

The Correlation Between the Intratumoral Thymidylate Synthase Levels and Clinicopathological Factors in Colorectal Cancer Patients

**Arihiro UMEHARA, Kazuhiko YOSHIMATSU, Shungo ENDO,
Hiroyuki KATO and Shunsuke HAGA**

Department of Surgery (Director: Prof. Tetsuro KAJIWARA)

Tokyo Women's Medical University Daini Hospital

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We determined the thymidylate synthase (TS) levels in colorectal cancer tissue specimens and then investigated the potential of using the TS levels as a prognostic factor in colorectal cancer. The subjects consisted of 44 patients with colorectal cancer who had been treated by a resection. The specimens were assayed by Western blotting using anti-human recombinant TS polyclonal antibody. The TS level was then determined semiquantitatively by an image analysis of the obtained bands. When the TS levels were viewed in the context of clinicopathological factors, low TS levels were seen in patients with positive serum carcinoembryonic antigen, lymph node metastases, liver metastases, and high grade lymphatic invasion. In patients with advanced Dukes stage, the TS levels were also low, thus suggesting that some correlation may exist between the stage and the TS levels. We concluded that the TS levels in colorectal cancer thus predicted the outcome of the disease, and therefore the TS levels may be useful as a prognostic factor.

Introduction

In recent years, the number of colorectal cancer (CRC) cases has been increasing in Japan. After the initial diagnosis, 77.2% of these patients undergo curative surgical treatment¹⁾. Over 50% of all patients show a favorable prognosis. However, 15~20% of these patients develop recurrent disease and die. Therefore, after a curative resection, many CRC patients are treated with 5-FU-based adjuvant chemotherapy.

Thymidylate synthase (TS) catalyzes the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). This reaction is an essential step in DNA biosynthesis²⁾. In addition, TS is also a target enzyme for

fluoropyrimidine drugs³⁾ which are often used during chemotherapy in CRC patients in order to induce biochemical modulation^{4)~6)}. The effect of 5-FU is mediated by its metabolite 5-fluoro-2'-deoxyuridine 5-monophosphate (FdUMP). The availability of FdUMP determines the extent of TS inhibition, which is thus mediated by the formation of a covalent ternary complex consisting of TS, FdUMP, 5,10-methylene-tetrahydrofolate (CH₂FH₄)³⁾. Several papers have reported that an over expression of TS in tumors may thus represent an important protective mechanism in response to fluoropyrimidine⁷⁾⁸⁾.

On the other hand, recent studies have reported a correlation between the TS levels in hu-

man cancer and the prognosis. However, the assessment of the intratumoral TS levels regarding prognosis have been diverse, because of differences in both the objectives and the methodologies for measuring the TS levels^{9)~11)}. We therefore measured the intratumoral TS levels in surgically resected specimens from CRC patients using the Western blotting method with anti-human recombinant TS (rTS) polyclonal antibody¹²⁾ because no previous study had ever measured the TS levels using this method. We also investigated whether or not a correlation exists between the intratumoral TS levels and other prognostic factors, to clarify the role of the TS levels in colorectal cancer patients.

Patients and Methods

Patients population

From 1989 to 1995, the TS levels were measured in a random sampling of 44 patients who underwent surgical resections for primary colorectal adenocarcinoma which had been confirmed histologically and had never been previously treated with anticancer drugs. All resected lesions were divided into two specimens; one was

processed for a routine pathological examination while the other extracted from the edge of tumor was frozen immediately in liquid nitrogen and then stored at -80°C until Western blotting was performed.

The clinicopathological features are summarized in Table 1. Dukes classification was used for the clinical stage and depth of tumor invasion. The histological typing, lymphatic invasion and venous invasion were all determined based on the criteria of the Japanese Classification of Colorectal Carcinoma¹³⁾. Thirty-five patients were also treated with fluoropyrimidine after surgery.

Preparation of tissue lysates for Western blotting

The tissue samples were placed in lysis buffer and homogenized immediately using a tissue homogenizer. The lysates were centrifuged at 14,000 rpm for 20 min at 4°C , and the supernatant was separated from the tissue. The protein content was measured and determined to be 6 mg/ml using the Bio-Rad protein assay kit (Veemendaal, Netherlands).

Table 1 Summary of clinicopathologic features

Variable		No. of patients (n = 44)
Age (yrs)		62.2 (30 ~ 83)
Gender	male	22
	female	22
Tumor site	right colon	13
	left colon	20
	rectum	11
Histological type	well differentiated	21
	moderately differentiated	20
	poorly differentiated	3
Dukes stage	A	4
	B	15
	C	17
	D	8
Chemotherapy (fluorouracil)	preoperative	0
	adjuvant	29
	therapeutic	6
	none	9

Anti-TS polyclonal antibody

Anti-human rTS polyclonal antibody was provided by the Second Cancer Research Laboratory (Taiho Pharmaceutical Co, Tokyo). The specificity of the purified antibodies was examined by a Western blot analysis using CCRF-CEM cytosol, a human leukemia cell line producing TS¹².

Western blot analysis

According to the method of Okabe et al¹², aliquots of the above described supernatant were heated for 2 min in a boiling water bath and then 30 µg protein/lane were placed in 12.5% polyacrylamide gel. A 5 ng/lane of rTS protein was applied as a positive control. After electrophoresis, the proteins were electrically blotted to the polyvinylidene difluoride (PVDF) membrane at 4 °C. The TS proteins in the membrane were immunochemically detected by the Avidin-Biotin-Complex (ABC) method. The anti-human rTS polyclonal antibody was used as a primary antibody. The blots were then air-dried and covered by plastic foil. Densitometry scanning of the membrane was performed using a Canoscan 600 (Canon Inc, Tokyo) and the findings were analyzed using an image analysis software program (NIH IMAGE v. 1.61; GWayne Rasband, National Institute of Mental Health, NIH). The TS score was determined by densitometry scanning.

All investigators were blinded to the clinical and pathological data. The TS densitometry score was calculated as follows: (Densitometry of sample TS)/(Densitometry of positive control) × 1000. We investigated any correlations between the TS densitometry score and such clinicopathological prognostic factors as age, gender, tumor site, histological type, depth of tumor invasion, serum carcinoembryonic antigen (CEA) levels, lymph node metastasis, hepatic metastasis, lymphatic invasion, venous invasion, Dukes stage and survival parameters.

Statistical analysis

A statistical analysis was performed using the nonparametric Mann-Whitney U ranking test and the Kruskal-Wallis test. The survival rate was determined based on the Kaplan-Meier estimates. The log-rank test was used to test the difference in survival. Statistical significance was considered to exist when $p < 0.05$.

Results

The membrane immunochemically detected TS proteins are showed in Fig. 1. The analyzing densitometry of the membrane are showed in Fig. 2. The TS densitometry scores of all patients are showed in Fig. 3. The average TS level was 71.4.

Regarding the clinicopathological features, neither age, gender, tumor site, histological type, nor venous invasion correlated with the TS densitometry score (Table 2).

Regarding the preoperative serum CEA level, the average TS densitometry score of the CEA negative group (< 2.5 ng/ml) was 77.1, while the CEA positive group (≥ 2.5 ng/ml) was 53.1 ($p = 0.0325$). As for lymph node metastasis, the negative group was 87.4, while the positive group was 56.7 ($p = 0.0022$). Regarding liver metastasis, the TS densitometry score was 77.8 in the negative group, and 42.6 in the positive group ($p = 0.0106$). For lymphatic invasion, the negative or low grade (ly0, ly1) group was 77.7 while the high grade (ly2, ly3) group was 49.7 ($p = 0.0269$). Regarding Dukes staging, the TS densitometry scores were as follows: Dukes A: 117.4, Dukes B: 81.7, Dukes C: 64.9, Dukes D: 42.6 ($p = 0.0030$).

To determine whether or not any correlation existed between the survival and the TS densitometry score, the patients were divided into three groups consisting of: a low TS group ($TS < 60$), a middle TS group ($60 < TS < 100$), and a high TS group ($TS \geq 100$). As a result, a correlation was observed between the TS densitometry

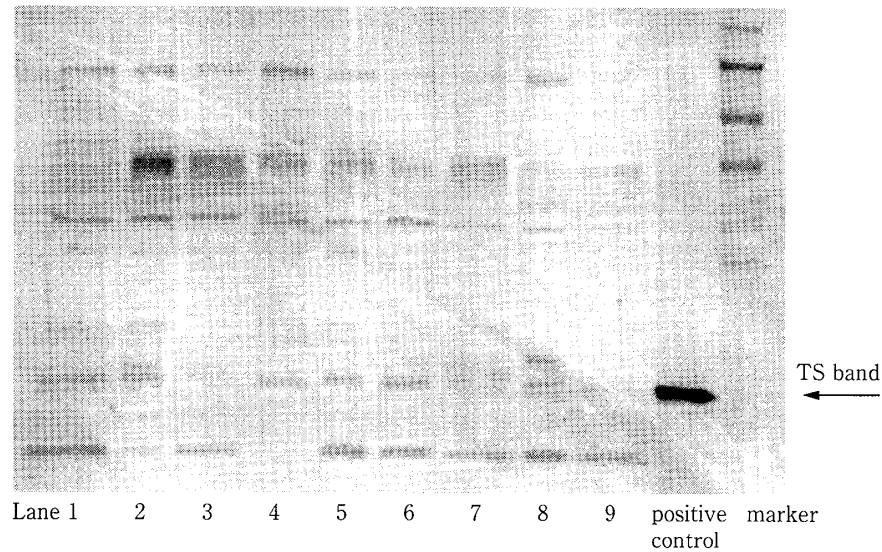
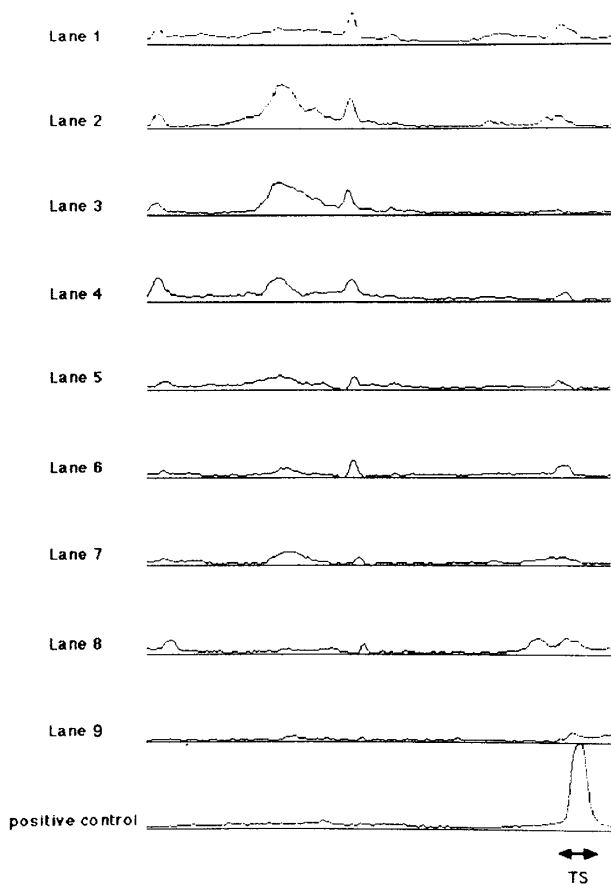


Fig. 1 Western blot analysis

The TS proteins in the PVDF membrane were immunochemically detected by the ABC method with anti-human rTS polyclonal antibody. → : TS protein of positive control.



score and prognosis (Fig. 4).

Discussion

Recent anticancer chemotherapy modalities designed to achieve biochemical modulation have helped to improve both the efficacy of chemotherapy and the survival rate of colorectal cancer patients⁴⁾⁵⁾. We used low-dose leucovorin/5-FU therapy in both patients with unresectable advanced CRC and those with recurrent disease. To prolong the survival time, the optimal administration procedure of this therapy should thus be determined. If the sensitivity of tumor cells to 5-FU can be predicted, then the use of inappropriate anticancer therapy can be reduced. The resistance of tumor cells for 5-FU is considered to be regulated by the expression levels of TS in tumor

Fig. 2 Densitometry of the membrane

These were analyzed using NIH IMAGE. ↔ : TS densitometry of positive control. The TS densitometry scores were calculated as follows :

$$\left(\text{Area of densitogram of sample TS} \right) / \left(\text{Area of densitogram of TS of positive control} \right) \times 1000.$$

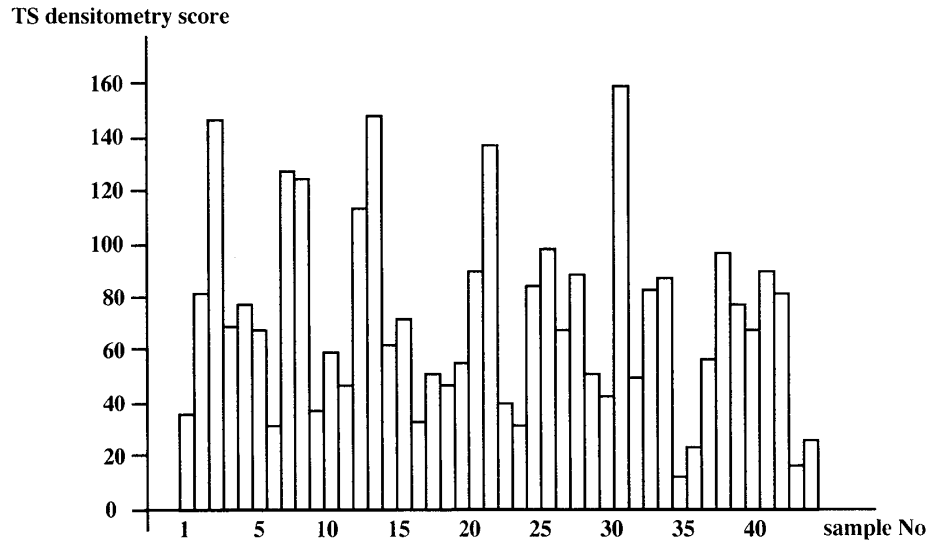


Fig. 3 TS densitometry scores in 44 patients
The mean of score was 71.4 and range was 12.4~159.8.

Table 2 Relation between clinicopathologic features and TS densitometry score

Variable		n	mean of TS	p value
Age(yrs)	≤ 62	20	70.2	NS
	> 62	24	72.2	
Gender	male	22	76.4	NS
	female	22	66.3	
Tumor site	right colon	13	55.9	NS
	left colon	20	81.6	
	rectum	11	73.2	
CEA	negative	25	77.1	0.0325
	positive	16	53.1	
Lymph node metastasis	negative	21	87.4	0.0022
	positive	23	56.7	
Liver metastasis	negative	36	77.8	0.0106
	positive	8	42.6	
Depth of invasion	limited to the wall	6	94.3	NS
	extended through the wall	38	67.7	
Histological type*	well differentiated	21	74.5	NS
	moderately differentiated	20	64.1	
	poorly differentiated	3	97.1	
Lymphatic invasion*	ly0, ly1	34	77.7	0.0269
	ly2, ly3	10	49.7	
Venous invasion*	v0, v1	39	72.3	NS
	v2, v3	5	63.8	
Dukes stage	A	4	117.4	0.0030
	B	15	81.7	
	C	17	64.9	
	D	8	42.6	

* : Japanese classification of colorectal carcinoma, NS : not significance.

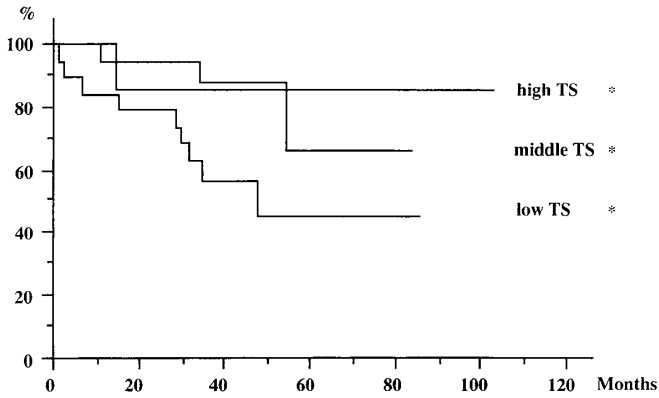


Fig. 4 Overall cumulative survival curves at each TS levels

The 5-year cumulative survival rates were 45.2% in the low TS group, 66.1% in the middle TS group and 85.7% in the high TS group. *: significant difference was observed between each groups at $p=0.0336$.

cells.

Berger et al^[14] and Swain et al^[15] reported that an overproduction of TS, as a result of gene amplification, has been shown to be associated with 5-FU resistance. In addition, Chu E et al^[16] reported, in a study of human breast and colon cancer cell lines exposed to 5-FU, that resistance was associated with increased levels of TS. Peters et al^[17] reported in a clinical study that colon cancers with upregulated TS levels were noted to be resistant to 5-FU. There are differences between the patients who received chemotherapy and the patients who did not, then we can not compare the two groups. There were nine patients in the untreated group, three patients were too old for chemotherapy, three were Dukes A and three were Dukes D.

Although the relationship between the TS levels in tumor cells and the patients' prognoses has been recently discussed in the literature, this hypothesis still remains controversial. In all studies reported to date, binding assays, immunohistochemical studies, and semiquantitative assays of mRNA have been used to determine the TS lev-

els. However, Western blotting, which is a semiquantitative procedure, has so far never been used to determine the TS protein levels.

We, therefore, semiquantitatively assayed the TS of tumor cells in patients with colorectal cancer using Western blotting, and thus estimated the TS level from the calculated TS densitometry score, in order to investigate the relationship between the TS levels and clinicopathological factors. The results of this investigation showed the TS levels to decrease in patients with increased serum CEA levels before operation, lymph nodes containing metastasis, liver metastasis, and a high-degree of lymphatic invasion, which thus indicated that a significant correlation existed between the TS levels and clinicopathological factors. The stage of cancer based on Dukes classification was inversely proportional to the TS levels. The TS levels significantly decreased in patients with advanced-Dukes stage cancer. When the patients were classified according to the TS levels, the lower TS group had a poorer outcome than the higher TS group. There was the correlation between intratumoral TS levels and the prognostic factors including serum CEA level, lymph node metastasis, liver metastasis, lymphatic invasion, and Dukes stage, but multivariate analysis showed TS was not independent variable for prognosis.

Our results contrasted sharply with those reported by Johnston et al^[9], Lenz et al^[18], Yamachika et al^[11]. Johnston et al^[7] qualitatively determined the TS level in cancer tissue obtained from patients with rectal cancer using an immunohistochemical method, and thus classified patients into a low intensity group and a high intensity group according to the intensity of the TS reaction assessed with a visual grading system. They reported the low intensity group to have a better outcome than the high intensity group. Lenz et al^[18] determined the TS mRNA level by poly-

merase chain reaction in biopsy specimens obtained from patients with gastric cancer, and also reported patients with low TS mRNA levels ($< 4.6 \times 10^{-3}$ TS/b-actin, and who were treated with 5-FU-based chemotherapy, to have a more favorable outcome than patients with a high TS mRNA level ($> 4.6 \times 10^{-3}$ TS/b-actin). Yamachika et al¹¹⁾ qualitatively determined the TS levels in tumor tissue specimens obtained from patients with CRC by immunostaining with polyclonal antibody, and classified the patients into a TS-negative group ($< 10\%$) and a TS-positive group ($\geq 10\%$) based on the percentage of positive cells. They also reported the TS-negative group to have a more favorable outcome than the TS-positive group. Western blotting thus appears to be a more objective method to determine the protein TS levels, although no definitive conclusions can be made until further comparison studies are performed.

On the other hand, Sanguedolce et al¹⁰⁾ obtained results consistent with ours. They determined the TS levels in tumor tissue specimens obtained from patients with colorectal cancer using a binding assay. They reported the TS levels to be higher in Dukes A tumors than in Dukes D tumors, and also described that patients with lower TS levels also had a significantly increased risk of death. We consider our results to be consistent with their results because the TS levels determined by Western blotting with polyclonal antibody correlated with the TS levels determined by a binding assay¹¹⁾.

Our results suggest that tumors with decreased TS levels appear to have an increased level of malignancy. Since highly malignant cells are known to have increased cell division and cell proliferation activities, DNA synthesis is thus considered to be activated in these cells. If so, then the TS levels would normally be expected to be elevated. We therefore think that the TS level

decreases as a result of cancer growth, either because the amount of TS is limited or since more TS is being used than is produced. As for the difference in the results of the measuring method, one must take into consideration the fact that the TS activity can only be measured by a TS binding assay and there is possibility that TS is not distributed homogeneously in cancer lesions. Therefore, it is necessary to evaluate the same specimens using different procedures in order to accurately determine the TS expression in patients with cancer.

We think another hypothesis that DNA biosynthesis depends on the actions of the de novo biosynthetic pathway, mediated by deoxy thymidylate synthase (TS), and the salvage pathway, mediated by thymidine (TdR) kinase. Another salvage enzyme levels, like TdR kinase, may be increased instead of TS levels in the patients with advanced colorectal cancer¹⁹⁾.

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大腸癌における腫瘍組織内 Thymidylate Synthase と臨床病理学的因子の関連についての検討

東京女子医科大学 附属第二病院 外科（指導：梶原哲郎教授）

ウメハラ アリヒロ ヨシマツ カズヒロ エンドウ シュンゴ カトウ ヒロユキ ハガ シュンスケ
梅原 有弘・吉松 和彦・遠藤 俊吾・加藤 博之・芳賀 駿介

Thymidylate synthase (TS) は DNA 合成に関わる酵素で、フッ化ピリミジン系抗癌剤の作用にも関与している。そこで大腸癌の腫瘍組織内 TS 量を測定し、臨床病理学的因子との関連から悪性度の指標としての可能性について検討した。対象は 1989～1995 年までの大腸癌切除例のうち、癌組織を新鮮凍結しておいた 44 例である。方法は凍結標本から蛋白を抽出し、一次抗体として抗ヒト recombinant TS ポリクローナル抗体を用いて、Western blot 法で TS を検出した。さらに検出されたバンドを画像解析し、positive control との比率から TS 量を半定量化し、臨床病理学的因子との関連を検討した。臨床病理学的因子のうち、年齢、性別、腫瘍径、組織学的分類、静脈侵襲については、TS 量との関連は認められなかった。術前 CEA 値からは陽性例で TS は低値であり、またリンパ節転移陽性例、肝転移陽性例、リンパ管侵襲が高度な症例で TS は低値であった。Dukes 分類からみると進行した症例ほど TS は低値であり、Dukes 分類と TS との間に関連を認めた。さらに対象を TS 値から低値群、中間群、高値群の 3 群に分けると、TS 値が低いほど予後は不良で、各群間に差を認めた。以上の結果から、大腸癌における腫瘍組織内 TS 量は悪性度の指標となると考えられる。