Messenger RNA expression of TS and ERCC1 in colorectal cancer and matched liver metastasis

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Abstract. 5-fluorouracil (5-FU) and oxaliplatin play important roles in chemotherapy for patients with colorectal cancer. The expression levels of thymidylate synthase (TS) and excision repair cross-complementing factor 1 (ERCC1) have been reported to be prognostic markers for patients with 5-FU/oxaliplatin chemotherapy. The aim of this study was to clarify the association between messenger RNA (mRNA) levels of TS and ERCC1 in primary colorectal cancer and those in corresponding liver metastasis. Formalin-fixed paraffin-embedded tumor specimens of 31 patients with resection for both colorectal cancer and liver metastasis were dissected by laser capture microdissection. After RNA extraction, TS and ERCC1 mRNA levels in both primary tumor and corresponding liver metastasis were measured by real-time reverse transcription-polymerase chain reaction. Both TS and ERCC1 mRNA levels in primary tumors were significantly associated with those in synchronous liver metastases (*TS*, rs=0.875, p=0.0024; *ERCC1*, rs=0.835, p=0.0038). TS mRNA levels in primary tumors were also associated with those in metachronous liver metastases (rs=0.659, p=0.0065), but not in ERCC1 (rs=0.319, p=0.19). In both genes, mRNA levels in metachronous liver metastases were higher than those in primary tumors (*TS*, p=0.0084; *ERCC1*, p=0.037). However, there was no difference in the TS and ERCC1 mRNA levels between primary tumors and synchronous liver metastasis. The measurement of TS and ERCC1 mRNA levels in primary colorectal cancer can predict those in synchronous liver metastases, but not in metachronous ones.

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Introduction

Malignant neoplasm is a leading cause of death in Japan. Colorectal cancer is the second leading cause of cancer deaths in the United States and the third in Japan (1). Colorectal cancer is still increasing in Japan (2) and it is considered as a result of westernized dietary habit. Metastasis is the main reason why colorectal cancer is fatal. The most frequent metastatic site from colorectal cancer is the liver (3). Therefore, it is of great importance to improve the treatment results for liver metastasis. The best way to cure liver metastasis from colorectal cancer is to resect the lesions completely. However, some patients are not eligible for surgical resection. In these cases, chemotherapy plays an important role for their treatments.

Chemotherapy for colorectal cancer has made great progress, since Moertel *et al* reported that adjuvant treatment with fluorouracil (5-FU) and levamisole reduced mortality rate by 33% in patients with Dukes' C colon cancer. Several studies have established as the standard chemotherapy with 5-FU and leucovorin (LV) for colorectal cancer (4-6).

Oxaliplatin, a new cytotoxic agent from the diamino-cyclohexane platinum family, forms cross-linking adducts and interferes with DNA replication and transcription (7). Several clinical trials have clarified the efficacy of FOLFOX, a combined chemotherapy with oxaliplatin, 5-FU, and LV, for metastatic colorectal cancer (8-10). FOLFOX improved the median survival in patients with metastatic colorectal cancer up to ~20 months. It was 4 to 5 months for patients who did not receive chemotherapy. At the present time, FOLFOX plays a vital role in chemotherapy for metastatic colorectal cancer. Although these cytotoxic combined therapies have a beneficial effect on the treatment for such patients, there is a possibility of severe adverse effects. In some cases, these adverse effects can be fatal. Therefore, it would be useful to predict the efficacy of FOLFOX before treatment. The

prediction of efficacy for such treatments would also avoid useless treatments.

Thymidylate synthase (TS) is the target enzyme of the antimetabolite 5-FU. Several studies reported that the gene expression level of intratumoral TS is a predictor of 5-FU-based chemotherapy (11,12).

The excision repair cross-complementing factor 1 (ERCC1) is an excision nuclease within the nucleotide excision repair pathway (13-15). It is well known that ERCC1 has an effect on repair of platinum-DNA damage. Human ovarian cancer cells that express ERCC1 mRNA to higher levels showed higher resistance to platinum drug exposures (16). Shirota *et al* reported that ERCC1 and TS mRNA levels predicted survival for colorectal cancer patients receiving chemotherapy with oxaliplatin and 5-FU (17).

FOLFOX is used for patients with advanced colorectal cancer, such as those with hematogenous metastasis. However, the association between mRNA levels of TS and ERCC1 in primary colorectal cancer and those in liver metastasis is still unclear. In this study, we evaluated the mRNA levels of TS and ERCC1 in both primary colorectal cancer and corresponding liver metastasis.

Materials and methods

Patients. Thirty-one patients, who underwent surgical resections for both primary colorectal cancer and liver metastasis between April 1997 and June 2005 at Tokyo Medical and Dental University Hospital, were enrolled in this study. The patient characteristics were: the male:female ratio was 18:13; median age was 62 years. The median post-operative follow-up period was 49 months. Of 31 patients, 18 had a metachronous liver metastasis and 13 had a synchronous metastasis. Median time from primary resection to hepatectomy was 20 months in patients with metachronous liver metastasis. Patients with ulcerative colitis, Crohn's disease, or familial adenomatous polyposis were excluded in this study. This study was approved by the Institutional Review Board of Tokyo Medical and Dental University and all patients gave written consent. Stage III patients with metachronous liver metastasis had 5-FU-based adjuvant chemotherapy before hepatectomy. Other clinicopathological characteristics are summarized in Table I.

Table I. Clinicopathological characteristics in 31 patients.

Primary site	
Cecum	1
Ascending	3
Transverse	3
Descending	2
Sigmoid	9
Rectosigmoid	9
Rectum	4
Pathology of primary cancer	
Well differentiated	13
Moderately differentiated	16
Poorly differentiated	1
Mucinous	1
Depth of primary tumor	
T1	0
T2	2
T3	21
T4	8
Lymph node metastasis in primary tumor	
N0	9
N1	13
N2	9
TNM stage	
Stage I	1
Stage II	7
Stage III	10
Stage IV	13

Laser capture microdissection. Formalin-fixed paraffin-embedded tissue blocks were cut into 10-μm thickness. Slides of 10 μm thickness were stained with nuclear fast red (American MasterTech Scientific, Lodi, CA). After this staining, laser capture microdissection (P.A.L.M. Microlaser Technologies AG, Munich, Germany) was performed to punch out only tumor tissue.

RNA isolation and cDNA synthesis. After laser capture microdissection, RNA isolation was done according to a proprietary procedure of Response Genetics, Inc (US patent number 6,248,535). After RNA isolation, cDNA was prepared from each sample, as previously described (18).

Quantitative reverse transcription-polymerase chain reaction (QRT-PCR). Quantification of TS, ERCC1 and an internal reference gene (ß-actin) was performed using a fluorescence-based real-time detection method [ABI PRISM 7900 Sequence Detection System (Taqman); Applied Biosystems, Foster City, CA], as previously described (19). The primers and probe sequences in this study are shown in Table II. The PCR mixture consisted of 1,200 nmol/l of each primer, 200 nmol/l probe, 0.4 units of AmpliTaq Gold Polymerase, 200 nmol/l each of dATP, dCTP, dGTP, dTTP, 3.5 mmol/l $MgCl_2$ and 1X Taq Man buffer A containing a reference dye, to a final volume of 20 μl (all reagents were supplied by Perkin-Elmer Applied Biosystems). Cycling conditions were 50˚C for 2 min, 95˚C for 10 min, followed by 46 cycles at 95˚C for 15 sec and 60˚C for 1 min. All the expression values (relative mRNA levels) are expressed as ratios between the genes of interest and an internal reference gene (ß-actin).

Statistical analysis. All data are expressed as mean ± standard deviation. To compare the mRNA levels of the genes of interest between primary colorectal cancer and corresponding liver metastasis, Wilcoxon signed-rank test was used. Spearman's rank correlation analysis determined the correlation of mRNA levels of the genes of interest between primary tumor and liver metastasis. Statistical significance was established at $P<0.05$ for all results.

Table II. Primer and probe sequences of the analyzed genes.

Primer and probe sequences	
TS	
Forward primer	5'-GCCTCGGTGTGCCTTTCA-3'
Reverse primer	5'-CCCGTGATGTGCGCAAT-3'
Probe	5'-(FAM)TCGCCAGCTACGCCCTGCTCA(TAMRA)-3'
ERCC1	
Forward primer	5'-GGGAATTTGGCGACGTAATTC-3'
Reverse primer	5'-GCGGAGGCTGAGGAACGA-3'
Probe	5'-(FAM)CACAGGTGCTCTGGCCCAGCACATA(TAMRA)-3'
ß-actin	
Forward primer	5'-TGAGCGCGGCTACAGCTT-3'
Reverse primer	5'-TCCTTAATGTCACGGACGATTT-3'
Probe	5'-(FAM)ACCACCACGGCCGAGCGG(TAMRA)-3'

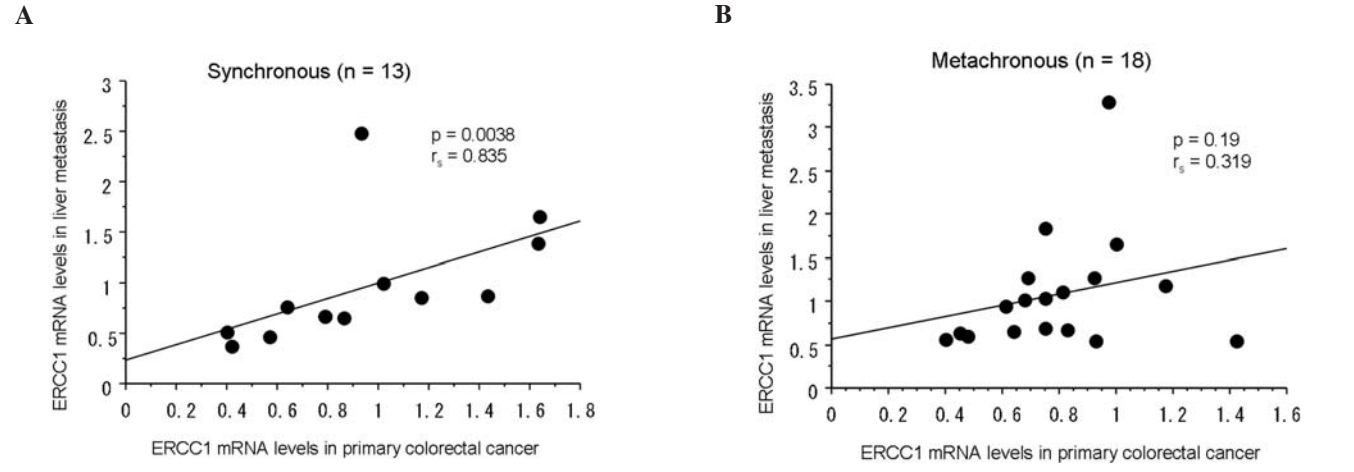

Figure 1. The correlation between ERCC1 mRNA levels in primary colorectal cancer and those in liver metastasis. (A) There was a significant positive correlation in case of synchronous liver metastasis. (B) ERCC1 mRNA levels in primary tumor were not significantly associated with those in metachronous liver metastasis.

Results

Correlation between ERCC1 mRNA levels in primary colorectal cancer and those in corresponding lever metastasis. In patients with synchronous liver metastasis, ERCC1 mRNA levels in primary tumor were significantly associated with those in liver metastasis (p=0.0038, r_s=0.84, Fig. 1A). In patients with metachronous liver metastasis, however, there was no significant correlation between ERCC1 mRNA levels in primary colorectal cancer and those in corresponding liver metastasis (Fig. 1B). There was no correlation between ERCC1 mRNA levels and clinicopathological features in Table I in this setting.

Correlation between TS mRNA levels in primary colorectal cancer and those in corresponding liver metastasis. TS mRNA levels in primary colorectal cancer were significantly associated with those in both synchronous and metachronoous liver metastasis (Fig. 2). There was no correlation between TS mRNA levels and clinicopathological characteristics in this setting.

Gene expression in primary tumor and matched liver metastasis. In synchronous liver metastasis, there were no significant differences in mRNA levels of both ERCC1 and TS between primary colorectal cancer and corresponding liver metastasis (*ERCC1*, p=0.20; *TS*, p=0.65; Fig. 3, Table III). On the other hand, mRNA levels of ERCC1 in metachronous liver metastasis were greater than those in primary colorectal cancer (p=0.037, Fig. 3A). There was the same tendency in TS mRNA expression in case of metachronous liver metastasis (p=0.0084, Fig. 3B).

Discussion

This study demonstrated that ERCC1 mRNA levels in synchronous liver metastasis were significantly associated with those in primary colorectal cancer. In metachronous liver

Table III. mRNA levels in primary tumor and corresponding liver metastasis.

	Synchronous metastasis			Metachronous metastasis		
	Primary tumor	Liver metastasis	P-value	Primary tumor	Liver metastasis	P-value
ERCC1	0.95±0.42	0.95±0.58	p=0.20	0.79±0.25	1.09±0.67	p=0.037
TS	1.74±1.12	1.59±0.74	p=0.65	1.52±0.74	2.04±0.91	p=0.0084

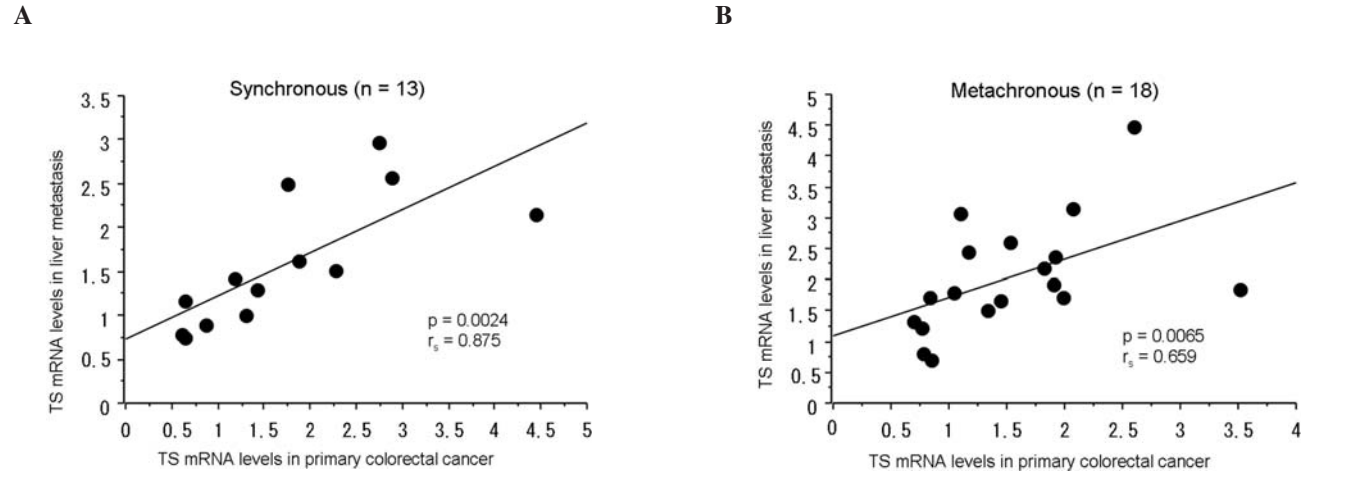

Figure 2. TS mRNA levels in primary colorectal cancer were significantly associated with those in both synchronous (A) and metachronous (B) metastasis, respectively.

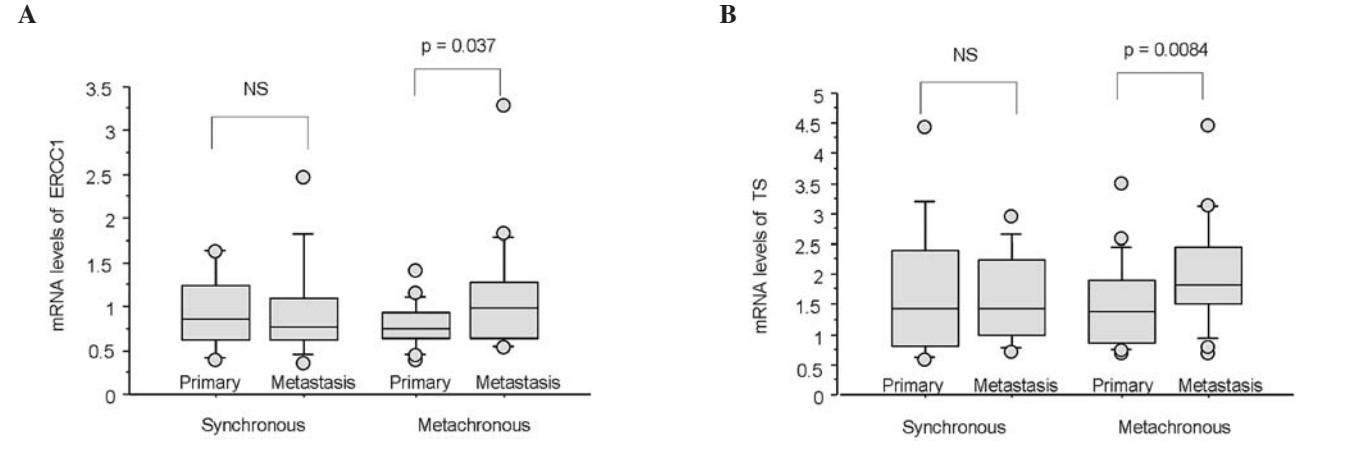

Figure 3. The expression of mRNA of ERCC1 (A) and TS (B) in primary colorectal cancer and corresponding liver metastasis. In both genes, there were no differences in mRNA levels between the primary tumor and synchronous liver metastasis. However, mRNA levels in primary tumor were significantly lower than those in metachronous liver metastasis in both ERCC1 and TS.

metastasis, however, ERCC1 mRNA levels were not correlated with those in the primary tumor. One of the reasons for this phenomenon may be the difference of circumstance for tumor growth. Median time from primary resection to hepatectomy was 2 years in this study. During this period, various factors might affect the ERCC1 mRNA levels.

Chemotherapy for advanced colorectal cancer including hematogenous metastasis, has been remarkably developed in the past several years. Oxaliplatin combined with 5-FU is one of the most promising therapies. It has been reported that FOLFOX improves survival in patients with metastatic colorectal cancer (8,10,20,21). In some cases, these drugs are now used with molecular target agents such as bevacizumab (22).

Shirota *et al* reported that mRNA levels of TS and ERCC1 were independent predictive markers of survival for 5-FU and oxaliplatin combination chemotherapy in 5-FU-resistant metastatic colorectal cancer (17). In their study, the tumor

samples were taken from metastatic sites of the liver or from a recurrent colorectal tumor mass. The association between ERCC1 mRNA levels in primary colorectal cancer and those in liver metastasis was not clarified.

In our study, ERCC1 mRNA and TS mRNA expression levels in patients with synchronous liver metastasis were both significantly associated with those in primary colorectal cancer. Thus, our result may indicate that in patients with synchronous liver metastasis, the gene expression levels of TS and ERCC1 in primary tumor can predict the prognosis of patients with 5-FU/oxaliplatin therapy. In patients with metachronous liver metastasis, however, ERCC1 mRNA levels in primary tumor were not associated with those in corresponding liver metastasis. Therefore, it will be required to clarify the predictors of response to FOLFOX in patients with metachronous liver metastasis.

Since 5-FU was used for the treatment of solid tumors, >40 years have passed. 5-FU still remains one of the most active agents used in the treatment of colorectal cancer. A number of studies have clarified that TS is a predictor of the efficacy of 5-FU-based chemotherapy as well as the prognosis in patients with those therapies, using several methods (12,23-29).

In this study, there were no differences in TS mRNA levels between primary tumor and corresponding liver metastasis. Furthermore, TS mRNA levels of primary colorectal cancer was significantly associated with those of both synchronous and metachronous liver metastasis. In metachronous liver metastasis, there was a difference in the results of gene expression between TS and ERCC1. That is, there was a positive correlation between TS mRNA levels in primary tumor and metachronous liver metastasis, but not in ERCC1. Based on these observations, the genes might respond differently to the surrounding environment, even if their expression levels were originally nearly equal.

TS expression in primary colorectal cancer and corresponding liver metastasis has been controversial. Yamada *et al* demonstrated that TS mRNA expression in hepatic metastases was significantly lower than in primary tumors (30). Backus *et al* showed that the expression of TS was significantly higher in metastases than in the matched primary tumor samples, using the method of immunohistochemical staining (31). On the other hand, Inokuchi *et al* reported that the TS mRNA level did not differ significantly between liver metastasis and primary tumor. In their study, no correlation was observed between primary tumor and liver metastasis (32). This discrepancy may be attributed to the stromal cells or normal tissue in their tumor specimens. To a varying degree, most of the specimens of liver metastases included stromal cells, normal liver tissue, or necrotic tissue in this study. It is considered that the technique of laser capture microdissection plays a potent role to punch out only cancer cells from specimens.

Our study also demonstrated that mRNA levels of both TS and ERCC1 in metachronous liver metastasis were higher than those in primary colorectal cancer, although those in synchronous liver metastasis were the same. This might be the result of the difference of circumstances where tumors increase in size. Cancer cells would be more modified in patients with metachronous liver metastasis than in those with synchronous one until it becomes visible as metastasis. On the other hand, in most of the stage III patients with metachronous liver metastasis, adjuvant chemotherapy was performed after curative resection for colorectal cancer. The increase of such gene expression levels might be caused by the 5-FU-based chemotherapy.

In conclusion, the measurement of mRNA levels of TS and ERCC1 in primary colorectal cancer may be useful in synchronous liver metastasis, but not in metachronous liver metastasis. These characteristics of expression of FOLFOX-related genes in primary colorectal cancer and corresponding liver metastasis should be taken into account. However, a large clinical study will be necessary to validate this preliminary result.

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