Prediction of Early Recurrence After Curative Resection of Colorectal Liver Metastasis and Subsequent S-1 Chemotherapy

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Abstract. *Background: S-1, an oral 5-fluorouracil (5-FU)-based medicine that combines tegafur, gimeracil and oteracil potassium is commonly used as an adjuvant chemotherapeutic drug for the treatment of colorectal cancer. Patients and Methods: We enrolled 53 patients who underwent curative resection for colorectal cancer and liver metastasis (synchronous, n=24; metachronous, n=29). The subsequent adjuvant chemotherapy with oral S-1 administration was initiated within 56 days after liver resection. Recurrence was evaluated by imaging studies, that were performed during the first year after liver resection. Of the 53 patients, 25 who did not recur within 1 year were defined as being in the no-recurrence (NREC) group and the remaining 18 patients were defined as being in the early-recurrence (EREC) group. There were no significant differences in gene expression profiling for drug resistance and metabolism between the NREC group and the EREC group. Results: In synchronous liver metastasis, there was no significant difference in early recurrence between serum carcinoembryonic antigen (CEA) ≤5 ng/ml and serum CEA >5 ng/ml (8/24 vs. 16/24, respectively). In metachronous liver metastasis, the early recurrence rate was significantly higher in patients with CEA >5 ng/ml compared to patients with CEA ≤5 ng/ml (15/29 vs. 14/29, p=0.05). The expression of cytochrome P450 2C19 (CYP2C19) and ATP-binding cassette, sub-family B member 1 (ABCB1) were significantly lower in the EREC group (6/15) compared to the NREC group (9/15) in colorectal cancer with metachronous liver metastasis and with serum CEA >5 ng/ml. Conclusion: Although the exact reason for down-regulation of these genes in the group with poor prognosis is unknown, the information obtained in this study may be useful in clinical practice for colorectal cancer.*

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Approximately 40-50% of patients with advanced colorectal cancer have distant metastasis (1, 2). The median survival time in patients with metastatic colorectal cancer is 4-6 months (3). S-1, an oral 5-fluorouracil (5-FU)-based medicine that combines tegafur, gimeracil and oteracil potassium is commonly used as an adjuvant chemotherapeutic drug for colorectal cancer (4). In two Phase II trials using S-1 chemotherapy in patients with metastatic colorectal cancer, the response rates were greater than 35% and the disease control rates were greater than 80% (5, 6). We also demonstrated that orally administered S-1 after curative liver resection for colorectal liver metastasis has an acceptable toxicity profile and a high completion rate of therapy (7). Therefore, adjuvant chemotherapy with S-1 is one of the treatment options after resection of colorectal liver metastasis in Japan (6, 8). However, some patients had an early recurrence even after an adjuvant chemotherapy with S-1. The objective of this study was to identify a novel biomarker that can be used as a predictor of early recurrence after the curative resection of colorectal liver metastasis and subsequent S-1 chemotherapy. RNA was extracted from formalin-fixed, paraffin-embedded primary colorectal cancer samples and a PCR array analysis was performed targeting human cancer drug resistance and metabolism genes.

Patients and Methods

Patients and samples. We enrolled 53 patients who underwent curative resection for colorectal cancer and liver metastasis (synchronous, n=24; metachronous, n=29) between October 2008 and August 2010. The subsequent adjuvant chemotherapy with oral S-1 administration was initiated within 56 days after liver resection and was repeated for 8 cycles. The oral S-1 was administered at the respective doses for 28 days, followed by a 2-week period of rest. Recurrence was evaluated by imaging studies, including chest and abdominopelvic computed tomography, which were performed at 4-

month intervals during the first year after liver resection. The patients also underwent hematologic testing and an assessment of clinical symptoms after liver resection.

Colorectal cancer samples for gene profiling were obtained from these 53 patients. A histological diagnosis of colorectal cancer was confirmed for all samples. An informed consent form, which was approved by the Institutional Review Board at Nagoya University, was obtained from all patients. The serum carcinoembryonic antigen (CEA) levels before the surgery for primary colorectal cancer were used for the analysis.

RNA extraction and cDNA preparation. The cancer sections were selectively isolated and obtained as formalin-fixed, paraffin-embedded specimens of primary colorectal cancer using laser capture microdissection (P.A.L.M. Microsystem, Leica, Wetzlar, Germany) (9). The dissected tissues were transferred to a reaction tube containing 400 μl of RNA lysis buffer and were homogenized. The RNA and cDNA were prepared based on the method described by Lord and colleagues (10). The quantification of beta-actin as an internal reference gene was performed using a fluorescence-based real-time detection system (ABI PRISM 7900 Sequence detection System, TaqMan®, Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The final volume of the reaction mixture was 20 μl and all reagents were from Perkin-Elmer Applied Biosystems.

PCR array analysis. The cDNA samples extracted from the colon cancer specimens were analyzed using the RT2 Profiler™ PCR Array for human cancer drug resistance and metabolism (QIAGEN, Hilden, Germany), which comprises a panel of 84 primer sets related to genes involved in cancer drug resistance and metabolism, such as DNA repair enzymes, cell cycle regulators, growth factors, hormone receptors and transcription factors. Among 84 genes, 47 genes were not detected due to inadequate amplification. Therefore, the remaining 37 genes were analyzed. Hypoxanthine phosphoribo-syltransferase 1 (*HPRT1*) and ribosomal protein L13a (*RPL13A*) were selected as the internal control genes. PCR arrays were analyzed using the 7300RT-PCR System (AB Applied Biosciences, Beverly, MA, USA). Up-regulated gene expression was defined as fold change ≥1.5, whereas down-regulated gene expression was defined as fold change ≤1.5. The procedure was performed according to the manufacturer's protocol.

Statistical analysis. All continuous data were presented as the median (range). Differences between the two groups were tested by the Mann-Whitney *U*-test for continuous data and the Chi-square or Fisher's exact test for categorical data using SPSS 20 (IBM, Armonk, NY, USA). A difference was considered statistically significant when $p<0.05$. The clusters were evaluated using the RT^2 profiler PCR array data analysis version 3.5 (QIAGEN) available on the web.

Results

Patients' characteristics. The patients' characteristics, including information on colorectal cancer and liver metastasis, are described in Table I.

Comparison of gene profiling for the NREC and EREC groups. For the 53 patients, 35 patients who did not recur within 1 year were defined as being in the no recurrence (NREC) group, while the remaining 18 patients were defined as being in the early recurrence (EREC) group. There were no significant differences in gene expression profiling for drug resistance and metabolism between the NREC group and the EREC group (Figure 1).

Table I. *Clinical characteristics of 53 patients who participated in the study.*

Age, year, (range)	63 (43-78)
Gender male/female	35/18
Location of primary cancer, n (%)	
Cecum	3 (6)
Ascending	5 (9)
Transverse	4 (8)
Descending	3 (6)
Sigmoid	13 (25)
Rectum	25 (47)
Surgical procedure for primary cancer	
Colectomy/Hemi-colectomy/Rectectomy	17/10/26
Liver metastasis	
Single/multiple	38/15
Synchronous/metachronous	24/29

Comparison of gene profiling for colorectal cancer having synchronous liver metastasis and colorectal cancer having metachronous liver metastasis. Next, we analyzed the gene expression profiling between colorectal cancer having synchronous liver metastasis (n=24) and colorectal cancer having metachronous liver metastasis (n=29) (Figure 2). Gene expression of the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon (*NFKBIE*) and topoisomerase I (*TOP1*) were higher in colorectal cancer with synchronous liver metastasis compared to colorectal cancer with metachronous liver metastasis. Eleven of the 24 colorectal cancer patients (46%) with synchronous liver metastasis recurred within 1 year, whereas 7 of the 29 colorectal cancer patients (24%) with metachronous liver metastasis recurred within 1 year. There were no significant differences in the recurrence rate within 1 year between colorectal cancer patients having synchronous liver metastasis and colorectal cancer patients having metachronous liver metastasis.

Impact of serum CEA levels on early recurrence. In patients with synchronous liver metastasis, there was no significant difference in early recurrence between patients with serum CEA ≤5 ng/ml and patients with serum CEA >5 ng/ml (8/24 *vs*. 16/24, respectively) (Table II). In metachronous liver metastasis, the early recurrence rate was significantly higher in patients with CEA >5 ng/ml compared to patients with CEA ≤5 ng/ml (15/29 *vs*. 14/29, respectively, $p=0.05$) (Table III).

Gene expression profiling differences between the NREC and EREC groups (for patients with colorectal cancer having

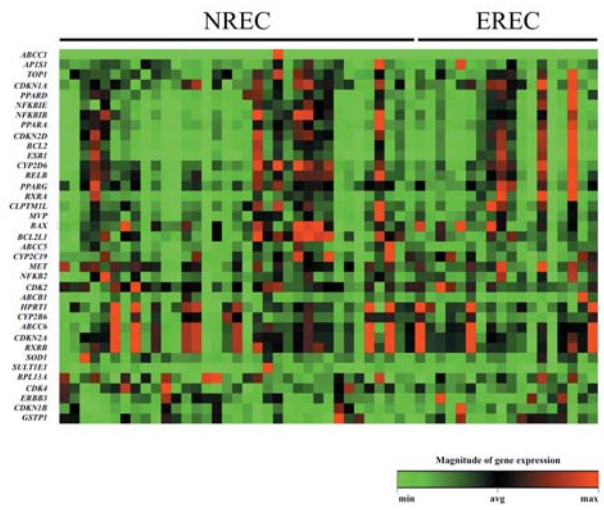

Figure 1. *One-dimensional expression of 43 genes in primary colorectal cancer between NREC (n=35) and EREC (n=18) of the 53 patients experiencing curative resection of colorectal liver metastasis and subsequent S-1 chemotherapy.*

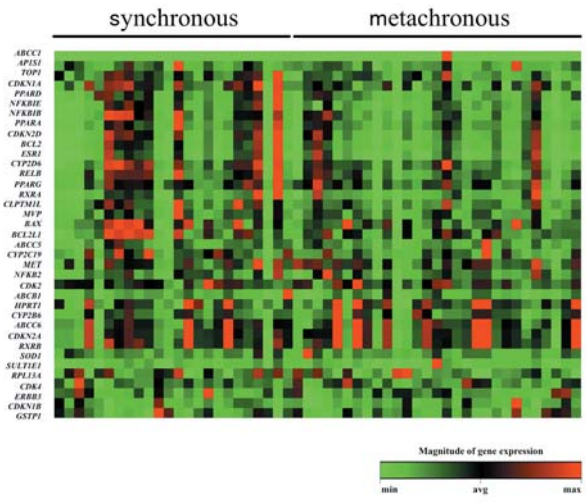

Figure 2. *One-dimensional expression of 43 genes between colorectal cancer patients having synchronous liver metastasis (n=24) and colorectal cancer patients having metachronous liver metastasis (n=29).*

Table II. *Impact of serum CEA levels on early recurrence in patients with synchronous primary colorectal cancer.*

	NREC (n=13)	EREC (n=11)	Total
CEA≤5 ng/ml	4	4	8
CEA>5 ng/ml	9	7	16

CEA, Carcinoembryonic antigen; NREC, no recurrence group; EREC, early recurrence group.

Table III. *Impact of serum CEA levels on early recurrence in patients with metachronous primary colorectal cancer.*

	NREC (n=22)	EREC (n=7)	Total
CEA≤5 ng/ml	13	1	14
CEA>5 ng/ml	9	6	15

CEA, Carcinoembryonic antigen; NREC, no recurrence group; EREC, early recurrence group.

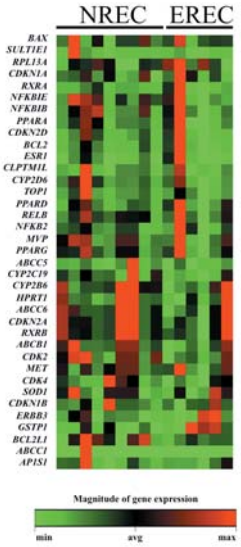

Figure 3. *One-dimensional expression of 43 genes for NREC (n=9) and EREC (n=6) in patients with colorectal cancer having metachronous liver metastasis and with high serum CEA levels (>5 ng/ml).*

metachronous liver metastasis and patients with serum CEA >5 ng/ml). Finally, we focused on patients with colorectal cancer having metachronous liver metastasis and with high serum CEA levels (>5 ng/ml). In this group, gene expression profiling was compared between the NREC (n=9) and EREC (n=6) groups (Figure 3). The expression of cytochrome *P450 2C19* (*CYP2C19*) and the expression of ATP-binding cassette, sub-Family B member 1 (*ABCB1*) were significantly lower in the EREC group compared to the NREC group (Table IV).

Discussion

For the last decade, advancements in surgical procedures and chemotherapy improved the prognosis of colorectal cancer (11). Surgical resection and perioperative chemotherapy also improved prognosis of colorectal liver metastasis (12). A Phase II clinical trial conducted in our Institution demonstrated an acceptable toxicity profile and a high rate of completion of the therapy in the patients who underwent a resection of colorectal

Table IV. *Gene expression profiling between the NREC and EREC groups; analysis in patients with metachronous primary colorectal cancer with CEA >5 ng/ml.*

Gene name	*p*-Value	Fold down-regulation
CYP2C19: Cytochrome *P450 2C19*	$p<0.03$	1.69
ABCB1: ATP-binding cassette, sub-family B (MDR/TAP), member 1	$p<0.05$	1.52

liver metastasis and subsequent adjuvant S-1 chemotherapy (7). However, approximately 35% of patients had to convert from S-1 chemotherapy to other regimens due to early recurrence. For these patients, S-1 chemotherapy was not suitable and another chemotherapeutic regimen should have been selected. In this study, we sought to determine a biomarker that can predict patients who are resistant to S-1 chemotherapy. However, among patients who underwent a resection of colorectal liver metastasis, there was no biomarker that could predict early recurrence despite S-1 chemotherapy.

Unexpectedly, there were no significant differences in gene expression between the NREC group (n=35) and the EREC group (n=18). Although there were genetic differences in the expression of *NFKBIE* and *TOP1* between colorectal cancer having synchronous liver metastasis and colorectal cancer having metachronous liver metastasis, there were no significant differences in early recurrence rate between the two groups. However, the early recurrence rates of patients with CEA >5 ng/ml was higher than that of patients with CEA ≤5 ng/ml in the group of patients with metachronous liver metastasis. *CYP2C19* and *ABCB1* expresson decreased in the EREC group with colorectal cancer having metachronous liver metastasis and serum CEA >5 ng/ml. These results indicated that an adjuvant chemotherapy with S-1 may not be effective after the curative resection of metachronous colorectal liver metastasis in patients with high serum CEA level (>5 ng/ml) and with lower expression of *CYP2C19* and *ABCB1*.

CEA is the most commonly used biomarker to assess the clinical effect of chemotherapy in gastrointestinal cancers. Koch *et al*. reported that there were no correlations between transient CEA elevations and cancer recurrence in patients with colorectal cancer (13) . Takahashi *et al*. reported that CEA expression was not associated with overall survival after adjuvant chemotherapy in patients undergoing repeated hepatectomy for colorectal liver metastasis (14). On the other hand, Chuang *et al*. reported that preoperative serum CEA (>5 ng/ml) is an independent prognosis factor in occurrence of metachronous liver metastasis (15). According to our results, there were no relationships between early recurrence and serum CEA level in synchronous liver metastasis. However, as was observed in this study, the early recurrence rate was significantly higher in patients with serum CEA >5 ng/ml compared to those patients with serum CEA ≤5 ng/ml in colorectal cancer with metachronous liver metastasis. In this subgroup of patients, serum CEA may be efficient in predicting early recurrence.

In this study, we found that the down-regulated expression of *CYP2C19* and *ABCB1* were correlated to the early recurrence in colorectal cancer with metachronous liver metastasis and with CEA >5 ng/ml. *CYP2C19* belongs to the CYP2C subfamily, which consists of four highly homologous genes, such as *CYP2C18, CYP2C19, CYP2C9* and *CYP2C8* (16). CYPs are the drug metabolizing enzymes, which are related to the metabolism of carcinogens or procarcinogens. Pharmacokinetics of CYP activity for anticancer drugs in tumors may be different from CYP activity in the liver (17) . CYPs in the tumor could lead to altered drug efficacy. Sachse *et al*. reported that reduced *CYP2C19* activity decreased the cancer risk in colorectal cancer (18). Shi *et al*. reported that poor metabolism of *CYP2C19* was significantly higher in lung cancer patients compared with local controls (19). Roddam *et al*. reported an increased risk of the development of adult acute leukemia for patients with poor metabolism of CYP2C19 (20). There are few studies of the influence of CYP2C19 on the recurrence of colorectal cancer. No carcinogenic substrates of CYP2C19 have been studied and identified in colorectal cancer. Further research is needed to address these issues.

ABCB1 (ATP-binding cassette, sub-family B (MDR/TAP) member 1) is a membrane-bound transporter, which belongs to the ATP-binding cassette superfamily (21). Its function involves multidrug resistance of cancer cells by an energy-driven efflux pump (22). The correlations of *ABCB1* expression and early recurrence of colorectal cancer are controversial. Weinstein *et al*. reported that *ABCB1* expression occasionally increased in recurrent colon cancer patients after exposure to chemotherapeutic drugs (23). Lazarius *et al*. reported that there are no significant differences in ABCB1 expression between primary carcinomas and their recurrence (22). The substrates of ABCB1 are hydrophobic drugs with neutral or positive charge (21). As 5-FU is not a substrate of ABCB1, it is not thought to confer resistance to 5-FU. Although the detailed mechanism is unclear, decreased expression of *ABCB1* is related to early recurrence after S-1 chemotherapy in patients with colorectal cancer having metachronous liver metastasis and with CEA >5 ng/ml.

Notably, the number of patients analyzed in this study was too small to reach a definitive conclusion. Although the exact

reason for the down-regulation of these genes in the group with poor prognosis is unknown, the information obtained in this study may be useful in clinical practice for colorectal cancer. Further studies are required to characterize the role of each gene in colorectal cancer and determine the potential utility of each gene as a biological marker for predicting early recurrence or the responsiveness to S-1 chemotherapy.

Conflicts of Interest

This study was funded by Taiho Pharmaceutical Co. Ltd (Tokyo, Japan). All Authors declare that they have no conflicts of interest. The sponsor had no role in the study design, performance of the study, data collection, data management and interpretation, preparation of this article or approval of the article.

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