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Expression level of thymidylate synthase is a good predictor of chemosensitivity to 5-fluorouracil in colorectal cancer

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Background. It is important to seek the appropriate chemotherapy drugs to effectively eliminate colorectal cancers. To avoid unnecessary medication and uncomfortable side effects, it is important to estimate the chemosensitivity of cancers to 5-fluorouracil (5-FU) before chemotherapy. ***Methods.*** We examined thymidylate synthase (*TS*) and dihydropyrimidine dehydrogenase (*DPD*) gene expressions in 23 colorectal cancers, using quantitative reverse transcription-polymerase chain reaction (RT-PCR). We then evaluated the relationship between *TS* and *DPD* gene expression levels and the sensitivity of colorectal cancers to 5-FU, as determined by histoculture drug response assay (HDRA). ***Results.*** A significant increase in the TS expression score was observed in 5-FU-sensitive colorectal cancers (0.57 ± 0.19) compared to 5-FU-resistant ones (1.16 ± 0.98; $P = 0.029$), whereas no significant differences in DPD expression scores were observed in 5-FU-sensitive colorectal cancers (0.86 ± 1.19) compared to 5-FU-resistant ones (0.56 ± 1.05; $P = 0.603$). ***Conclusions.*** TS mRNA may be useful as a predictor of the 5-FU chemosensitivity of colorectal cancers.

Key words: TS, DPD, 5-FU, HDRA, colorectal cancer

Introduction

Colorectal cancer is one of the most aggressive cancers and has a high incidence rate in most countries.[1] To rid patients of this potentially fatal cancer, we perform surgical operations and subsequent chemotherapy and radiotherapy. For this purpose, it is important to seek appropriate chemotherapy drugs that will be effective in eliminating colorectal cancers.

5-Fluorouracil (5-FU) has been used as the standard treatment for various cancers. However, the response rate is less than 20% in colorectal cancers,[2] suggesting that some cancers are resistant to 5-FU. If leucovorin, a 5-FU modulator, is administered with 5-FU, the response rate would be still around 30%. To avoid unnecessary medication and uncomfortable side effects, it is important to estimate the chemosensitivity of cancers to 5-FU in advance of treatment. Several reports have indicated that intratumoral levels of expression of the mRNA for the 5-FU target enzyme, thymidylate synthase (TS),[3] and the rate-limiting catabolic enzyme of 5-FU, dihydropyrimidine dehydrogenase (DPD),[4,5] were predictors of the sensitivity of colorectal cancers to 5-FU. However, it would be difficult to estimate the correlation of sensitivity of colorectal cancers themselves to 5-FU with the expression levels of these enzymes if colorectal cancers in vivo were used to analyze the exact correlation. Difficulties arise not only due to intratumoral conditions but also due to extratumoral conditions, such as tumor size, location, and vascularity, which could influence the sensitivity of cancers to 5-FU.

We previously investigated whether the sensitivity of 5-FU to cancers could be predicted by a histoculture drug response assay (HDRA).[6,7] Viewing the results of determinations by HDRA, from the standpoint of clinical efficacy, sensitivity was 88% and specificity was 80%, suggesting that HDRA significantly reflected the clinical effects of 5-FU ($P = 0.001$ by χ^2 test).[8]

In the present study, we examined *TS* and *DPD* gene expressions in 23 different colorectal cancers, using quantitative reverse transcription-polymerase chain reaction (RT-PCR). We then evaluated the relationship between *TS* and *DPD* gene expression levels and the sensitivity of colorectal cancers to 5-FU, as determined by HDRA.

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Patients materials, and methods

Tissue specimens

The study group consisted of 23 colorectal cancer patients who underwent surgical operations at the Gastroenterological Surgery Section, Nagoya University Graduate School of Medicine. All tumors were collected on surgical resection and stored at −80°C.

RNA preparation and reverse transcription

Total RNA was extracted from colorectal cancers with guanidium thiocyanate, as described previously.[9] The amount of RNA was measured spectrophotometrically by absorbance at 260 nm. First-strand cDNA was generated from RNA as described previously.[10]

Quantitative RT-PCR

Quantitative RT-PCR was performed in an ABI 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Thermocycling was done in a final volume of 50 μl, containing 2.0 μl of the cDNA sample, 200 nM each of the TS or DPD primers (forward and reverse), 5 nM of the TS or DPD probe, and 25 μl of qPCR Mastermix, which consists of Taq DNA polymerase, reaction buffer, and deoxynucleotide triphosphate mixture (Applied Eurogentec, Seraing, Belgium). The quantitative RT-PCR primers and probes for TS and DPD were described previously.[11] The PCR amplification consisted of 45 cycles (95°C for 15 s and 60°C for 60 s) after an sdenaturation step (95°C for 12 min). To correct for differences in both quality and quantity between samples, ribosomal 18 S was used as an internal control. TS, DPD, and ribosomal 18 S mRNA variabilities were determined from triplicate samples. The quantity of all triplicate samples was in error by less than 10%. We applied an average quantity of the triplicate samples. These targets were obtained from the same mRNA preparations.

TS and DPD expression scores

We calculated the relative amounts of TS or DPD mRNA in colorectal cancers that were normalized to an internal control ribosomal 18 S mRNA. The TS or DPD expression score in each tissue was defined as follows: amount of TS or DPD/amount of ribosomal 18 S mRNA.

Histoculture drug response assay (HDRA)

Tumor tissue was cultured and the effectiveness of the anticancer agents was determined using succinate dehydrogenase inhibition test (SDI). After a washing in Hank's solution with 2.5% PSA (penicillin, 100 U/ml; streptomycin, 100 μg/ml; amphotericin B, 25 U/ml; Gibco BRL, Grand Island, NY, USA), 10 mg of tumor tissue was placed in 24-well microplates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) into which 1-cm square gelatin sponges (Upjohn, Kalamazoo, MI, USA) had been immersed in 20% fetal calf serum (FCS) RPMI 1640 (Nihon Seiyaku, Tokyo, Japan) containing the anticancer agent. The tissue was then cultured for 7 days at 37°C with 5% CO2. A mixed solution of 100 μl of 0.6 mg/ml collagenase (230 U/mg; Worthington Biochemical, Freehold, NJ, USA) and 100 μl of 0.4% MTT (3-[4,5-dimethyl-2-thiazolyl]-2-5-dimethyl-2H-tetrazolium bromide) 0.1 M Na succinate was added, and after 3 h the optical density (OD) at 540 nm was measured, using an Easy Reader (SLT-Lab instruments, Salzburg, Austria). A 5-FU concentration of 50 μg/ml was used for HDRA.

Determining drug efficacy

The efficacy of each individual drug was calculated according to the inhibition index (II), using the formula below. When the II was 50% or above, there was considered to be sensitive; however, the efficacy of drugs that caused infections, or those with an OD of 0.1 or less in the control group could not be determined.

$$\text{Inhibition index (II)} = (1 - \text{OD of treated tumor} / \text{OD of control}) \times 100$$

Statistical analysis

Differences between the means of analyzed TS and DPD expression scores were calculated by Welch's *t*-test; $P < 0.05$ was considered significant.

Results

We first analyzed TS, DPD, and ribosomal 18 S expression levels in 23 colorectal cancers using quantitative RT-PCR. Figure 1 shows the histogram of the TS and DPD expression scores, described in "Patients, materials, and methods." The average TS and DPD expression scores were 1.01 ± 0.88 and 0.64 ± 1.01, respectively. For individual TS and DPD expression scores, see Table 1.

To determine the role of TS and DPD expressions in colorectal cancers, we examined the correlation of TS and DPD expression scores with sensitivity to 5-FU measured by HDRA. Figure 2 shows the differences in TS and DPD expression scores according to sensitivity to 5-FU. A significant increase in the TS expres-

Table 1. TS and DPD expression scores, using quantitative RT-PCR, and chemosensitivity to 5-FU measured by HDRA in 23 colorectal cancers

Case no.	TS expression score	DPD expression score	Sensitivity to 5-FU
1	0.82	1.51	+
2	1.39	0.08	−
3	0.42	0.02	+
4	1.26	0.12	−
5	0.44	0.14	+
6	0.62	0.14	−
7	0.73	0.10	−
8	1.14	0.19	−
9	0.40	0.04	−
10	0.59	0.08	−
11	0.45	0.06	−
12	1.73	0.10	−
13	1.36	0.23	−
14	0.56	0.29	−
15	0.71	0.07	+
16	1.19	0.45	−
17	0.68	3.00	+
18	4.37	2.30	−
19	0.18	0.31	−
20	0.36	0.42	+
21	0.55	0.46	−
22	1.10	4.08	−
23	2.14	0.55	−

TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase; HDRA, histoculture drug response assay

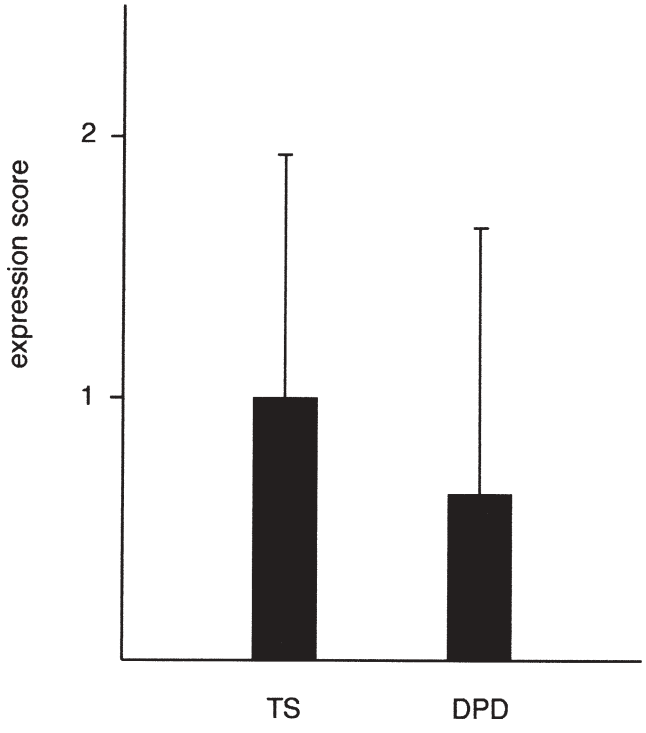

Fig. 1. Distribution of thymidylate synthase (*TS*) and dihydropyrimidine dehydrogenase (*DPD*) expression scores in colorectal cancers. The average TS and DPD expression scores were 1.01 ± 0.88 and 0.64 ± 1.01, respectively

sion score was observed in 5-FU-sensitive colorectal cancers (0.57 ± 0.19) compared to 5-FU-resistant ones (1.16 ± 0.98; $P = 0.029$), whereas no significant differences in the DPD expression scores were observed in 5-FU-sensitive colorectal cancers (0.86 ± 1.19) compared to 5-FU-resistant ones (0.56 ± 1.05; $P = 0.603$).

Discussion

Estimation of the sensitivity of cancers to 5-FU before chemotherapy, in order to prevent useless medication in patients, is important. Although TS and DPD expressions have been examined for this purpose, they have not always reflected the chemosensitivity of cancers, because cancers in vivo were often used to analyze the correlations between their expression and cancer sensitivity to 5-FU. As noted above, extratumoral conditions, such as tumor size, location, and vascularity, would influence the chemosensitivity of cancers if cancers in vivo were used. To understand the exact correlation of intratumoral TS and DPD expressions to chemosensitivity, cancers in vitro should be used. Moreover, if cancers in vitro do not show chemosensitivity, there is obviously no possibility that cancers in vivo will. Therefore, we applied HDRA to estimate the correlation of TS and DPD expressions with the sensitivity of colorectal cancers to 5-FU. However, HDRA may over-

Fig. 2a,b. Differences in TS (**a**) and DPD (**b**) expression scores according to sensitivity to 5-fluorouracil (*5FU*). The *upper and lower limits of the boxes*, and the *lines across the boxes*, indicate the 75th and 25th percentiles and the medians, respectively. The *upper and lower horizontal bars* indicate the maximal and minimal scores, respectively. A significant increase in the TS expression score was observed in 5FU-sensitive colorectal cancers (*plus sign*, +; 0.57 ± 0.19) compared to 5-FU-resistant ones (*minus sign*, −; 1.16 ± 0.98; $P = 0.029$), whereas no significant differences in DPD expression scores were observed in 5-FU-sensitive colorectal cancers (0.86 ± 1.19) compared to 5-FU-resistant ones (0.56 ± 1.05; $P = 0.603$)

estimate the response rate of 5-FU to colorectal cancers in vivo because it is an in vitro assay and could exclude the extratumoral conditions that disturb the 5-FU effect on cancers.

As described previously, HDRA significantly reflected the clinical effects of 5-FU.[8] HDRA is based on a technique originally developed by Hoffman.[11,12] The special characteristic of this technique lies in the use of a histoculture instead of a culture of free cells, which makes tissue culture from a surgically resected specimen possible. Despite infection being a source of trouble in this procedure, contamination occurred in very few cases.

In this study, we examined the correlation of TS and DPD expressions with sensitivity to 5-FU measured by HDRA. We found a significant correlation between TS overexpression and chemosensitivity to 5-FU in colorectal cancers. This result suggested that TS mRNA may be useful as a predictor of colorectal cancer chemosensitivity to 5-FU. Previously, Johnston et al.[13] showed a close linear relationship between *TS* gene expression and TS protein expression. They also showed that both the TS protein level and *TS* gene expression were significantly associated with response to 5-FU-based therapy, suggesting that TS protein may also be useful as a predictor of colorectal cancer chemosensitivity to 5-FU.

In this modest study, we could not detect any correlation between DPD expression and chemosensitivity to 5-FU. This result may have been due to sampling bias arising from the heterogeneity of TS and DPD expression in tumors, because some investigators have clearly indicated that cancers with DPD overexpression did not respond to 5-FU therapy.[14] Additional larger studies are needed to determine the clinical relevance of identifying TS and DPD expressions in colorectal cancers for the appropriate use of 5-FU.

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