehydrogenase mRNA levels by real-time RT-PCR assay using LightCycler. TYMS/GAPDH mRNA levels of the 56 esophageal cancer tissue samples were 1.553±0.275. The relationship between *TYMS/GAPDH* mRNA and the patient clinicopathological characteristics were examined (Table I). No significant differences were noted in *TYMS/GAPDH* mRNA with respect to age, gender, pathological differentiation, tumor status, lymph node status, stage or vessel invasion. The *TYMS/GAPDH* mRNA expression levels in patients positive with lymphatic invasion were significantly higher compared to those in patients who exhibited negative lymphatic invasion (P=0.0127).

The *DPYD/GAPDH* mRNA expression levels were 5.463±1.807. By contrast, the *DPYD/GAPDH* mRNA expression levels in patients positive with lymphatic invasion were significantly lower compared to those in patients who exhibited negative lymphatic invasion (P=0.0417). Moreover, no significant differences were noted with respect to other factors (Table II).

No significant clinicopathological differences were noted in patients classified into groups with *TYMS* levels higher

Table II. Correlation of *DPYD* mRNA expression in esophageal cancer with clinicopathological characteristics.

Characteristics	No. of patients (n=56)	*DPYD* expression relative to *GAPDH*	P-value
Age at surgery			0.8581
≤65 years	33	5.190	
>65 years	23	5.855	
Gender			0.1240
Male	44	6.265	
Female	12	2.525	
Pathological subtype			0.2431
Well	16	3.217	
Non-well	40	6.515	
Tumor status			0.3333
T1/2	21	7.741	
T3/4	35	4.096	
Lymph node status			0.4785
n^+	41	4.680	
n^-	15	7.605	
Pathological stage			0.5420
0/I/II	20	6.959	
III/IV	36	4.632	
Lymphatic invasion			0.0418
Negative	11	13.225	
Positive	42	3.717	
Blood vessel invasion			0.3017
Negative	19	8.347	
Positive	34	4.206	

DPYD, dihydropyrimidine dehydrogenase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase

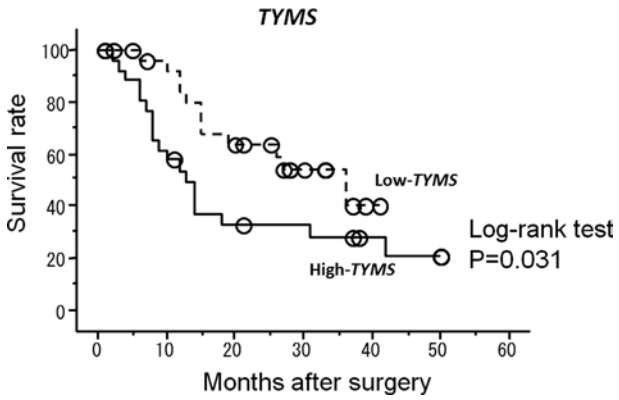

Figure 1. The overall survival rate of esophageal squamous cell carcinoma patients and the expression levels of thymidylate synthetase (*TYMS*). The expression levels of *TYMS* were high/low.

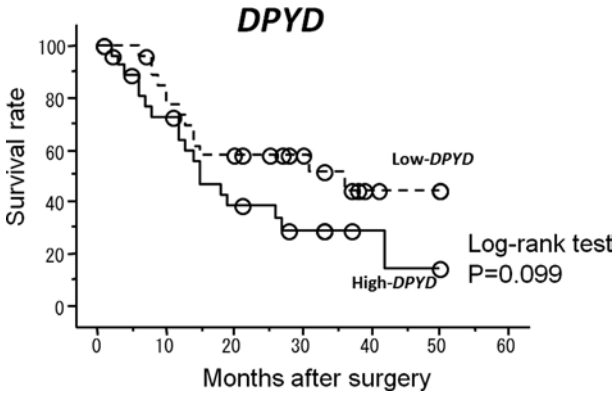

Figure 2. The overall survival rate of esophageal squamous cell carcinoma patients and the expression levels of dihydropyrimidine dehydrogenase (*DPYD*). The expression levels of *DPYD* were high/low.

(n=27) and lower (n=29) than 0.855. However, a significantly higher risk of lymph node metastasis was noted in patients with higher levels of *DPYD* (n=28, *DPYD*/*GAPDH* mRNA levels >1.70).

Relationship between TYMS and DPYD and survival. The correlation between the *TYMS* and *DPYD* mRNA expression levels and the survival of ESCC patients following surgery (median follow-up 19.7 months) was investigated. Patients with high *TYMS* mRNA expression levels had a significantly shorter survival after surgery compared to patients with low *TYMS* mRNA expression levels (P=0.031) (Fig. 1). Patients with high *DPYD* mRNA expression levels had a shorter survival, but no significant difference was found (P=0.099) (Fig. 2).

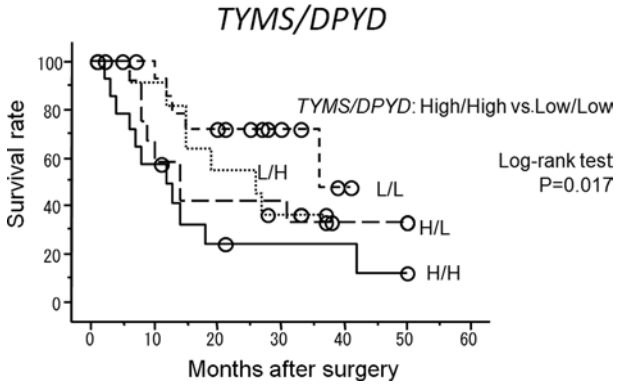

Figure 3. The overall survival rate of esophageal squamous cell carcinoma patients and the expression levels of thymidylate synthetase (*TYMS*) and dihydropyrimidine dehydrogenase (*DPYD*). The expression levels of *TYMS* and *DPYD* were divided into the groups: high *TYMS*/*DPYD*, high *TYMS* but low *DPYD*, low *TYMS* but high *DPYD* and low *TYMS*/*DPYD*.

Of the four groups of patients (high *TYMS*/*DPYD*, high *TYMS* but low *DPYD*, low *TYMS* but high *DPYD* and low *TYMS*/*DPYD)*, the best survival rate was found in the group with low *TYMS*/*DPYD* and the worst survival rate was observed in the group with high *TYMS*/*DPYD* (P=0.017) (Fig. 3). The univariate analysis showed that among the clinicopathological characteristics, local invasiveness (tumor status) (risk ratio 7.38; P=0.0003), lymph node metastasis (node status) (risk ratio 6.07; P=0.0032), lymphatic invasion (risk ratio 5.48; P=0.021), blood vessel invasion (risk ratio 3.56; P=0.0069) and *TYMS* mRNA expression (risk ratio 2.19; P=0.038) were statistically significant prognostic factors. The multivariate analysis showed that local invasiveness (P=0.045) and *TYMS* mRNA expression (P=0.041) were independent prognostic factors (Table III).

Table III. Univariate and multivariate analysis of the expression levels of thymidylate synthetase and dihydropyrimidine dehydrogenase and various clinical characteristics.

A, Univariate		
Characteristics	HR (95% CI)	P-value
Tumor status	7.38 (2.52-21.66)	0.0003
Lymph node status	6.07 (1.83-20.14)	0.0032
Pathological stage	1.48 (0.71-3.11)	0.3000
Lymphatic invasion	5.48 (1.29-23.39)	0.0210
Blood vessel invasion	3.56 (1.42-8.96)	0.0069
TYMS	2.19 (1.05-4.57)	0.0380
DPYD	1.79 (0.88-3.67)	0.1100
B, Multivariate		
Tumor status	4.67 (1.03-21.08)	0.0450
Lymph node status	2.37 (0.62-9.10)	0.2100
Lymphatic invasion	1.08 (0.13-9.17)	0.9500
Blood vessel invasion	1.42 (0.47-4.32)	0.5400
TYMS	2.33 (1.03-5.24)	0.0410

CI, confidence interval; *TYMS*, thymidylate synthetase; *DPYD*, dihydropyrimidine dehydrogenase.

Discussion

Esophageal cancer is a digestive cancer with poor prognosis and the mortality rate is steadily increasing. Three types of treatment are currently available, i.e., operation, chemotherapy and radiation therapy. Frequently, chemotherapy and radiation therapy are combined, both before and after surgery. In esophageal cancer, tumor growth is extremely rapid. Consequently, prompt and correct diagnosis and staging, including identification of remote metastases, and individual treatment are required. The prediction of sensitivity to chemotherapeutic agents prior to therapy is relevant. Chemotherapy for esophageal cancer relies heavily on 5-fluorouracil (5-FU) and cisplatin. However, individual variations in responsiveness to these chemotherapies exist. Therefore, the susceptibility testing of the anti-cancer drug treatment in esophageal cancer was reported. We also examined the relationship between the expression of *TYMS*, *DPYD*, thymidylate synthetase (*TYMP*) and orotate phosphoribosyl transferase (*OPRT*) and 5-FU sensitivity in 25 ESCC cell lines. Our findings showed that the *TYMS* and *DPYD* mRNA expression levels may aid in predicting the anti-tumor activity of 5-FU in ESCC (4). In colorectal cancer, Salon *et al* and Nishimura *et al* reported a correlation between the clinical effect of 5-FU and the expression of those genes (5,6). Oguri *et al* reported that the degradation of 5-FU via *DPYD* is a significant determination of 5-FU sensitivity, while the induction of *TYMS* contributes to acquired resistance against 5-FU in lung cancer (7).

On the other hand, certain authors have reported that *TYMS* and *DPYD* exhibit the malignant potential of gastric and colon cancers. Terashima *et al* reported that in a group of patients who did not receive adjuvant chemotherapy, survival was poor in patients with high *TYMS* activity (8). Shirota *et al* investigated the correlation between *DPYD* and malignant potential in colon cancer, reporting that higher *DPYD* levels were associated with higher pathological classification, micro-scopic lymph node metastasis and liver metastasis (9). Suda *et al* found that the expression of *TYMS* in gastric cancer correlated with recurrence and survival rate (10).

Therefore, *TYMS* and *DPYD* affect the clinical outcome of esophageal cancer in two ways. One possibility is that *TYMS* and *DPYD* affect the malignancy of cancer, the other is that they affect the outcome of the anti-cancer drug treatment. Therefore, in the present study cases in which anti-cancer drug treatments were used pre- and post-surgery were excluded. Additionally, Tanaka *et al* reported that the expression of *TYMS* and *DPYD* was altered by chemoradiation therapy (CRT) in residual tumor cells of esophageal cancer, when comparing mRNA levels in pre-CRT biopsies and post-CRT specimens (11). Brucher *et al* found no significant correlation between clinical or histological factors and the relative gene expression of *TYMS*, *TYMP*, *DPYD* or *Her-2/neu*. However, patients exhibiting these factors underwent pre-operative, combined radiochemotherapy (12). Therefore, not only were the cases with anti-cancer treatment excluded, but also those cases with radiation therapy. As a result, we examined the

correlation between the malignant potential of esophageal cancer and the expression of *TYMS* and *DPYD*.

In this study, *TYMS* mRNA expression was significantly correlated with lymphatic invasion. However, no other clinicopathological characteristics correlated with *TYMS* mRNA levels. With regard to post-surgical survival, a high expression of *TYMS* was associated with a poor prognosis. Only the parameter and tumor status were noted in the multivariate analysis. Comparable results were reported by Suda *et al* in gastric cancer. These authors reported that the survival curve for the *TYMS*-positive group was significantly lower compared to that of the *TYMS*-negative group in the immunohistochemical study (10). In addition to *TYMS*, *DPYD* mRNA expression was statistically correlated with lymphatic invasion. Nevertheless, no other factors, including prognosis, correlated with *DPYD*. Certain studies reported the usefulness of the combination analysis. Suda *et al* reported that the *TYMS*-positive *TYMP*-positive group was more inhibited than in other groups (8). Beck *et al* reported that in cultured cells from colorectal cancers, those with low *DPYD* and *TYMS* expression were experimentally more sensitive, while the patients were clinically more sensitive to 5-FU (13). Danenberg *et al* reported that in colorectal cancer, patients with low *TYMS*, *DPYD* and *TYMP* levels exhibited the best survival curves (14). Ichikawa *et al* reported that the combined expression of *TYMS* and *DPYD* predicted the efficacy of chemotherapy (15). In the present study, combination analysis was useful. The low *TYMS*/*DPYD* group showed the best survival curves statistically. A combined evaluation of the expression of other genes, such as *TYMP*, is required for a more accurate prediction of the response.

In conclusion, the present study showed that there was a significant correlation between *TYMS* and *DPYD* mRNA levels in esophageal cancer and the survival of patients presenting with type of cancer. Based on the present data and the relationship between gene expression and 5-FU sensitivity in esophageal carcinoma cell lines, more effective treatment should be established for individual patients.

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