

Prognostic Significance of Thymidylate Synthase Expression in the Adjuvant Chemotherapy after Resection for Pulmonary Metastases from Colorectal Cancer

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Abstract. *Background:* Recent studies have discussed on the prognostic value of thymidylate synthase (TS) assessment in metastases of colorectal cancer (CRC). The aim of this study was to evaluate the prognostic significance of molecular biomarkers including TS expression, after pulmonary metastasectomy followed by 5-fluorouracil-based adjuvant chemotherapy. *Patients and Methods:* A total of 80 patients who underwent metastasectomy for pulmonary recurrence from CRC were included in this study. Tumor sections were stained by immunohistochemistry for TS, orotate phosphoribosyltransferase (OPRT), dihydropyrimidine dehydrogenase (DPD), excision repair cross-complementation group 1 (ERCC1), breast cancer susceptibility gene 1 (BRCA1), vascular endothelial growth factor (VEGF), microvessel density (MVD) assessed by CD34 and p53. *Results:* Survival after pulmonary metastasectomy was significantly longer in patients treated by adjuvant chemotherapy than those without adjuvant therapy. TS, OPRT, ERCC1, BRCA1 and CD34 expression was significantly associated with better outcome from the use of adjuvant chemotherapy. *Conclusion:* Expression level of TS, OPRT, ERCC1, BRCA1 and MVD in resected colorectal lung metastases may play an important role for predicting outcome after 5-fluorouracil-based adjuvant therapy.

Colorectal cancer (CRC) is a major cause of cancer mortality worldwide, and spreads either regionally into draining lymph nodes, or systemically to the liver or lungs. Pulmonary metastasectomy in CRC patients is widely accepted, and has

been described to improve survival rates in selected patients with CRC metastasis to the lung (1, 2). However, metastasectomy alone does not offer a satisfactory survival benefit in CRC patients with metastatic disease to the liver or to the lung. At present, there is still no data available on the use of adjuvant chemotherapy after pulmonary metastasectomy.

5-Fluorouracil (5-FU)-based chemotherapy has been described to be effective for the treatment of various solid tumors, including lung cancer, gastric cancer and colon cancer (3). The anticancer activity of 5-FU has been described to be closely associated with the intratumoral expression of thymidylate synthase (TS), orotate phosphoribosyltransferase (OPRT) and dihydropyrimidine dehydrogenase (DPD) (4). The expression of TS is the target enzyme for 5-FU-based chemotherapy, and previous reports demonstrated that TS expression is a significant prognostic indicator and a predictor of the response to 5-FU based chemotherapy in CRC patients (5, 6). In contrast, several reports have documented that TS expression is not predictive of survival in the adjuvant setting with 5-FU regimens of CRC (7, 8). Thus, it is unclear whether TS expression has significant prognostic value in CRC treated by adjuvant chemotherapy with 5-FU regimens. However, there are still no useful molecular markers that can identify these patients with CRC who may benefit from adjuvant chemotherapy with 5-FU regimens.

Nucleotide excision repair (NER) has been described to be involved in the repair of platinum-induced DNA damage (9). Several researchers have investigated the prognostic and predictive significance of NER pathway biomarkers (10-12). Excision repair cross-complementation group 1 (ERCC1) and breast cancer susceptibility gene 1 (BRCA1) are involved in the NER system, and these proteins are known to be associated with resistance to platinum-based chemotherapy (10-13). A recent report demonstrated that ERCC1 expression may be useful for the prediction of outcome in patients with advanced CRC treated by 5-FU and oxaliplatin chemotherapy (14). However, it is unknown

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whether expression of ERCC1 is a predictive marker for survival in the adjuvant setting of CRC patients.

It remains controversial whether 5-FU-based adjuvant chemotherapy can improve outcome after metastasectomy in patients with pulmonary recurrence from CRC. We retrospectively investigated the prognostic significance of 5-FU-based adjuvant chemotherapy after pulmonary metastasectomy in patients with CRC metastasis to the lung, and an immunohistochemical study was conducted to evaluate whether TS expression can predict the outcome of adjuvant 5-FU chemotherapy. In addition, outcome after adjuvant chemotherapy was correlated with p53 and tumor angiogenesis as assessed by vascular endothelial growth factor (VEGF) and microvessel density (MVD).

Patients and Methods

Patients. We analyzed data for 92 consecutive patients who were evaluated for resection of CRC pulmonary metastases at Shizuoka Cancer Center between May 2003 and September 2008. Six patients who received induction of chemotherapy or radiation therapy were excluded. Specimens of 6 patients were not available. Therefore, a total of 80 patients were enrolled in the study. The study protocol was approved by the Institutional Review Board. In this study, the definition of adjuvant chemotherapy is not neoadjuvant chemotherapy but postoperative adjuvant chemotherapy.

Immunohistochemical staining. Immunohistochemical staining was performed according to the procedure described in the previous reports (15, 16). The following antibodies were used: a rabbit polyclonal antibody against TS (clone RTSSA, 1:1600 dilution; Taiho Pharmaceutical, Saitama, Japan); a rabbit polyclonal antibody against OPRT (1:1200 dilution; Taiho Pharmaceutical, Saitama, Japan); a rabbit polyclonal antibody against DPD (clone RDPDPA, 1:500 dilution; Taiho Pharmaceutical, Saitama, Japan); a mouse monoclonal antibody against ERCC1 (AB12356, 1:200 dilution; Abcam, Tokyo, Japan); a mouse monoclonal antibody against BRCA1 (AB116780, 1:50 dilution; Abcam, Tokyo, Japan); a mouse monoclonal antibody against TUBB3 (MMS-435P, 1:500 dilution; Convince, California); a monoclonal antibody against VEGF (1:200 dilution; Immuno-Biological Laboratories Co., Ltd., Japan); a mouse monoclonal antibody against CD34 (1:800 dilution; Nichirei, Tokyo, Japan); a mouse monoclonal antibody against p53 (D07, 1:50 dilution; DAKO). Antibodies against TS, OPRT and DPD were kindly donated by Taiho (Tokyo, Japan).

The expression of TS, OPRT and DPD was considered if nuclear or cytoplasmic staining was present. For TS, OPRT and DPD, a semi-quantitative scoring method was used: 1= <10%, 2=10-25%, 3=25-50%, 4=51-75% and 5=>75% of cells stained positively. The tumors in which stained tumor cells made up more than 25% of the tumor were graded as positive.

ERCC1 and BRCA1 were assessed semiquantitatively by estimating the percentage of tumor cells with positive nuclear and/or cytoplasmic staining on whole tumor slides: 0=no staining, 0.1=positive staining in 1-9% of the tumor cells, 0.5=positive staining in 10-49% of the tumor cells and 1=positive staining in > 50% of the tumor cells. The staining intensity was also evaluated in a semiquantitative method representing the average intensity of the stained tumor cells: 0=no staining, 1=weak

Table I. Patient characteristics.

Patient characteristic	N=80
Median age (range, years)	66 (31-81 years)
Gender	
Male/female	53/27
Performance status (PS)	
0/1	68/12
Smoking history	
Yes/No	46/34
Primary tumor site	
Colon/rectum/colorectal	31/39/10
Primary tumor stage	
I/II/III/IV/unknown	7/16/24/15/18
Maximal tumor size	
≤13/>13 mm	43/37
Preoperative CEA level	
≤3.3/>3.3 µg/l	42/38
No. of resected metastases	
1/2-4	49/31
Extent of resection	
Limited resection/lobectomy	65/15
Mode of resection	
Unilateral/bilateral simultaneous	66/12

CEA, Carcinoembryonic antigen; 5-FU, 5-fluorouracil.

staining, 2=moderate staining and 3=strong staining. The proportion and intensity scores were then multiplied to obtain a total score, which ranged from 0 to 3 (H-score).

The expression of VEGF was quantitatively assessed according to the percentage of immunoreactive cells in a total of 1000 neoplastic cells. The number of CD34-positive vessels was counted in four selected hot spots in a 0.26 mm² field area. MVD was defined as the mean count of microvessels per 0.26 mm² field area.

For p53, microscopic examination for the nuclear reaction product was performed and scored. According to a previous report (15), p53 expression in more than 10% of tumor cells was defined as high expression. Sections were assessed using a light microscope in a blinded fashion by at least two of the authors.

Statistical analysis. Probability values of <0.05 indicated a statistically significant difference. Fisher's exact test was used to examine the association of two categorical variables. Correlation of different variables was analyzed using the nonparametric Spearman's rank test. The Kaplan-Meier method was used to estimate survival as a function of time, and survival difference were analyzed by the log-rank test. Multivariate analyses were performed using stepwise Cox proportional hazards model to identify independent prognostic factors. Statistical analysis was performed using JMP 8 (SAS, Institute Inc., Cary, NC, USA) for Windows.

Results

Patient characteristics. Patient characteristics are listed in Table I. The median age of the patients was 66 years (range, 33-81 years). Almost twice as many patients were smokers as were non-smokers, and had a single metastasis as

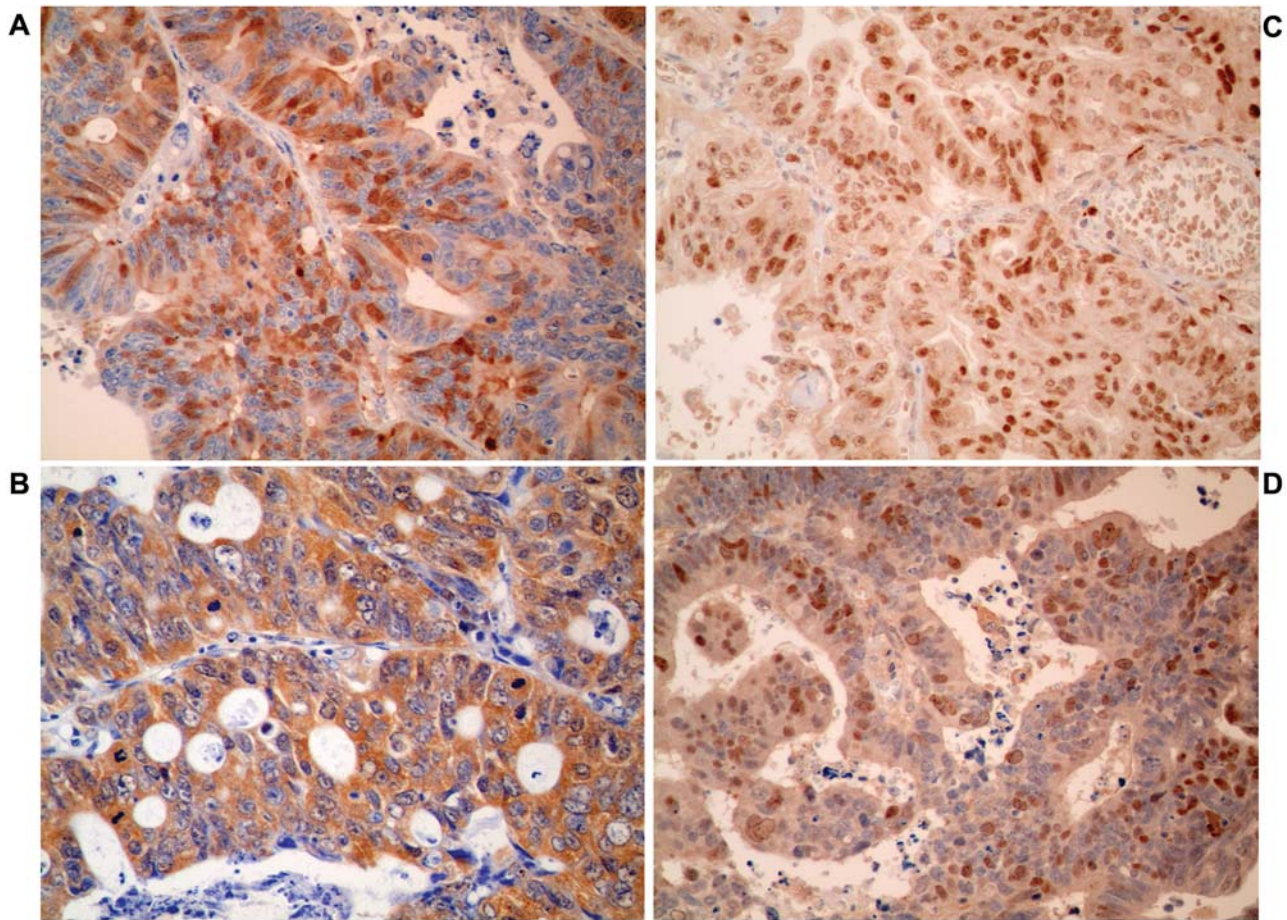


Figure 1. Example of immunohistochemical staining of thymidylate synthase (TS), orotate phosphoribosyltransferase (OPRT), excision repair cross-complementation group 1 (ERCC1) and breast cancer susceptibility gene 1 (BRCA1) in pulmonary metastatic colorectal cancer. A. TS is stained mainly in nuclei (score 5). B. OPRT is stained mainly in cytoplasm (score 5). C. ERCC1 is stained in nuclei (H-score 3). D. BRCA1 is stained in nuclei (H-score 3).

compared to multiple metastases. Thirty-eight patients had a high level of preoperative carcinoembryonic antigen (CEA) and 42 a low level of CEA (median, 3.3 µg/l). The maximal tumor size of resected pulmonary metastases ranged from 7 to 95 mm (median, 13 mm).

Forty-two patients (53%) received adjuvant chemotherapy after pulmonary metastasectomy and 38 patients (47%) had no adjuvant therapy. In 42 patients receiving adjuvant chemotherapy, 29 patients were treated with 5-FU regimens with oxaliplatin and 13 receiving 5-FU regimens without oxaliplatin. The adjuvant chemotherapy for these patients was conducted under the guideline of National Comprehensive Cancer Network (NCCN) (17) and American Society of Clinical Oncology (ASCO) (18). Briefly, the chemotherapeutic regimens included oxaliplatin/leucovorin/5-FU (FOLFOX) and uracil-tegafur (UFT)/leucovorin. The duration of chemotherapy varied among patients but was decided in compliance with the NCCN/ASCO guidelines.

Immunohistochemical staining. Each protein revealed a profile pattern of the unique expression. The immunohistochemical staining of the biomarkers was evaluated for the 80 pulmonary metastatic lesions (Figure 1). A positive expression of TS, OPRT and DPD was recognized in 65% (52/80), 75% (60/80) and 8.8% (7/80), respectively. The median H-score of ERCC1 and BRCA1 was 0.10 and 0.75, respectively. Positive expression of ERCC1 and BRCA1 was recognized in 25% (20/80) and 50% (40/80) of cases, respectively. The staining pattern of VEGF was uniformly localized in the cytoplasm and/or membrane of neoplastic tissue. The median rate of VEGF positivity was 21% (range, 2-58%). High expression was recognized in 50% of cases (40/80). The median number of CD34-positive vessels was 23 (6-42). High expression of CD34 was seen in 50% of cases (40/80). High expression of p53 was recognized in 55% of cases (44/80).

Table II shows a comparison of the different variables according to TS expression. TS expression was not significantly associated with these different variables.

Table II. Patient characteristics according to Thymidylate synthase expression.

Patient characteristic	TS (+) (n=52)	TS(–) (n=28)	p-Value
Age			
≤65/>65 yr	23/29	15/13	0.489
Gender			
Male/female	37/15	16/12	0.224
Performance status (PS)			
0/1	42/10	26/2	0.198
Smoking history			
Yes/no	32/20	14/14	0.351
Maximal tumor size (mm)			
≤13/>13 mm	28/24	15/13	>0.999
Preoperative CEA level			
≤3.3/>3.3 µg/l	27/25	15/13	>0.999
No. of resected metastases			
Single/multiple	34/18	15/13	0.341
Extent of resection			
Limited resection/lobectomy	42/10	23/5	>0.999
Mode of resection			
Unilateral/bilateral simultaneous	45/7	21/7	0.226
OPRT			
Positive/negative	39/13	21/7	>0.999
DPD			
Positive/negative	3/49	4/24	0.232
ERCC1			
Positive/Negative	13/39	7/21	>0.999
BRCA1			
Positive/negative	29/23	11/17	0.157
p53			
Positive/negative	32/20	12/16	0.157
VEGF			
High/low	29/23	11/17	0.241
CD34			
High/low	28/24	12/16	0.482

Table III. Comparison of different variables between patients undergoing adjuvant chemotherapy and those not undergoing adjuvant therapy.

Variables	Adjuvant chemotherapy (n=42)	No adjuvant therapy (n=38)	p-Value
Age			
≤65/>65 year	23/19	15/23	0.187
Gender			
Male/female	29/13	24/14	0.640
PS			
0/1	38/4	30/8	0.212
Smoking history			
Yes/no	29/13	17/21	0.041
Maximal tumor size			
≤13/>13 mm	27/15	16/22	0.072
No. of resected metastases			
Single/multiple	23/19	26/12	0.254
Preoperative CEA			
≤3.3/>3.3 µg/l	27/15	15/23	0.043
TS			
Positive/negative	24/18	28/10	0.160
OPRT			
Positive/negative	31/11	29/9	>0.999
DPD			
Positive/negative	6/36	1/37	0.117
ERCC1			
Positive/negative	12/30	8/30	0.606
BRCA1			
Positive/negative	22/20	18/20	0.823
p53			
Positive/negative	25/17	19/19	0.500
VEGF			
High/low	20/22	20/18	0.823
CD34			
High/low	24/18	16/22	0.263

Comparison of different variables according to adjuvant therapy. We divided our study population into two groups of patients: those who received adjuvant chemotherapy (n=42) and those without adjuvant therapy (n=38). The results of the statistical comparison between the two groups according to the different variables are listed in Table III. Smoking history and lower preoperative CEA level yielded a statistically significant positive correlation with undergoing adjuvant chemotherapy. The other parameters were not significantly differently distinguished between adjuvant chemotherapy (n=42) and no adjuvant therapy (n=38).

Survival analysis according to the expression of biomarkers. The median follow-up time for all patients was 71.0 months, and the 5-year survival rate was 55.8%. A statistically significant difference in the progression-free survival (PFS) and overall survival (OS) after pulmonary metastasectomy

was observed between patients with or without adjuvant chemotherapy (Figure 2). The median PFS of patients without adjuvant therapy was 30.6 months, but that with adjuvant chemotherapy was not reached ($p=0.016$). The median OS of patients without adjuvant therapy was 56.5 months, but that with adjuvant chemotherapy was not reached ($p=0.035$).

Next, we analyzed outcome according to the expression of biomarkers. No statistically significant difference in the PFS and OS was observed between patients with or without positive (high) expression of TS, OPRT, DPD, VEGF, CD34, p53, ERCC1 and BRCA1, as defined by the cut-off points (median values). Survival analysis was performed among patients with positive (high) expression of these biomarkers. Patients whose tumors stained positively for TS, OPRT, ERCC1 and BRCA1 had a statistically significant longer PFS from the use of adjuvant chemotherapy compared with

those who were not treated (Figure 3). Moreover, we compared the outcome between patients treated by adjuvant chemotherapy including oxaliplatin (n=29) and those without adjuvant therapy (n=38), and evaluated the relationship between outcome and the expression of ERCC1 and BRCA1. The present study indicated that patients with positive staining for ERCC1 had a statistically significant longer PFS from the use of adjuvant chemotherapy including oxaliplatin than no adjuvant therapy ($p=0.042$), but not these with positivity for BRCA1 ($p=0.809$). No statistically significant difference in the outcome according to other biomarkers was observed between patients with or without adjuvant chemotherapy. Survival analysis was also performed among patients with low expression of these biomarkers. Patients with a low CD34 expression had a statistically significant longer PFS from the use of adjuvant chemotherapy compared with those who were not treated (Figure 3). No statistically significant difference in the survival was recognized for the other biomarkers.

The other potential prognostic factors were age, sex, PS, smoking history, extent of resection, bilaterality of the lesion, numbers and sizes of metastases, and preoperative CEA level. Multivariate and univariate analysis did not show any of have to be significant prognostic factors for recurrent metastasis.

Discussion

This is the first study to investigate the relationship between these molecular markers including TS expression and outcome after adjuvant therapy in patients with resected pulmonary metastases from CRC. Our results demonstrated that survival after metastasectomy was significantly longer in patients treated by adjuvant chemotherapy than those without adjuvant therapy. Patients who had positive expression for TS, OPRT, ERCC1 and BRCA1 and a low CD34 expression seemed to benefit substantially from the use of 5-FU-based adjuvant chemotherapy compared with those who were not treated. 5-FU/oxaliplatin chemotherapy may play an important role in improving outcome after pulmonary metastasectomy in the adjuvant setting of patients with positive ERCC1 expression.

Previous reports revealed the clinical relevance of low TS expression as a predictor of the response to 5-FU-based chemotherapy and the long survival of the patients with advanced CRC (5, 6). Several researchers described that TS expression in the primary CRC tumor is of limited value in predicting the clinical response to 5-FU-based adjuvant chemotherapy (19, 20). In contrast, several previous reports documented that patients with high TS expression had a longer disease-free survival after adjuvant 5-FU chemotherapy and those with low TS expression did not (19, 21). The present study also indicated that patients whose

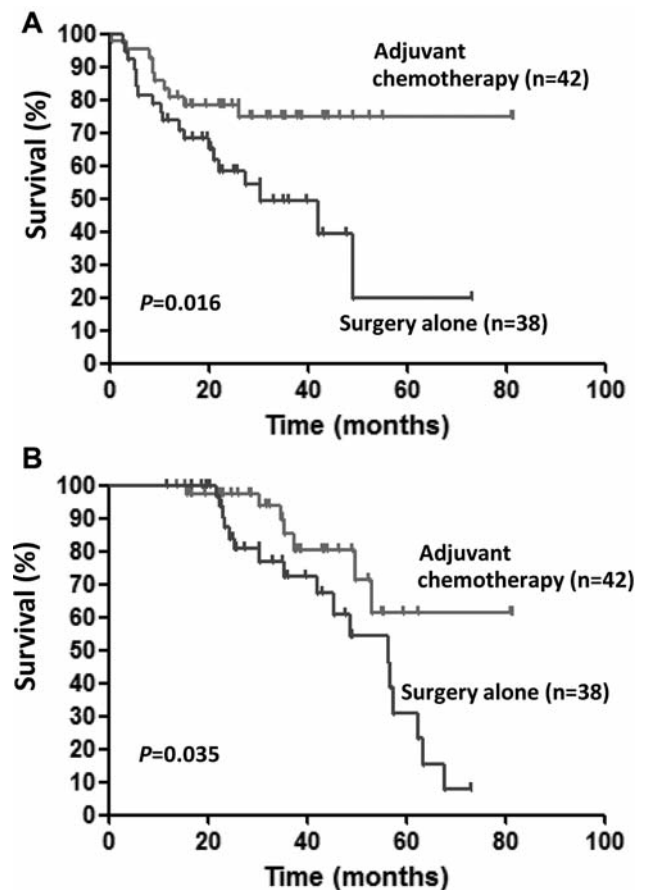


Figure 2. Statistically significant difference in the progression-free survival (A) and overall survival (B) was observed between patients with adjuvant chemotherapy (n=42) and those undergoing surgery alone (n=38).

pulmonary metastatic tumors had positive TS expression may benefit from 5-FU-based adjuvant treatment compared with those who were not treated. In these studies including our study (19, 21), the percentage of cases with high TS expression was more than 60-65%, compared with less than 40-50% in the studies which have documented the survival benefit of adjuvant chemotherapy in patients with low TS levels (5, 6). Such a difference among these studies may account for the contradictory results. Pre-clinical data suggests that high TS levels are closely associated with 5-FU resistance of cancer cells (22). However, the clinical outcome according to TS expression is still a matter of debate for CRC patients who received 5-FU-based chemotherapy.

Corsi *et al.* reported high TS levels in resected metastatic sites to be associated with poor outcome after metastasectomy plus adjuvant chemotherapy in CRC patients (23). Their data demonstrated a significantly higher TS expression in the primary sites than metastatic

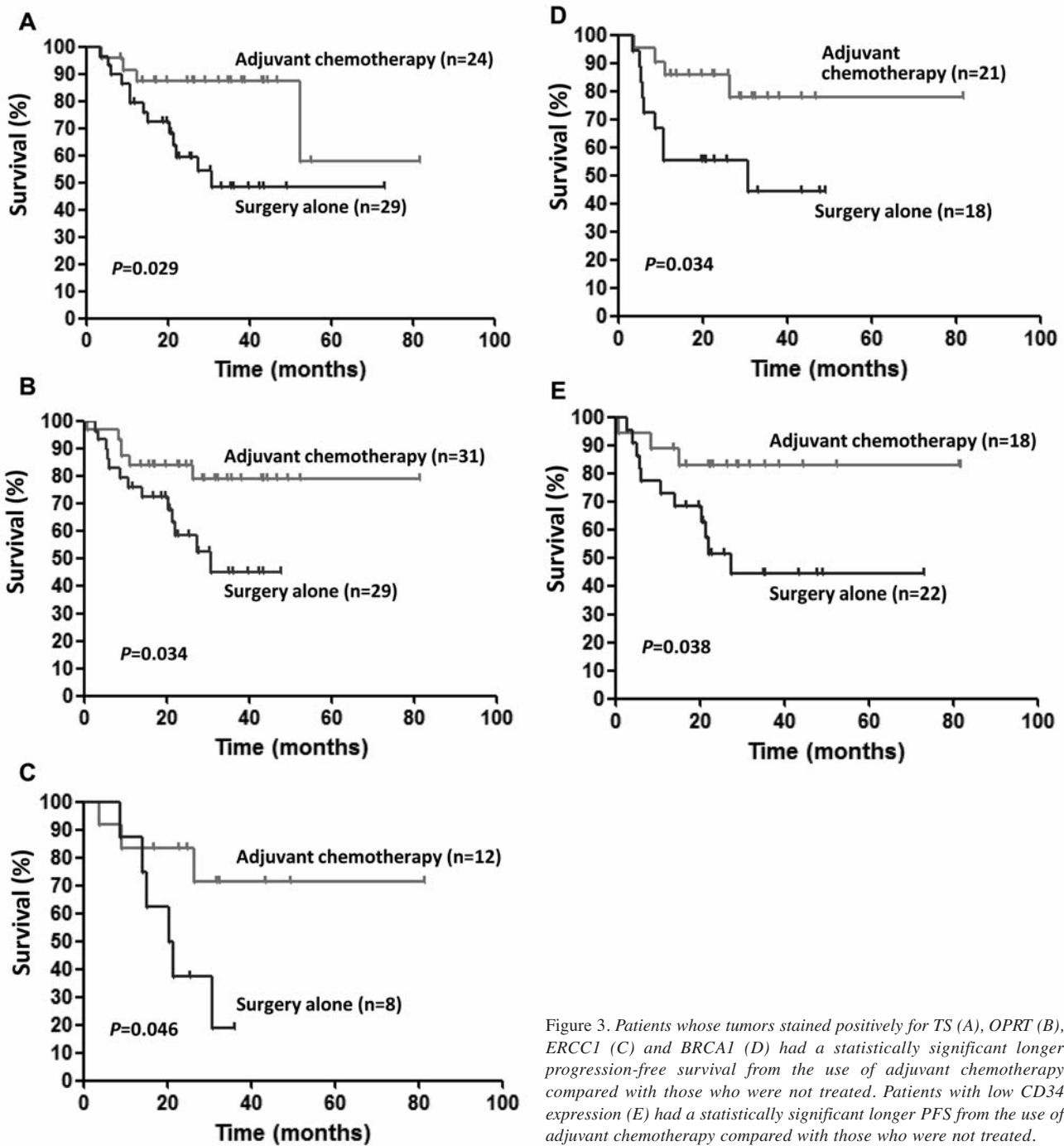


Figure 3. Patients whose tumors stained positively for TS (A), OPRT (B), ERCC1 (C) and BRCA1 (D) had a statistically significant longer progression-free survival from the use of adjuvant chemotherapy compared with those who were not treated. Patients with low CD34 expression (E) had a statistically significant longer PFS from the use of adjuvant chemotherapy compared with those who were not treated.

sites, and 53% of patients showed high TS levels in primary disease compared with 33% in the metastases. Their study population consisted of 60 patients: 48 underwent liver and 12 lung resection. The incidence of high TS expression in their study (33%) was lower compared with that of our study (65%), and only 12 patients underwent resection for pulmonary metastases

from CRC. The other reports have documented that TS expression was useful for predicting clinical outcome of 5-FU-based chemotherapy in patients with liver metastases from CRC (5, 6). However, little is known whether TS expression in pulmonary metastatic tumors can predict outcome of 5-FU-based adjuvant chemotherapy after pulmonary metastasectomy.

OPRT is an important enzyme in 5-FU cytotoxicity and DNA synthesis. A high OPRT expression in human neoplasm has been associated with 5-FU activity (24). In studies using human CRC cancer tissues, a higher OPRT enzyme activity was observed in 5-FU sensitive tissue specimens than nonsensitive ones based on an *in vitro* chemosensitivity test (25). Although it remains controversial whether OPRT expression is closely associated with outcome after adjuvant 5-FU-based chemotherapy in CRC, our study suggests that CRC patients with a high OPRT expression have a survival benefit from 5-FU-based adjuvant treatment compared with those who were not treated.

Recently, high ERCC1 expression was described to be associated with poor outcome in patients with advanced CRC treated by 5-FU and oxaliplatin combination chemotherapy (14, 26). In patients with non-small cell lung cancer (NSCLC), adjuvant chemotherapy significantly prolonged survival among patients with negative ERCC1 expression, but not among patients with positive ERCC1 expression. In NSCLC patients with observation (control group), those with positive ERCC1 expression survived longer than those with negative ERCC1 expression (11). However, our results suggest that 5-FU/oxaliplatin-based adjuvant chemotherapy confirmed a survival benefit in patients with metastatic CRC with positive expression of ERCC1. In contrast, Grabsch *et al.* described that patients with BRCA1-negative CRC had significantly shorter OS and disease-free survival, and loss of BRCA1 expression was significantly associated with poor outcome in the CRC patients who received adjuvant therapy (27). Adjuvant therapy in their study was not limited to regimens including oxaliplatin, and their results suggest that BRCA1 protein expression levels may influence response to adjuvant therapy. Our study finding also corresponds to the results of their study. However, ERCC1 expression levels may strongly influence clinical outcome of adjuvant therapy in the small number of patients who received 5-FU regimens including oxaliplatin. However, it is unknown whether ERCC1 and BRCA1 expression levels in the metastatic tumors can predict survival in CRC patients who underwent pulmonary metastasectomy and adjuvant chemotherapy.

Angiogenesis plays a key in tumor growth and metastatic spread. A meta-analysis has documented that high MVD and VEGF expression, markers of angiogenesis, was able to predict poor outcome in CRC patients (28). Several investigators have reported high MVD to correlate with significantly lower OS following metastasectomy, with high MVD in metastases associated with increased risk of death over low MVD metastases (29). High VEGF expression correlated with increased metastatic spread and poor prognosis for primary CRC. However, studies of resected CRC liver metastases have failed to show any correlation between VEGF expression and survival (30, 31). Our results

suggest that CRC patients with low MVD had a favorable outcome after adjuvant therapy as compared with those with high MVD, but VEGF expression was not useful for predicting benefit from the use of adjuvant therapy. One retrospective study documented that VEGF and MVD expression did not predict a favorable response to anti-angiogenic agent (bevacizumab) in metastatic CRC (32). Although it is unclear whether angiogenic markers in resected CRC lung metastases could predict the outcome after pulmonary metastasectomy followed by adjuvant chemotherapy, the present study showed that adjuvant therapy may have a survival benefit in patients with metastatic CRC with low MVD.

The present study has several limitations. Firstly, our population had a small sample size. Although the patients' characteristics were not significantly different between those receiving adjuvant chemotherapy (n=42) and those without adjuvant therapy (n=38), this is a retrospective study. Adjuvant therapy after lung resection was also not uniform, thus the outcome may be influenced by the content of adjuvant therapy. Another limitation is that the follow-up period was not long enough for CRC after pulmonary metastasectomy.

In conclusion, this retrospective study showed that 5-FU-based adjuvant chemotherapy can improve clinical outcome after metastasectomy for pulmonary metastases from CRC. Patients with high TS levels may benefit from adjuvant 5-FU-based chemotherapy. However, TS expression was not an independent prognostic factor for predicting poor outcome. Moreover, patients who had positive expression for OPRT, ERCC1 and BRCA1, and low MVD seemed to have a favorable outcome when treated with adjuvant therapy. Our results suggest that the expression level of TS, OPRT, ERCC1, BRCA1 and MVD in pulmonary metastases play an important role for predicting clinical outcome after 5-FU-based adjuvant therapy. Further prospective study is required for investigating predictive markers of adjuvant chemotherapy after pulmonary metastasectomy in CRC patients.

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Conflicts of interest statement

None of the Authors have any financial or personal relationships with other people or organizations that could inappropriately influence our work.

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