RESEARCH ARTICLE

Editorial Process: Submission:11/09/2021 Acceptance:03/12/2022

Nucleic Acid Metabolizing Enzyme Levels Predict Chemotherapy Effects in Advanced and Recurrent Colorectal Cancer

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Abstract

Background: Thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) predict the effects of fluoropyrimidine. However, the effects of FOLFOX therapy from the perspective of fluorouracil plus leucovorin (FL) remain underexplored. Hence, the relationship between mFOLFOX6 therapy (mFOLFOX6) and therapeutic efficacy was evaluated in patients with advanced/recurrent colorectal cancer (CRC). **Methods:** Correlations between TS and DPD and primary and metastatic lesions in recurrent CRC were analyzed. Univariate and multivariate analyses of TS and DPD in combination with response rate (RR), progression-free survival (PFS), and overall survival (OS) were performed. **Results:** A positive correlation between DPD and primary and metastatic lesions; correlations between TS and RR, DPD and RR, and PFS and OS; and significant differences for RR and DPD and TS, PFS and DPD, and OS and DPD were obtained. **Conclusion:** Nucleic acid metabolizing enzymes in primary lesions can be used to predict mFOLFOX6 efficacy in patients with recurrent CRC.

Keywords: Recurrent and advanced colorectal cancer- thymidylate synthase (TS)- mFOLFOX6 therapy

Asian Pac J Cancer Prev, **23 (3)**, 1005-1011

Introduction

In colorectal cancer (CRC) with lymph node metastases, postoperative adjuvant chemotherapy has been shown to inhibit recurrence, and the IDEA study demonstrated that administering a fluoropyrimidine anticancer drug in combination with oxaliplatin for 6 months is the standard therapy (Shi et al., 2017). Previously, the efficacy of fluorouracil plus leucovorin (5FULV2) therapy was evaluated, and recurrence was shown to be inhibited at 18% in the IMPACT study compared to the untreated group (International Multicentre Pooled Analysis of Colon Cancer Trials (IMPACT) investigators, 1995), with equivalence also being shown for oral uracil/tegafur/Yuzel and capecitabine (Twelves et al., 2005). Hence, the clinical significance of predicting the effects of fluoropyrimidine enzymes is important, and various studies have been conducted. On the other hand, there have been no clinical studies to date demonstrating the efficacy of 5-fluorouracil (5-FU)/leucovorin with irinotecan (FOLFIRI) in combination with molecular-targeted therapies (Allegra et al., 2011; Alberts et al., 2012) or FOLFIRI as adjuvant chemotherapy (Saltz et al., 2007). Conversely, for advanced/recurrent CRC, 5-FU/leucovorin with oxaliplatin (FOLFOX) or FOLFIRI in combination with molecular-targeted drugs reportedly prolonged the life expectancy by 33 months (Saltz et al., 2008; Van Cutsem et al., 2009). However, oxaliplatin is not very effective as a single agent (Rothenberg et al., 2003), and high efficacy was observed for the first time in combination with FL therapy, demonstrating that fluoropyrimidine anticancer agents are highly involved. Since the effect of fluoropyrimidine anticancer agents is affected by the nucleic acid metabolizing enzymes, thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD), the significance of these as predictors of the effects of the modified FOLFOX 6 regimen (mFOLFOX6) was investigated.

Materials and Methods

Patients and Methods

This retrospective study was approved by the Institutional Review Board (IRB) of Tokyo Medical University Hospital and Tokyo Medical University Hachioji Medical Center (IRB Approval No. 2004-343).

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The study adhered to the principles of the Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects. The target population included 69 patients with advanced/recurrent CRC in whom TS and DPD were measured in primary CRC lesions using enzyme-linked immunosorbent assays (ELISAs) from February 2004 to May 2007. The breakdown of patients with advanced/recurrent CRC was as follows: mean age was 64.9 ± 10.6 years, 41 were mean and 28 were women; cancer was synchronous in 55 patients, metachronous in 14 patients; the tumor was localized in the right colon in 21 and the left colon in 48 patients; target lesions were liver metastases in 23 patients, lung metastases in 18 patients and other (lymph nodes, peritoneal dissemination, etc.) recurrence in 28 patients. In addition, 22 (R0: 9 patients) received localized treatment.

In patients who received chemotherapy followed by localized treatment of metastases, response was determined based on the final extent of shrinkage regardless of the duration.

In accordance with a previous report, patients were divided into an LL group with low TS and DPD, an HL group with high TS and low DPD, an LH group with low TS and high DPD, and an HH group with high TS and DPD, based on a mean TS of 20.9 g/mg protein (±9.8 g/mg protein) and mean DPD of 130.6 ng/mg protein (±52.38 g/mg protein), for inter-group comparisons. All patients underwent mFOLFOX6 therapy and had a performance status of ≤1.

Moreover, DPD and TS were measured in the primary and metastatic lesions of 10 patients, whose mean age was 62.3 ± 7.7 years. The cancer was synchronous in two patients and metachronous in eight patients, and the sites of metastases were liver metastases in nine patients and lung metastases in one patient.

Enzyme activity assay: TS and DPD protein expressions were determined in the tissues with ELISA utilizing a two-step sandwich method. First, the tissues were homogenized and centrifuged, and the supernatant was collected. The supernatant was then diluted as appropriate, and TS antibodies (or DPD antibodies) were added to solid plates for reaction. Thereafter, TS antibody (or DPD antibody)-peroxidase conjugates were added to form a sandwich complex of TS antibody-TS-TS antibody-peroxidase conjugate (or DPD antibody-DPD-DPD antibody-peroxidase conjugate). To this solution, ortho-phenylenediamine, a chromogenic substrate, was added together with hydrogen peroxide solution, which is a substrate of peroxidase, and the intensity of the color generated from the reaction with peroxidase in the complex was measured. The TS or DPD protein expression level in the tissue samples were measured from a calibration curve prepared using a standard (Ishibashi et al., 2009).

The TS and DPD measurements were used to compare the mean values between the two groups of primary and metastatic lesions and to obtain correlation coefficients. In addition, multivariate and survival analyses were performed by combining TS and DPD in the primary lesions with response rate (RR), progression-free survival (PFS), and overall survival (OS).

Statistical analyses were performed using "EZR" (version 1.54). Furthermore, t-tests were used for comparisons of means between two groups, analysis of variance for comparison of means in three or more groups, Pearson's correlation coefficient for correlation coefficients, the Kaplan–Meier method and log-rank tests for survival analyses, and multiple regression analyses for multivariate analyses. A P-value of ≤0.05 was considered significant.

Results

1) TS and DPD were analyzed in primary and metastatic lesions. DPD was significantly high (P = 0.014), while there was no significant difference for TS at P = 0.159. When analyzed by Pearson's correlation coefficient, the coefficient was 0.775 for DPD, showing a positive correlation (P = 0.00846). On the other hand, the coefficient for TS was 0.0275, showing no correlation (P = 0.94) (Table 1).

2) There were six patients who were not evaluable. The RR (n = 63) was 38.0%, with 5 patients (7.9%) experiencing a complete response and 19 patients (30.1%) experiencing a partial response, and the mean OS in all patients was 31.5 ± 16.3 months.

TS and DPD were evaluated along with RR, PFS, and OS.

Pearson's correlation coefficient was −0.299 (P = 0.0125) for RR, 0.079 (P = 0.515) for PFS, and 0.327 (P = 0.79) for OS, showing a significant difference for RR.

For DPD, the coefficient was −0.381 for RR (P = 0.00), 0.577 for PFS (P = 0.00), and 0.452 for OS (P = 0.00), showing significant differences for RR, PFS, and OS (Table 2).

3) Multivariate analyses of RR revealed significant differences for TS (P = 0.0042), DPD (P = 0.018),

Table 1. Comparison of Thymidylate Synthase (TS) and Dihydropyrimidine Dehydrogenase (DPD) in Primary and Metastatic Lesions (n = 10)

	Mean	standard deviation	P-value	Correlation coefficient	P-value
DPD					
Primary lesion	94.45	44.57			
Metastatic lesion	259	187.5	0.0149	0.755	0
TS					
Primary lesion	31.16	41.02			
Metastatic lesion	11.78	7.32	0.159	0.025	0.94

For DPD, a significant difference in the mean was seen and a strong positive correlation was observed

Table 2. Correlations of Thymidylate Synthase (TS) and Dihydropyrimidine Dehydrogenase (DPD) with Response Rate (RR), Progression-Free Survival (PFS), and Overall Survival (OS)

	TS		DPD	
	Correlation coefficient	P-value	Correlation coefficient	P-value
RR	−0.290	0.012	−0.380	0
OS	0.032	0.790	0.450	0
PFS	0.079	0.510	0.570	0

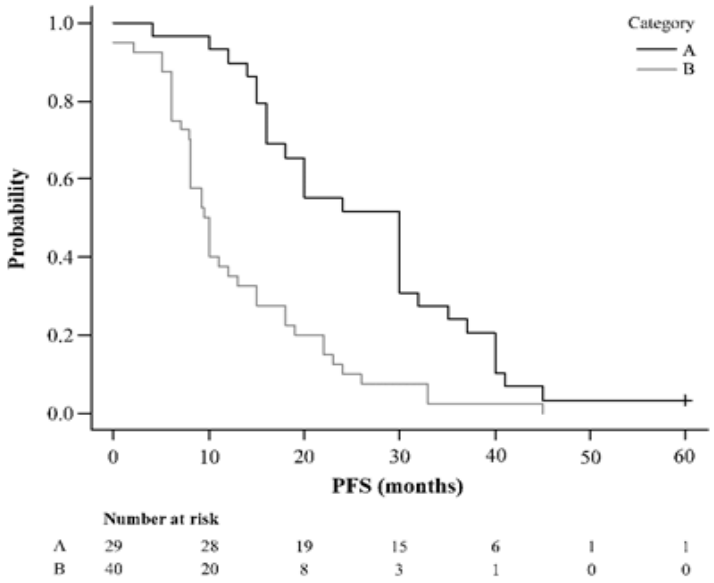

Figure 1. Kaplan–Meier Curve with Progression-Free Survival as the Objective Variable (Two Groups) Comparisons between Two Groups (A: LL + HL and B: LH + HH) Showed a Significant Difference (P = 0.000021)

localized treatment (P = 0.027), and sites of metastases (P = 0.045) but not for age, sex, tumor localization, and timing of metastasis due to the cancer being synchronous or metachronous (Table 3-1).

Multivariate analyses of PFS revealed significant differences for DPD (P = 0.0021) and localized treatment (P = 0.00); however, for TS, it was P = 0.073, and no significant differences were seen for age, sex, and tumor localization (Table 3-2).

Multivariate analysis of OS revealed significant differences for DPD (P = 0.010) and localized treatment (P = 0.00) but not for TS (P = 0.13), age (P = 0.40), sex (P = 0.46), tumor localization (P = 0.65), sites of metastases (P = 0.38), and timing of metastases (P = 0.33) (Table 3-3).

4) An evaluation was also performed for the combination of the two factors, TS and DPD. For RR, a significant difference was observed in the LL group among the four groups (P = 0.000021). For PFS and OS, the two groups of low DPD and high DPD were compared, and PFS was P = 0.00 and OS was P = 0.0011, showing a significant difference.

5) Comparisons of PFS and OS were performed using the Kaplan–Meier method. PFS in the low DPD group was significantly different at P = 0.000021 (Figure 1), and there was a significant difference among the four groups (P = 0.00018) (Figure 2). In addition, there was a significant difference in OS at P = 0.00042 (Figure 3) and P = 0.0026 (Figure 4).

Table 3-1. Multivariate Analysis of Response Rate (RR) as an Objective Variable

	95% confidence interval [CI] (lower limit to upper limit)		Standard error	P-value
Thymidylate synthase (TS)	−0.063	−0.012	0.012	0.0042
Dihydropyrimidine dehydrogenase (DPD)	−0.014	−0.0013	0.0031	0.018
Age	−0.020	0.021	0.010	0.97
Sex	−0.840	0.100	0.23	0.12
Tumor localization	−0.420	0.460	0.22	0.92
Sites of metastases	−0.470	0.110	0.14	0.21
Localized treatment	0.700	0.270	2.60	0.012
Timing of metastasis	−0.740	0.570	0.32	0.803

Significant differences were observed for TS, DPD, and localized treatment

Table 3-2. Multivariate Analysis of Progression-Free Survival as an Objective Variable

	95% confidence interval [CI] (lower limit to upper limit)		Standard error	P-value
Thymidylate synthase (TS)	−0.61	0.02	0.15	0.073
Dihydropyrimidine dehydrogenase (DPD)	−0.20	−0.04	0.03	0.0021
Age	−0.28	0.23	0.13	0.84
Sex	−8.20	3.6	2.95	0.43
tumor localization	−8.05	3.18	2.8	0.38
Sites of metastases	−4.27	1.65	1.48	0.37
localized treatment	10.9	26.6	3.91	0
timing of metastasis	−12.05	4.21	4.06	0.33

A significant difference was observed for DPD

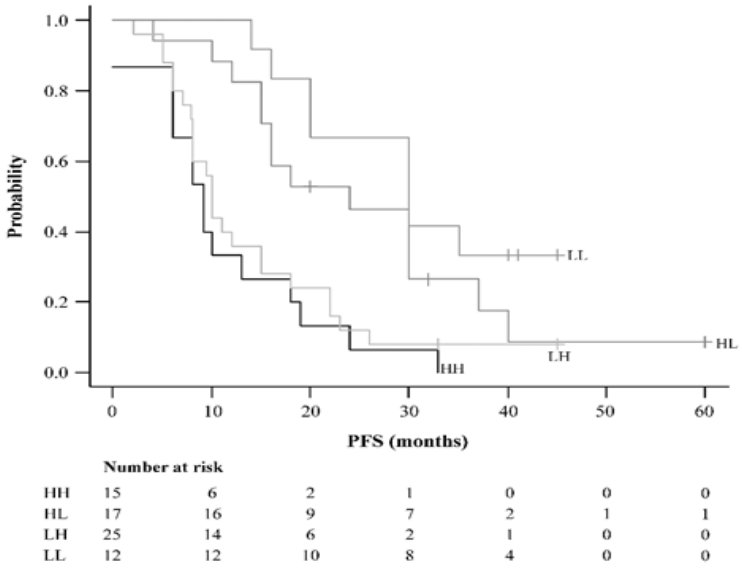

Figure 2. Kaplan–Meier Curve with Progression-Free Survival as the Objective Variable (Four Groups) Comparisons among the Four Groups Showed a Significant Difference (P = 0.00018)

Discussion

The efficacy of oxaliplatin (Ox) monotherapy in FOLFOX therapy is low in CRC, and it is highly effective in combination with 5FULV2 therapy. Although the therapeutic effect of Ox alone is poor, such an effect is inferred to be due to a synergistic effect with 5FULV2 therapy.

In the development of FOLFOX therapy, RR to Ox alone in 5-FU resistant CRC was approximately 10%; however, the RR to mFOLFOX6 in patients who became resistant to the deGramont regimen was 27% (De Gramont et al., 1997; Andret et al., 1999; Maindrault-Goebel et al., 1999).

In this manner, the high RR in patients who acquired resistance to 5FULV2 therapy or were treated with first-line Ox cannot be said to be a simple additive effect, and the inference is that some biochemical modulation is acting on Ox.

The ERCC1 gene that regulates the effect of Ox is a DNA repair gene. The hypothesis is that the higher the expression, the stronger the resistance to Ox (Yin et al.,

Table 3-3. Multivariate Analysis of Overall Survival as an Objective Variable (P-value 0.00)

	95% confidence interval [CI] (lower limit to upper limit)		Standard error	P-value
Thymidylate synthase (TS)	−0.82	0.11	0.23	0.13
Dihydropyrimidine dehydrogenase (DPD)	−0.27	−0.037	0.05	0.01
Age	−0.54	0.22	0.19	0.4
Sex	−11.9	5.55	4.36	0.46
Tumor localization	−10.16	6.47	4.15	0.65
Sites of metastases	−6.34	2.46	2.2	0.38
Localized treatment	11.9	35.4	5.87	0
Timing of metastasis	−22.1	1.27	5.85	0.07

Significant differences were observed for DPD and localized treatment.

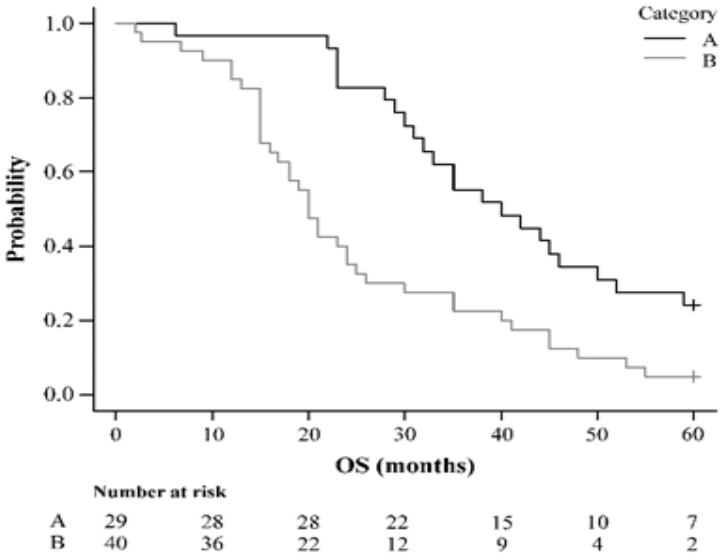

Figure 3. Kaplan–Meier Curve with Overall Survival as the Objective Variable (Two Groups) Comparisons between Two Groups (A: LL + HL and B: LH + HH) Showed a Significant Difference (P = 0.00042)

2011). However, no clear conclusions on predictors of the effect of Ox have been reported to date. In addition, as described in a previous report, there are indices such as glutathione S-transferase Pi (GSTPi) for neurotoxicity associated with platinum-based anticancer agents (Katayanagi et al., 2019) that can be used as an index of adverse reactions, but this is not a predictor of efficacy.

On the other hand, TS and DPD can predict the effect of postoperative adjuvant chemotherapy based on the results of measurements of primary CRC lesions we had performed in the past (Shi et al., 2017), and there have also been reports of these as prognostic factors. However, there are few reports on their significance in combination chemotherapy for CRC. Although the "ESMO Consensus Guidelines for the Management of Patients with Metastatic Colorectal Cancer" in the 2016 Annals of Oncology specify "DPD testing prior to 5-FU as an option but not routinely recommended," the inference is that it was primarily due to evaluations focusing on the pharmacological activity of Ox and irinotecan. However, to date, no clear predictive factors of effects have been identified for FOLFOX. Thus, a decision was made to perform a retrospective analysis from the perspective of 5FULV2 therapy in patients with recurrent advanced CRC in whom TS and DPD had been measured in the past.

The anticancer agent 5-FU inhibits cell division by exploiting the differences in nucleic acid metabolizing enzymes in tumors when compared to normal tissues. In matched samples of primary CRC and normal colorectal tissues, TS levels in primary CRC were reportedly significantly higher than in normal colorectal tissues, while DPD levels were significantly lower than in normal tissues. 5-FU inhibits cell division by utilizing the differences in nucleic acid metabolizing enzymes in tumor and normal tissues, and target molecules of 5-FU, including TP, (Nishimura et al., 2002) are also reported as indices that influence the prognosis of CRC (Schüller et al., 2000; Ichikawa et al., 2003; Katsumata et al., 2001). Moreover, the antitumor effect of 5-FU is to inhibit the synthesis of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). In RNA dysfunction, the phosphorylation of 5-FU results in the formation of fluorouridine triphosphate (FUTP), which antagonizes uridine triphosphate (UTP), a normal metabolite from uracil, and is incorporated into the RNA, causing dysfunction in cancer cells. In DNA dysfunction, phosphorylation of 5-FU results in 5-fluorodeoxyuridicacid (FdUMP), which binds to dUMP, a part of the DNA synthesis pathway, at 1700-fold higher strength and inhibits DNA synthesis. Since DPD is a rate-limiting enzyme that acts in the first step in the degradation of 5-FU and >80% of 5-FU entering the body is degraded by DPD, 5-FU may be less effective in cancers in which DPD is elevated. In addition, TS is an enzyme that targets FdUMP and, if the expression level of TS in tumors is high, FdUMP cannot inhibit TS completely and resistance to 5-FU arises.

In the present analysis, first, the correlation was high when the DPD measurement results were compared between the primary and metastatic lesions, and the hypothesis was that DPD would be higher than in primary lesions and reflect the pathology of metastatic lesions. KRAS gene measurements in primary CRC lesions reportedly correlate with metastatic lesions, but a positive correlation with increased enzyme expression levels was also observed in DPD (Watanabe et al., 2011). On the other hand, TS was expressed at a decreased level, a correlation was not observed, and heterogeneity was observed in metastatic lesions, which were interesting findings. The clinical efficacy was re-evaluated after referring to the results on TS and DPD reported by Sumi et al., (2010). Although TS was a prognostic factor in adjuvant chemotherapy, as reported by Katsumata et al. (Katsumata et al., 2018), a strong relationship between DPD and PFS and OS was suggested for FOLFOX therapy in advanced/recurrent CRC. According to previous reports, sensitivity to 5-FU treatment is higher when DPD

expression is low (Kuwahara et al., 2011); however, in this study, significant differences were observed for RR, PFS, and OS despite high DPD expression in metastatic lesions. For TS, there is a report that patients with low TS expression are more likely to respond to treatment than those with high TS expression (Ichikawa et al., 2003). In this study, a significant difference was observed in RR for TS, but no significant differences were observed in OS or PFS. TS in metastatic lesions was low, and the assumption is that patients are likely to respond. 5FULV2 therapy seems to take advantage of these factors with the addition of leucovorin (LV) administration to continuous administration and rapid intravenous infusion. Thus, 5FULV2 therapy can be said to be effective for metastatic lesions with low TS and high DPD.

In addition, 9,073 genes are highly sensitive to 5-FU, and DPD occupies the 167th position as a negatively correlated gene and TS is in the 1,430th position. Thus, DPD was proven to be a metabolic enzyme that determines the effect of 5-FU.

FOLFOX therapy is associated with an issue wherein the neurotoxicity of Ox persists for a long period of time. Katayanagi et al., (2019) reported a relationship between peripheral neuropathy and GSTPi involved in the metabolism of platinum-based anticancer drugs. The genetic polymorphism has been shown to be associated with neurological disorders. It may be therapeutically beneficial to investigate other chemotherapies by measuring GSTPi, DPD, and TS for GSTPiAG-type and HH groups that are susceptible to peripheral neuropathy. In addition, DPD deficiency presenting with serious adverse reactions has been reported to be associated with the administration of 5-FU (Sakata et al., 2017) and, although TS/DPD measurements are not highly evaluated at present, measurement may show significance in the choice of prior chemotherapy and the prevention of adverse reactions in some patients.

Although this study was performed at a single institution, it was a collaborative involving various medical departments. However, the small sample size limits the clinical significance. In addition, this study was conducted around the time when FOLFOX therapy was being introduced in Japan, and there being little need to take factors related to molecular-targeted drugs into account led to a simple and clear conclusion of the pharmacological effect.

At present, target factors for chemotherapy such as the primary lesion site, indication of anti-epidermal growth factor receptor, RAS/BRAF, and microsatellite instability are increasing. Although TS and DPD have been evaluated in this study, they have not received much attention in recent years. However, as long as fluoropyrimidine anticancer agents form the basis of chemotherapy for CRC, the significance of measurement is considered to be high.

Considering that the efficacy of Ox monotherapy is low and that it is highly effective in synergy with 5FULV2 therapy, there is a possibility that DPD and TS measurements may predict the efficacy of FOLFOX therapy, and these are considered to be useful factors. In this study, TS values in primary lesions were found to be important predictors of the efficacy of chemotherapy with FOLFOX for advanced/recurrent CRC in terms of RR. DPD values were found to be important predictors of efficacy in terms of RR, PFS, and OS. In addition, the combination of TS and DPD was a stronger predictor of efficacy and prognosis in terms of RR, PFS, and OS.

To conclude, the significance of measuring TS and DPD is likely to be high until the predictors for the efficacy of Ox can be identified in the future and until biochemical modulation with 5FULV2 therapy can be clarified; thus, there appears to be sufficient room for further research.

Author Contribution Statement

Conceived and designed the analysis: Mitsuru Watanabe, Kenji Katsumata, Akihiko Tsuchida. Data collection: Tetsuo Sumi, Tetsuo Ishizaki, Masanobu Enomoto, Masatoshi Shigoka, Takahiro Wada, Hiroshi Kuwabara, Junichi Mazaki, Kenta Kasahara, Tomoya Tago, Ryutaro Udo,Yuichi Nagakawa,Shigeyuki Kawachi. Wrote the paper: Mitsuru Watanabe.

Acknowledgements

We would like to express our sincere gratitude to Department of Gastrointestinal Surgery, Tokyo Medical University Hospital, Hachioji Medical Center. Tokyo Medical University Medical Ethics Committee. Approval number is No. 2004-343.

Funding statement

Research funding was provided by the Department of Gastrointestinal Surgery, Tokyo Medical University.

Conflict of interest

None.

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