

# Phase II study of tailored chemotherapy for advanced colorectal cancer with either 5-fluorouracil and leucovorin or oxaliplatin and irinotecan based on the expression of thymidylate synthase and dihydropyrimidine dehydrogenase

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**Background:** Thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) are essential enzymes for 5-fluorouracil (5-FU) metabolism. In patients with advanced colorectal cancer (ACRC), retrospective studies have shown that low expression levels of TS and DPD correlated with response to 5-FU. We performed a prospective study in which the choice of first-line chemotherapy with either 5-FU or a non-5-FU containing regimen was based on TS and DPD expression.

**Patients and methods:** Fresh-frozen samples of metastases were obtained from 58 previously untreated patients with ACRC. mRNA expression of TS and DPD was quantified using an RT-PCR assay. Patients with low tumor expression of both TS and DPD received weekly bolus 5-FU/leucovorin (LV) 500 mg/m<sup>2</sup> (group A); patients with high TS and/or DPD received 3-weekly oxaliplatin 85 mg/m<sup>2</sup> and irinotecan 200 mg/m<sup>2</sup> (group B). After progression, cross-over to the alternative regimen was attempted.

**Results:** Of 53 eligible patients, 31 had tumors with both low TS and low DPD, and were treated in group A. A response was observed in 11 patients [35%; 95% confidence interval (CI) 19% to 54%]. Cross-over to second-line oxaliplatin/irinotecan resulted in a partial response in two out of 16 patients (13%; 95% CI 1% to 38%). In group B, four out of 22 patients responded (18%; 95% CI 5% to 40%), while no responses were observed in 12 patients after cross-over to 5-FU/LV (0%; 95% CI 0% to 28%).

**Conclusions:** Prospective selection of 5-FU/LV chemotherapy based on low TS and DPD expression in patients with ACRC did not confirm the high response rates reported in retrospective studies. The procedure of obtaining metastatic tissue and quantitation of enzymes appeared feasible but cumbersome. Before assessing the clinical utility of a predictive marker in a randomized trial, future studies should focus on prospective validation of the assay in a large and well defined population.

**Key words:** colorectal cancer, dihydropyrimidine dehydrogenase, predictive marker, thymidylate synthase

## introduction

Colorectal cancer is the second most common cause of cancer-related death in the Western world. Surgery is the primary treatment of localized disease, but approximately half of all

patients eventually die from metastatic disease. In patients with advanced colorectal cancer (ACRC), 5-fluorouracil (5-FU) has been the mainstay of chemotherapy since its introduction almost 50 years ago. As the addition of leucovorin improved its efficacy, treatment with 5-FU modulated with leucovorin (LV) has remained the standard therapy for many years [1], despite a modest response rate of only 20–25%. Recently, the number of therapeutic options in ACRC has increased tremendously with the introduction of new cytotoxic agents such as irinotecan and oxaliplatin, oral 5-FU pro-drugs such as capecitabine, and targeted therapies such as bevacizumab and cetuximab. Current combination chemotherapy in first-line

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treatment of ACRC results in a response rate of about 40–60%, with a median survival approaching 2 years.

Predictive markers identify tumors as being responsive or resistant to a specific treatment and may be of value in selecting the optimal treatment for the individual patient. A well-known example is the estrogen receptor in breast cancer, which is widely used to make a decision on hormonal treatment. Much research has focused on predictive markers in colorectal cancer [2]. Being the target enzyme for 5-FU, thymidylate synthase (TS) is the rate-limiting enzyme for the conversion of deoxy-uridine monophosphate (dUMP) to deoxy-thymidine monophosphate (dTMP), essential for DNA synthesis. In various retrospective clinical studies in ACRC, low levels of expression of TS correlated with an improved response rate and overall survival in patients treated with 5-FU [3–8]. Interestingly, Aschele et al. [7] reported a poor correlation between TS levels measured in the primary colorectal tumor and in its metastases. They observed a predictive value for clinical response to 5-FU of TS levels in the metastatic lesions, but not of TS levels in primary tumors. Response rates were 71% and 23% in patients with low and high TS, respectively, measured in metastatic lesions from 27 ACRC patients. Likewise, Findlay et al. [9] observed that TS expression in primary colorectal tumors, assessed by immunohistochemistry, did not predict response to 5-FU. Backus et al. also reported a significantly higher expression of TS in metastases than in the matched primary colon cancer samples [10], and found a correlation between low TS levels in liver metastases and prolonged patient survival after hepatic arterial therapy with 5-FU [11].

Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme for 5-FU catabolism, accounting for 80–90% of the drug's clearance. Owing to an increased exposure to active 5-FU metabolites, patients with a DPD deficiency are likely to suffer from severe toxicity when treated with 5-FU [12]. Interestingly, however, clinical studies also revealed a higher tumor response to 5-FU in patients with low DPD tumor levels [8, 13, 14]. In a retrospective study, Salonga et al. [8] observed a response rate of 92% to 5-FU in 11 ACRC patients with low levels of TS and DPD in metastatic lesions, and no responses in 22 patients with high TS and/or DPD.

In patients who have a low chance of responding to 5-FU (high levels of TS and/or DPD) other chemotherapeutic agents may be of clinical value. Irinotecan, a topoisomerase I inhibitor, and oxaliplatin, a platinum analog, have modes of action and metabolism different from 5-FU. Theoretically, TS and DPD should not predict the efficacy or the toxicity of these agents. Besides, a large Japanese population study did not show any correlation between the activity of TS and that of DPD in patients with various solid tumors, including colorectal cancer [15]. Indeed, the antitumor efficacy of irinotecan was found to be independent of TS levels [16]. No data were reported on TS and DPD as predictive markers for single-agent oxaliplatin, although low TS levels were predictive for a better response to the combination of oxaliplatin and 5-FU [17].

Based on the high response rates with 5-FU in retrospective studies, we performed this first prospective study in previously untreated patients with ACRC in which the selection of chemotherapy was based on mRNA expression of TS and DPD in metastatic lesions. Patients received either 5-FU/LV

or irinotecan and oxaliplatin based on the expression levels assessed by reverse transcription (RT)–PCR on RNA extracted from fresh metastatic tumor biopsies. The major aim was to assess prospectively the actual response rate of 5-FU/LV as first-line treatment in patients with low expression levels of both enzymes. Other end points of the study were the response rate of the combination of irinotecan and oxaliplatin in first-line and the response rate with 5-FU/LV in second-line in patients with high expression of one or both enzymes.

## patients and methods

### patients

Patients with histologically confirmed metastatic colorectal cancer, aged between 18 and 75 years, with a performance status of 0 to 2 according to the WHO scale were eligible for the study. Furthermore, eligibility criteria included: biotable metastatic lesion, no prior palliative chemotherapy, completion of any adjuvant chemotherapy more than 6 months before, measurable or evaluable metastatic disease, and bone marrow reserve, hepatic function and renal function within normal limits [defined as an absolute neutrophil count  $>2 \times 10^9/l$ , platelets  $>150 \times 10^9/l$ , serum bilirubin  $<1.5 \times$  upper normal limit (UNL) in case of liver metastases and serum creatinine  $<1.25 \times$  UNL]. Pretreatment assessment included a full medical history, physical examination, complete blood cell count, serum chemistry, electrocardiogram, chest X-ray and computed tomography scan of the abdomen. All patients gave written informed consent, and the study protocol was reviewed and approved by the local medical ethical committee.

### methods

In all patients, a pretreatment ultrasound-guided needle biopsy of metastatic tissue was performed, which yielded two to three cores, each 10 mm long. In patients with multiple metastatic lesions, a biopsy was performed from the best accessible lesion only. Each core was immediately frozen in liquid nitrogen before storage at  $-70^\circ\text{C}$ . The interval between biopsy and storage was  $<1$  min. The level of TS was measured using a quantitative RT–PCR assay with competitive templates. In this assay a defined amount of competitive templates of the target gene *TS* or *DPD*, and of a reference gene,  $\beta$ -*actin*, was added to the reaction mixture, enabling simultaneous amplification of the native template and the competitive template in the same reaction tube. Care was taken that the amplification efficiencies were optimal. This enabled exact measurements of the number of transcripts [18]. This type of assay has been cross-validated among multiple laboratories, including ours, and has shown good reproducibility [19]. The relative mRNA levels of the samples were expressed as the ratio of the target gene over the reference gene,  $\beta$ -*actin*. Validation of the assay for *TS* mRNA expression has been described previously [20]. The assay for *DPD* expression was described earlier by Johnston et al. [21], who generously provided the competitive templates for our study. In order to determine the cut-off values for this study, 16 cDNA samples prepared from colorectal cancers were assayed both by the competitive template procedure and by real-time PCR using the Taqman assay as described by Salonga et al. [8]. Cut-off points for response to 5-FU have been defined by Salonga for the *TS*/ $\beta$ -*actin* ratio at  $3.5 \times 10^{-3}$  and for the *DPD*/ $\beta$ -*actin* ratio at  $2.5 \times 10^{-3}$ , respectively. A significant correlation was observed between the two assays, although the relative expression values were different as a result of the use of different primers and PCR conditions, leading to different efficiencies. The cut-off points used in our study were  $5 \times 10^{-3}$  and  $45 \times 10^{-3}$  for *TS* and *DPD* mRNA expression, respectively [22]. Care was taken that the ratio of the expression of the competitive template and native template was between 0.1 and 10, in order to prevent the absorption of the bands being either too low or in a non-linear absorption range [20].

The results of TS and DPD levels of expression were available within 2 weeks after biopsy. Patients with both low TS and low DPD were treated with weekly bolus 5-FU/LV infusion at a dose of 500 mg/m<sup>2</sup> according to the Roswell Park regimen (treatment group A). Patients with a high TS and/or high DPD were treated with oxaliplatin at a dose of 85 mg/m<sup>2</sup> as a 2-h infusion followed by irinotecan at a dose of 200 mg/m<sup>2</sup> as a 30-min infusion, every 3 weeks (treatment group B). This combination regimen was reported to be feasible and active in patients resistant to 5-FU [23]. Standard antiemetics consisted of 10 mg metoclopramide in group A and of 8 mg dexamethasone, 8 mg ondansetron and 0.25 mg atropine (to prevent the occurrence of the cholinergic syndrome) in group B. Prophylactic use of growth factors was not allowed. Subsequent cycles of chemotherapy required a white blood cell count of  $>3 \times 10^9/l$  and a platelet count of  $>100 \times 10^9/l$ . Treatment was continued until progressive disease or unacceptable toxicity. During therapy, laboratory analysis and evaluation of toxicity according to the National Cancer Institute Common Toxicity Criteria version 2.0 were performed every 3 weeks. Responses were evaluated every 9 weeks according to WHO criteria. A complete response was defined as the disappearance of all evidence of disease for  $>4$  weeks. Partial response was defined as a  $>50\%$  decrease in the sum of the products of the two longest perpendicular diameters of all measured lesions for  $>4$  weeks, with no evidence of progressive disease. Stable disease was defined as no significant change in measurable and non-measurable disease. Progressive disease was defined as a  $>25\%$  increase in the product of the longest perpendicular diameters of any measurable lesion or in the estimated size of non-measurable disease, the appearance of a new lesion, or the reappearance of old lesions.

Upon disease progression, cross-over to the alternative schedule was attempted after a new biopsy from a metastatic site.

### statistical analysis

The primary outcome of interest was the response rate of 5-FU/LV in previously untreated patients with low levels of TS and DPD (group A). Other end points of the study were the response rate of the combination of irinotecan and oxaliplatin in first-line and the response rate with 5-FU/LV in second-line in patients with high expression of one or both enzymes (group B). The sample size of group A was calculated on an assumed absolute increase of the response rate of 5-FU/LV of at least 20%, as compared with the published response rate with 5-FU/LV of 23% in a meta-analysis including 803 unselected advanced CRC patients treated [1]. For a two-sided significance level  $\alpha$  of 0.05 with a power  $(1 - \beta)$  set at 80%, a sample size of 31 evaluable patients was required. We compared proportions of patients in both treatment groups with the  $\chi^2$ -test, reporting two-sided  $P$  values. A  $P$  value of  $<0.05$  was considered as significant. Since it was not expected that patients with a high TS and/or DPD would respond to 5-FU in second-line (estimated response rate  $<20\%$ ), an interim-analysis of response was planned for the first 14 patients after cross-over in group B to 5-FU/LV. Cross-over in group B would be stopped if no responses were observed in these patients, because the chance that a therapy with an actual response rate of 20% results in no objective response in 14 patients is  $<5\%$ . Kaplan–Meier plots of time to progression and overall survival were constructed. The time to progression lasted from the start of chemotherapy until the first observation of tumor progression and the overall survival time ended at the date of death or of last follow-up. The cut-off point for survival analysis was November 2004. All data were analysed using the statistical program SPSS for Windows version 9.0.

## results

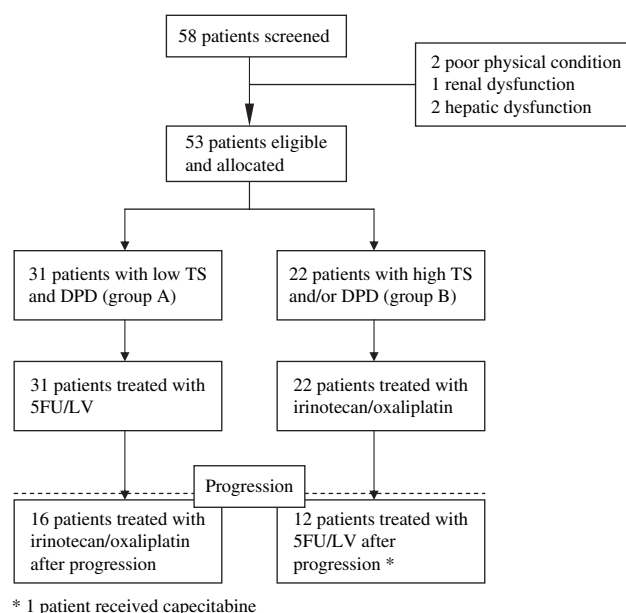
This prospective, single-center parallel phase II study accrued patients between February 2000 and May 2003. A total of 58 patients with ACRC were screened and registered, and

underwent a biopsy of metastatic tumor tissue for analysis of expression of TS and DPD (Figure 1). Five patients were considered ineligible at the time of treatment allocation because of poor physical condition ( $n = 2$ ), or hepatic or renal dysfunction ( $n = 3$ ). Based on TS and DPD levels, 31 out of 53 entered patients were assigned to treatment with 5-FU/LV, while 22 were assigned to the irinotecan/oxaliplatin combination. All entered patients were eligible for evaluation of response and toxicity, except one patient in treatment group B, who rapidly progressed and went off-study after start of therapy not being evaluable for response or toxicity. Patient characteristics are reported in Table 1.

The site of biopsy was the liver in 46 patients, a lymph node in three patients, a pelvic mass in three patients and a peritoneal tumor deposit in one patient. The TS/ $\beta$ -actin ratio ranged from 0.09 to  $16.84 \times 10^{-3}$  (median 1.06). The DPD/ $\beta$ -actin ratio had a wider range of expression, from not detectable to  $598 \times 10^{-3}$  (median 2.16). The majority of patients (45 of 53; 85%) had a low level of TS (Figure 2; Table 2). Thirty-one out of 53 patients (58%) had low levels of both TS and DPD, and were allocated to receive treatment with 5-FU/LV.

### responses

No complete responses were observed in either treatment group. Partial responses were seen in 11 out of 31 patients receiving 5-FU/LV [response rate by intention to treat 35%; 95% confidence interval (CI) 19% to 54%] (Table 3). As compared with a historical response rate of 23%, this study showed a trend towards an improved response rate ( $P = 0.10$ ,  $\chi^2$ -test). Disease control, including partial responses, minor responses and stable diseases lasting longer than 6 months, was observed in 22 out of 31 patients (71%; 95% CI 52% to 86%). Only four out of 22 patients treated with irinotecan/oxaliplatin combination regimen obtained a partial response (response rate by intention to treat 18%; 95% CI 5% to 40%),



**Figure 1.** Trial design. TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase; 5-FU, 5-fluorouracil; LV, leucovorin.

**Table 1.** Patient characteristics

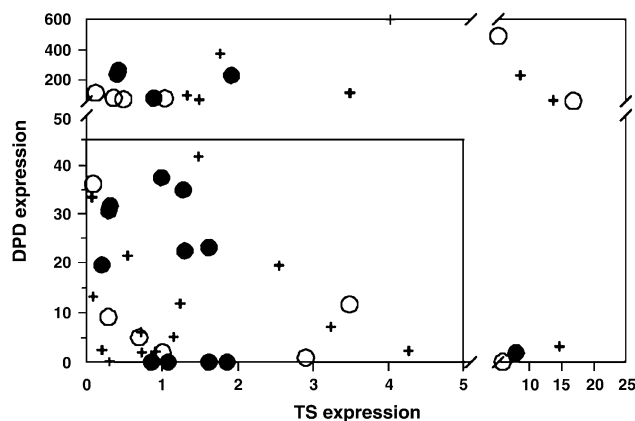
	Group A	Group B
Eligible	31	22
Evaluable		
For toxicity	31	21
For response	31	21
Gender		
Male	21	11
Female	10	11
Age (years)		
Median	56	59
Range	33–71	45–74
WHO performance status		
0–1	30	20
2	1	2
Site of primary tumor		
Colon	12	11
Rectosigmoid	19	11
Metastatic sites		
Liver	29	20
Lung	8	6
Lymph nodes	4	7
Peritoneum	4	3
Other	2	1

with disease control being achieved in 11 patients (50%; 95% CI 28% to 72%).

After progression on first-line chemotherapy, 28 patients crossed over to the alternative regimen as second-line chemotherapy, while 12 patients received further chemotherapy outside the study protocol. In group B, no responses were observed in 12 patients who received second-line 5-FU/LV at cross-over (one patient received capecitabine instead of 5-FU/LV) (0%; 95% CI 0% to 28%). Of note, four out of five patients with stable disease on second-line 5-FU/LV had low TS and high DPD, while four out of six patients with both low TS and DPD had progressive disease. One patient was not evaluable for response on second-line 5-FU/LV. Two patients refused further chemotherapy, and seven were too ill for further chemotherapy due to progressive disease. Cross-over to second-line oxaliplatin/irinotecan in group A resulted in a partial response in two out of 16 patients (13%; 95% CI 1% to 38%).

With a median follow-up of 30 months, the median time to progression was 6.4 months (95% CI 5.1–7.7) and the median overall survival time was 23.4 months (95% CI 9.4–37.4) in the 5-FU/LV group. For the irinotecan/oxaliplatin group, the median time to progression and the median overall survival were 13.2 months (95% CI 8.4–18) and 6.1 months (95% CI 1.5–10.7), respectively (Figure 3).

At progression and before the cross-over, 10 patients underwent a second biopsy of metastatic tissue to measure TS and DPD (Figure 4). DPD levels after chemotherapy decreased significantly in the irinotecan/oxaliplatin group, compared with the levels before start of chemotherapy. An increase in DPD expression was observed in the 5-FU/LV group, but this did not



**Figure 2.** Distribution of thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) expression in 53 advanced colorectal cancer patients receiving either 5-fluorouracil/leucovorin (5-FU/LV) or irinotecan/oxaliplatin treatment. Lines represent the cut-points for TS and DPD expression. Patients receiving 5-FU/LV are left of the TS cut-off lines and below the DPD cut-off; all other patients received irinotecan/oxaliplatin. Filled symbols represent patients responding to treatment and open symbols represent patients with progressive disease. Crosses represent patients with either a minor response or stable disease. These were considered as non-responders in the calculation of the response rate.

reach significance. No consistent changes could be detected in the levels of TS as compared with pretreatment levels.

**side-effects**

The median number of chemotherapy courses administered in first-line was 21 (range eight to 72) in group A and eight (range one to 23) in group B. Considering that three courses of weekly 5-FU/LV are comparable in duration to one course of 3-weekly irinotecan/oxaliplatin, the median duration of chemotherapy was similar in both groups. The actually delivered dose per unit of time was more than 90% of the planned dose per time for both regimens.

Observed severe side-effects of both first-line regimens are depicted in Table 4. There were no toxic deaths, and only one patient in each treatment group was admitted to the hospital because of neutropenic fever. One patient treated with 5-FU/LV suffered from a lung embolism and two patients developed severe diarrhea. Two patients treated with irinotecan/oxaliplatin required hospitalization due to dehydration; in addition, one patient experienced severe fatigue and one patient developed severe diarrhea. In both groups, only one patient discontinued chemotherapy because of toxicity (diarrhea).

**discussion**

This is the first prospective study using the expression levels of TS and DPD in metastatic lesions to tailor the choice of first-line chemotherapy with 5-FU/LV in patients with advanced colorectal cancer. Although several retrospective studies have suggested the value of the expression of TS in predicting the response to 5-FU, this has never been evaluated prospectively. Although the response rate of 35% that we obtained appears

**Table 2.** Thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) expression levels, according to predefined thresholds (see Patients and methods)

	TS low (%)	TS high (%)	Any TS (%)
DPD low	31 (58)	4 (8)	35 (66)
DPD high	14 (26)	4 (8)	18 (34)
Any DPD	45 (85)	8 (15)	53 (100)

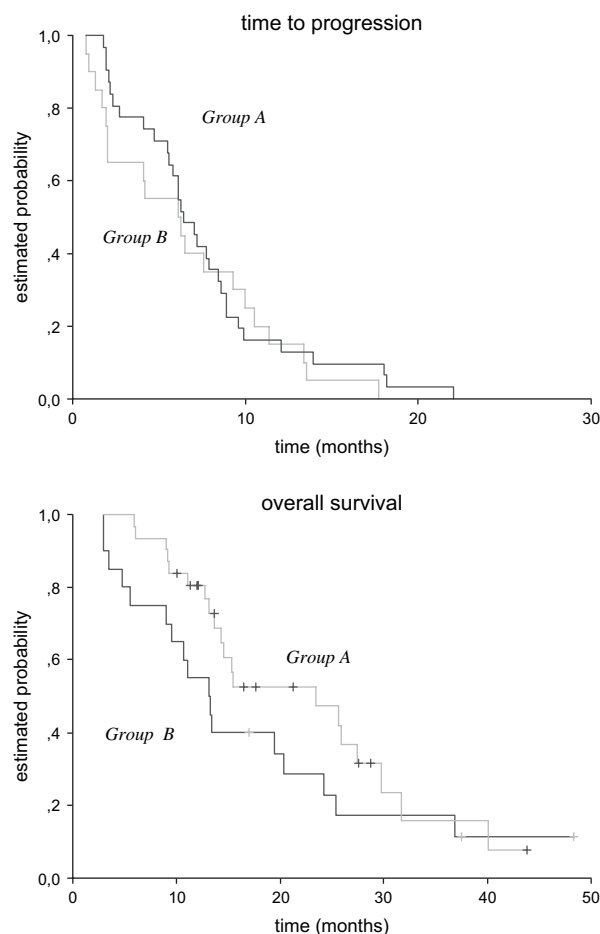
**Table 3.** Tumor response to first-line treatment

Response	5-FU/LV (n = 31)		Irinotecan/oxaliplatin (n = 22)		P value
	n	%	n	%	
Partial response	11	35	4	18	0.20
Stable disease	14	45	10	45	
Progressive disease	6	19	7	32	
Not evaluable	0	0	1	5	

5-FU, 5-fluorouracil; LV, leucovorin.

higher than the expected response rate in unselected patients, it was lower than what we had hoped for. The recently updated meta-analysis on 5-FU and LV reported a response rate of 21% (95% CI 19% to 23%) in unselected patients [24]. However, the criteria for response assessment used in these relatively old studies may have been less strict than used nowadays and the actual response rate of 5-FU/LV in unselected patients may be even <20%, as reported in more recent trials using an identical schedule of 5-FU/LV as in our study [25]. It may well be that a larger sample size could have detected a more convincing improvement in response rate in group A in our study. Nevertheless, our response rate did not come near to the very high response rates (>70%) reported in retrospective studies in patients with low TS treated with 5-FU. It is very unlikely that our relatively low response rate is due to the schedule of bolus infusion of 5-FU, because Aschele et al. [26] reported high response rates of 66% and 50% in patients with a low level of TS, treated with continuous infusion or bolus 5-FU, respectively.

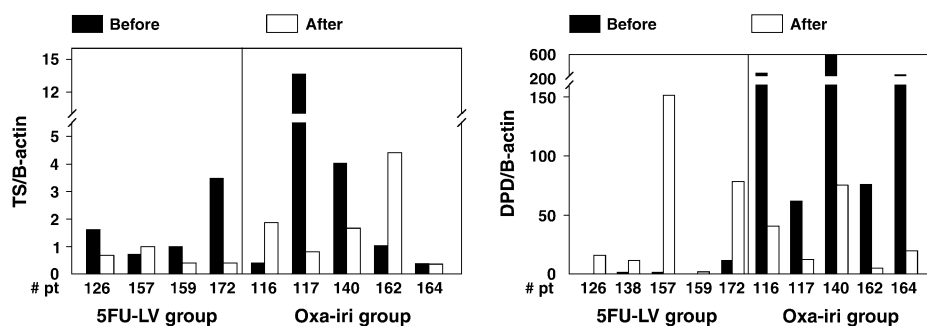
Interestingly, the group of patients who had low TS and low DPD not only had a higher response rate to 5-FU/LV as compared with the combination of irinotecan/oxaliplatin, but they also had a better prognosis in terms of overall survival. It is unlikely that the longer survival was due to a higher response rate in this group, as the time to progression was the same in the two groups. Apart from being a predictor of response to 5-FU, TS expression is known to be a prognostic factor in ACRC. In a recent review, Popat et al. [27] analyzed 20 retrospective studies which assessed the value of TS expression as prognostic factor in colorectal cancer. In general, low TS expression was associated with a better survival than high TS, not only in patients treated with adjuvant or palliative chemotherapy including 5-FU, but also in patients who did not receive any chemotherapy. In this analysis important methodological issues were raised. Of the 20 studies identified in the review



**Figure 3.** Kaplan–Meier curves for time to progression and overall survival.

[27], six studies quantified TS expression by RT–PCR in relation to the expression of  $\beta$ -actin or GAPDH. The threshold level of TS was defined as the one most likely to predict disease outcome and varied from study to study. Similarly, the cut-off values used in immunohistochemistry studies or studies employing biochemical assays varied greatly.

This leads us to the question of the optimal assay for these determinations [28]. Immunohistochemistry has major advantages over RT–PCR, because it is suitable for widely available paraffin-embedded archival material, allowing assessment of the quality and content of the sample, but has limitations related to the difficulty of quantitation, partly due to variable fixation procedures. Although the biochemical assays measuring the enzymatic activity are probably the most relevant assays, because they measure function instead of gene expression they require relatively large amounts of fresh tissue. We elected to use fresh tumor material obtained from a metastatic site before start of chemotherapy, in order to be able to perform RT–PCR, because this technique was the one that appeared to have given the most consistent results. The RT–PCR technique, as used in our study, provides a highly sensitive and quantitative assay. The limitations of this method, however, are the need for fresh tumor samples and the lack of a control of the material that is processed. In our study, we attempted to evaluate the histology of the slices obtained from the peripheral areas of



**Figure 4.** Thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) expression before (filled bars) and after (open bars) treatment with either 5-fluorouracil/leucovorin (5-FU/LV) (left groups of bars) or irinotecan/oxaliplatin (right groups of bars). The vertical line separates these groups. The decrease in DPD after irinotecan/oxaliplatin (oxa-iri) treatment was significant ( $P = 0.03$ ); the increase in DPD expression after 5-FU/LV was just not significant. The numbers indicate the number of the patients (#pt).

**Table 4.** Grade 3–4 toxicity in first line (number of patients)

Toxicity	5-FU/LV ( $n = 31$ )	Irinotecan/oxaliplatin ( $n = 22$ )
Anaemia	0	0
Leukopenia	2	5
Thrombocytopenia	0	0
Neutropenic fever	1	1
Nausea	1	3
Vomiting	0	2
Diarrhea	8	2
Fatigue	3	5
Neuropathy	0	2

5-FU, 5-fluorouracil; LV, leucovorin.

the core tissue that was used for PCR analysis. Although for the majority of the samples tumor material was confirmed to be present, the assessment was not possible in all samples and the percentage of viable tumor cells varied greatly from sample to sample. These considerations underscore the difficulty of this type of prospective studies using fresh tumor material.

Retrospective studies using TS expression have failed to fully predict response to 5-FU in all studies [5–7]; therefore, other factors accounting for sensitivity or resistance to 5-FU may play important additional roles. One of these is the expression of DPD, an enzyme involved in the catabolism of 5-FU. DPD expression has indeed been correlated with clinical outcome in a few studies [8, 13, 14, 29]. One study in ARCR patients reported a striking difference in response rate between patients whose tumors expressed low DPD (50%) as compared with those with high DPD (0%) [8]. In our study, most responders had low TS, whereas expression of DPD was less predictive (Figure 2). If we had omitted DPD expression as a selection marker for 5-FU/LV chemotherapy in this study, the number of patients in group A would have increased from 31 to 45 (see Table 2), with a response rate somewhere between 24% (11/45) and 56% (11 + 14/45) at most. Other molecular markers, such as thymidine phosphorylase, p53, Bcl-2, microsatellite instability and retention of heterozygosity of chromosome 17p or 18q sites have been correlated with 5-FU sensitivity [30, 31], although a mechanistic relation was not always clear in these studies.

In our study the response rate and overall survival with irinotecan/oxaliplatin (18%) in patients with a high level of TS and/or DPD was clearly lower than in other studies employing this combination in unselected patients [23, 32]. The high level of TS in our group B may also have been a marker of poor prognosis, accounting for the worse outcome in this group irrespective of the allocated chemotherapy regimen [27]. Considering the high level of TS and/or DPD in group B, it was not unexpected that none of the 12 patients responded to second-line 5-FU/LV. Owing to this result, and as the study was closed after entering 31 evaluable patients in group A, we refrained from completing second-line 5-FU/LV in group B in further patients.

Since this study was initiated, major improvements in the systemic treatment of ACRC have rapidly occurred, and 5-FU/LV is no longer a standard chemotherapy regimen for the majority of fit patients with ACRC; nonetheless, 5-FU-based combination regimens still represent the standard systemic treatment for this disease. It would be worthwhile to investigate the predictive value of TS, DPD and other markers in nowadays commonly used 5-FU combination regimens. In a retrospective analysis, TS levels in metastatic lesions failed to predict clinical outcome in patients with ACRC treated with a combination of bolus 5-FU with MTX [26]. However, one study reported a better survival in patients whose tumors had a low level of TS and excision cross-complementing gene 1 (*ERCC1*) expression in 5-FU-pretreated ACRC patients receiving oxaliplatin and 5-FU [17].

An important addition to the systemic therapy of ACRC is the recent introduction of two targeted agents, cetuximab and bevacizumab, which respectively target epidermal growth factor receptor (EGFR) and vascular endothelial growth factor [33, 34]. The selection of patients for regimens including these agents poses formidable challenges, as with these targeted agents also, no selection is performed. Patients selected for cetuximab treatment suffice to have EGFR-positive samples (one positive tumor cell) and no selection is performed for patients being treated with bevacizumab. Although the combination of multiple agents can increase response rate and improve overall survival, this is at the cost of important toxicities. Furthermore, the recently introduced biological agents, in addition to chemotherapy, represent a significant challenge to the financial

management of cancer patients' care. Adequate markers of sensitivity may allow to reduce the number of drugs needed to treat patients, and in doing so, importantly limit side-effects and costs.

Only recently, the issue of clinical trial design for predictive markers in cancer studies has received attention in medical literature [35, 36]. Sargent et al. [35] extensively described two classes of clinical trial designs that allow the assessment of the clinical usefulness of a predictive marker. At the time of writing our study protocol, much of this information was lacking. In retrospect, our study design has been hampered by the fact that the study population is split into two groups with different marker and different treatment status, which makes any comparison across the groups impossible. In conclusion, our prospective selection of 5-FU/LV chemotherapy based on low TS and DPD expression in patients with ACRC did not confirm the high response rates reported in retrospective studies. Before assessing the clinical utility of a predictive marker in a randomized trial, future studies should focus on prospective validation of the assay in a large and well defined population.

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