Thymidylate Synthase and Dihydropyrimidine Dehydrogenase mRNA Expression Levels: Predictors for Survival in Colorectal Cancer Patients Receiving Adjuvant 5-Fluorouracil


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ABSTRACT

Purpose: Despite adjuvant 5-fluorouracil (5-FU) therapy, ~30% of patients with International Union Against Cancer stage II and III colorectal cancer develop recurrence. In this study, we determined the prognostic value of thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) expression in colorectal cancer patients treated with adjuvant 5-FU.

Experimental Design: A real-time reverse transcription-PCR technique for quantitation of relative gene expression from paraffin-embedded specimen was established first. In a second step, archival paraffin-embedded primary tumor tissue of 309 patients who participated in adjuvant colorectal cancer trials was analyzed for TS and DPD mRNA expression.

Results: TS mRNA expression determined by real-time reverse transcription-PCR correlated with TS protein levels determined by TS immunoblot and immunohistochemistry in cultured colon cancer cell lines and paraffin-embedded primary tumor tissue. TS mRNA levels in fresh-frozen tissue also correlated with TS mRNA levels in corresponding paraffin sections. Among the patients receiving adjuvant 5-FU therapy, those with high TS survived longer than those with low TS, and in each TS subgroup, the ones with low DPD survived longer than the ones with high DPD levels. Multiple Cox regression analysis showed that besides tumor stage (P = 0.010), only the combination of TS and DPD expression turned out to be an independent prognostic factor for survival (P = 0.030).

Conclusions: This suggests that TS and DPD quantitation may be helpful to evaluate prognosis of patients receiving adjuvant 5-FU and that patients with high TS and low DPD may benefit from adjuvant 5-FU chemotherapy.

INTRODUCTION

CRC remains a significant health problem in the western world (1). Advances in early detection and surgical therapy have contributed to constantly decreasing death rates (2). Despite complete clearance (R0 resection) of the primary tumor, ~50% of the patients with R0 resection develop local or distant recurrence presumably attributable to disseminated micrometastases present at the time of surgery (1). The recurrence rate is significantly reduced using adjuvant treatment with 5-FU in UICC stage III colon cancer and UICC stage II and III rectal cancer in combination with local irradiation (3). Nevertheless, ~30% of the patients receiving adjuvant treatment still develop tumor recurrence within 5 years. Thus, it would be highly desirable to identify most patients who are likely to benefit from adjuvant 5-FU treatment before the initiation of such therapy.

5-FU irreversibly blocks TS after conversion to its active metabolite 5-fluorodl-UMP (4). TS catalyzes the methylation of dUMP to dTMP with 5,10-methylenetetrahydrofolate as a co-factor and provides the sole intracellular de novo source of thymidylate, one of the rate-limiting steps in DNA synthesis (5). Several preclinical and clinical studies demonstrated that high TS levels correlate with 5-FU resistance in various malignancies (6, 7). TS has also been identified as an independent prognostic marker for survival in R0-resected rectal cancer, suggesting that TS may also function as a biomarker for the malignant potential of each tumor independent of 5-FU treatment (8, 9).

Another mechanism contributing to 5-FU resistance is its inactivation in the tumor cells. DPD catalyzes the first and
rate-limiting step of the pyrimidine catabolic pathway (10). DPD activity has been identified as a critical determinant of the metabolism and pharmacology of 5-FU (10). Several studies demonstrated that a high DPD level was also associated with 5-FU resistance (11). Recently, Salonga et al. (12) reported that only CRCs with low DPD and low TS mRNA levels responded to 5-FU treatment in advanced unresectable disease.

The aim of this study was to analyze the importance of TS and DPD mRNA expression in the primary tumor for the survival of patients receiving adjuvant 5-FU therapy. Therefore, we established a mRNA quantitation technique using real-time RTPCR and determined TS and DPD levels from paraffin-embedded primary CRC tissue sections. We now report that patients with high TS and low DPD levels had a significantly longer survival than patients with low TS and high DPD levels.

MATERIALS AND METHODS

Materials. The following were purchased: enhanced chemiluminescence substrate, random hexamers, and Escherichia coli tRNA from Amersham Pharmacia (Freiburg, Germany); Taqman Core Reagent Kit and TaqMan probes from Applied Biosystems (Weterstida, Germany); HT29 cells from the American Type Culture Collection (Manassas, VA); biotinylated secondary antibody, StreptABCComplex, and normal serum from Dako (Glostrup, Denmark); FBS, antibiotics, media, Trizol reagent, Tris buffer, EDTA, distilled water, water-saturated phenol, MMLV reverse transcriptase, RNaseOut, primers, and a 1-kb plus DNA standard from Invitrogen GmbH (Karlsruhe, Germany); BioMax ML from Kodak (Rochester, NY); Immobilon-P nitrocellulose membranes from Millipore (Bedford, MA); mouse monoclonal TS antibody (clone DCS-6) from NeoMarkers, Inc. (Union City, CA); guanidine isothiocyanate from Roth GmbH (Karlsruhe, Germany); and N-lauroyl-sarcosine, β-mercaptoethanol and all other chemical from Sigma (Deisenhofen, Germany).

Cell Culture and Immunoblotting. HT29 and NM66484 human colon cancer cells were grown in Ham’s F-12 medium supplemented with 10% FBS, penicillin G (100 units/ml), and streptomycin (100 μg/ml). Cells were maintained at 37°C in humidified air with 5% CO₂. For immunoblot analysis of TS protein, pulverized placenta or exponentially growing (pT 3 50 mg/day for 3 days every 14 days) for a total of 1 year (arm – 60

Clinical evaluation was performed in a prospective manner in each of the participating centers. Data were readily transferred to the study center in Ulm after each evaluation point. The quality of the data management was monitored by an independent study monitor (14).

Tumor Tissue. We tried to obtain paraffin-embedded primary tumor specimens from all patients who entered the FOGT-1 or FOGT-2 trial between 1992 and 2000 in five major FOGT centers (Ulm, Magdeburg, Rotenburg, Gießen, and Gera). Eventually, paraffin blocks of 429 patients of these centers were available for RNA extraction. In some cases, a small piece of the primary tumor was additionally frozen in liquid nitrogen immediately after resection and stored at −80°C for TS mRNA quantification.

Immunohistochemistry. Paraffin-embedded 5-μm tissue sections were stained using the streptavidin-peroxidase technique as described previously (15). After deparaffinization and blocking endogenous peroxidase activity, the sections were incubated for 20 min at 23°C with 1% normal horse serum and for 20 h at 4°C with the mouse monoclonal antibody against TS (4 μg/ml) that was used for immunoblotting. This antibody has been shown to specifically recognize TS (16, 17). Bound antibodies were detected with biotinylated horse universal secondary antibodies and streptavidin-peroxidase complex, using diaminobenzidine tetrahydrochloride as the substrate. Sections were counterstained with Mayer’s hematoxylin. Omission of primary antibodies did not yield any immunoreactivity. The scoring of TS staining was performed as described previously (8, 9).

Total RNA extraction from Fresh-frozen Tissue and Cell Lines. Total RNA from pulverized fresh-frozen primary tumor tissue and cell lines was extracted using Trizol reagent and transcribed into cDNA using MMLV reverse transcriptase according to the manufacturer’s instructions as described in detail below.

Total RNA Extraction from Paraffin-embedded Tissue. Three 10-μm sections were prepared from primary tumor blocks that contained at least 50% tumor cells and directly transferred into a microcentrifuge tube. RNA was extracted according to a previously described method with minor modifications (18). Briefly, 1 ml of xylene was added to each tube followed by an incubation in a shaking water bath for 5 min at 50°C. After centrifugation for 10 min at 14,000 rpm, the supernatant was discarded, and the washing step was repeated twice. The deparaffinized sections were rehydrated in xylene:ethanol:water at the following ratios (95:95:5, 95:90:10, 95:80:20; 95:75:25, and 95:70:30) at 50°C for 5 min each. After each step, the rehydration medium was removed after centrifugation for 10 min at 14,000 rpm. After discarding the last supernatant, the pelletted sections were resolved in 70% ethanol and dried in a speed-vac for 15-min centrifugation and removal of the supernatant. Then 500 μl of buffer [4 μM guanidine isothiocyanate, 0.5% sarcosyl, 25 mM sodium citrate, 100 mM 2-mercaptoethanol (pH 7.0)]
were added to the dried tissue and incubated at 4°C for 30 min. After that, 1 μg of E. coli rRNA and 1 mM of EDTA were added, and the remaining tissue was homogenized mechanically. For RNA demodification, homogenates were shaken at 85°C for up to 1 h (19). RNA was extracted from homogenates according to Chomczynski and Sacchi (20) by addition of 50 μl of 2 M sodium acetate (pH 4), 500 μl of water-saturated phenol, and 100 μl of chloroform-isoamyl mixture (49:1). RNA was recovered from the water phase by isopropanol precipitation and transcribed into cDNA at 39°C for 45 min using 400 units of MMLV reverse transcriptase, 1/1000 first strand buffer, 0.04 μg/μl random hexamers, 10 mM DTT, and 1 mM deoxyribonucleoside triphosphate.

**TS and DPD mRNA Quantitation.** A real-time fluorescence detection method (TaqMan PCR) based on the recently described procedure (21) was used. After RNA isolation and cDNA generation, real-time fluorescence PCR was carried out for β-actin and the genes of interest. TaqMan PCR results in an increase of fluorescent signal which correlates to the concentration of PCR amplified. When the fluorescent signal intensity is plotted versus PCR cycle number PCR amplification translates into a logarithmic curve, which exceeds the nonspecific background after several PCR rounds. The cycle at which the background is exceeded is defined as threshold cycle (21).

The use of β-actin as a reference gene avoids the need of RNA concentration measurement, which could be a major source of error for analysis. The β-actin real-time PCR analysis was also used to estimate the amount of extracted mRNA. The rise of the β-actin signal after cycle 33 using the described conditions indicated an insufficient amount of mRNA present for the subsequent TS and DPD quantitation. This was the case in 120 of the available 429 samples. Thus, the subsequent real-time RT-PCR analysis for TS and DPD was restricted to 309 specimens. Relative TS gene expression values using β-actin as denominator closely correlates with the TS and DPD protein content (17, 22). Thus, relative gene expression of TS and DPD was determined based on the threshold cycles of the gene of interest and of the internal standard β-actin, respectively (21). Positive controls (samples of known value) and negative controls (samples without cDNA templates) were performed in parallel for each PCR experiment to assure equivalent assay conditions.
Primer sequences were based on the GenBank accession nos. AB004047 (β-actin), U20938 (DPD), and X02308 (TS). PCR conditions used have been described previously (21).

**Statistical Analysis.** For descriptive statistical analysis, absolute and relative frequencies and median, minimum, and maximum were calculated. To describe the relationship between two continuous variables, Spearman’s rank correlation coefficient was computed.

Disease-specific survival time was defined as the number of days from start of adjuvant treatment to tumor-related death (failure), to death from other reasons (censored), or until data evaluation for the patients being alive (censored). Recurrence-free survival time was computed as the number of days from start of adjuvant treatment to the time of recurrence at any site (failure), to tumor related death (failure), to death from other reasons (censored), or until last follow-up for the patients being alive (censored).

Survival data were analyzed by the method of Kaplan and Meier. The log-rank test was used to define optimal TS and DPD cutoff values. Thus, log-rank testing was performed for TS and DPD with cutoffs ranging from 0.1 to 1.5 in steps of 0.1. As final cutoff, the value with the lowest P in the log-rank test was chosen for TS and DPD, respectively. Subsequently, multiple Cox regression analysis was performed to simultaneously assess the influence of several prognostic factors on survival and to select important prognostic factors. Backward elimination with a selection level of 5% was used for variable selection. For important prognostic factors, the hazard ratio with corresponding 95% CI and the P were computed.

Statistical analysis was performed using SAS version 6.12 (SAS Institute, Inc., Cary, NC).

**RESULTS**

**Establishment of Relative TS Quantitation from Paraffin-Embedded Tissue.** In a first step, relative TS mRNA quantitation was established as described previously (21). TS mRNA levels in HT29 and NMG6486 human colon cancer cell lines and in human placenta correlated with the corresponding TS protein levels as determined by immunoblot analysis (Fig. 1).

TS mRNA levels of fresh-frozen tumor specimens obtained during surgery (n = 28) and of corresponding paraffin-embedded material obtained after complete pathological examination were determined next. This analysis revealed a close correlation between TS mRNA levels using paraffin-embedded and fresh-frozen tissue (r = 0.70; Fig. 2A). In general, TS mRNA levels were ~3-fold lower in paraffin-embedded compared with the corresponding fresh-frozen tissues. Material of only one patient revealed a relatively low TS level (0.73) in the paraffin-embedded material but a relatively high TS level (6.93) in the fresh-frozen material (Fig. 2A).

Next, we compared TS mRNA levels from paraffin-
embedded tumor specimens \( n = 33 \) determined by RT-PCR with TS protein expression of corresponding sections determined by immunohistochemistry. TS immunohistochemical analysis using the specific monoclonal TS antibody TS 106 \( \text{(8, 9, 17)} \) revealed a close association of TS protein levels with the corresponding TS mRNA levels determined by RT-PCR (Fig. 2B). In three samples with TS mRNA levels > 1.0, the immunohistological score in the cancer cells was 1, and in one sample with a relatively low TS mRNA level (0.43), the score was 2. In Fig. 3, examples of low (Fig. 3A) and high (Fig. 3B) TS staining intensities are shown.

**Clinical and Pathological Parameters of the Study Population.** After establishment of relative mRNA quantitation from paraffin-embedded tumor tissue, this method was used to determine relative TS and DPD mRNA expression levels in 309 specimen of CRC patients enrolled in two German adjuvant multicenter trials. Fourteen of the 309 patients had to be excluded from the statistical analysis because these patients either never received adjuvant 5-FU therapy \( n = 5 \) or were registered for the studies despite a nonmatching tumor stage \( n = 3 \) or tumor localization \( n = 3 \). Quantitative RT-PCR analysis of the paraffin-embedded sections was carried out in quadruplicate with a mean SD of 15% (median, 13%; range, 1–89) and 22% (median, 18%; range, 1–97%) for TS and DPD, respectively. SDs > 40% were observed for TS and DPD in 2.7 and 11% of all specimens, respectively. A high SD was observed in samples with very low amounts of mRNA available for the RT-PCR analysis, indicated by a rise of the real-time PCR signal of the internal standard \( \beta \)-actin after cycle 30 and with very low TS or DPD mRNA levels. The quantitative PCR analysis revealed large variations in the mRNA expression levels of TS and DPD among the 295 primary CRCs. The median TS level was 0.75 (range, 0.12–7.21) and the median DPD level was 0.28 (range, 0.00–1.62). The characteristics and the treatment of these patients and the overall FO GT-1 and FO GT-2 study population are listed in Table 1. The median follow-up was 38.7 months (range, 4.4–98.3 months).

**Association of TS and DPD Levels with Disease-specific Survival.** Kaplan-Meier curves for age showed no association with disease-specific survival, whereas sex and tumor localization were marginally associated with survival. Women as well as patients with rectal cancer (because of lower UICC stage) tended to survive longer. Because of the low number of cases or events in some subgroups, an analysis of grading and invasion depth (T) was not performed (Table 1). Nodal stage \( (N_0 \text{ versus } N_1 \text{ versus } N_2) \) and tumor stage (Fig. 4A) were strongly associated with disease-specific survival. Interestingly, patients with higher TS levels turned out to survive longer than patients with lower TS levels. The difference in survival among the subgroups was most obvious at a TS cutoff level of 0.6 \( (P = 0.032; \text{Fig. } 4B) \). Also patients with lower DPD levels tended to survive longer. The impact of the DPD level on survival was most obvious at a cutoff DPD level of 0.4 \( (P = 0.229; \text{Fig. } 4C) \). Using a cutoff TS level of 0.6 and a DPD cutoff level of 0.4 resulted in four subgroups for the combination of TS and DPD. Patients with a high TS and a low DPD level had the best disease-specific survival from start of adjuvant treatment to tumor related death, whereas patients with a low TS and a high...
Table 2 Kaplan-Meier estimates for survival rates depending on TS and DPD mRNA levels

<table>
<thead>
<tr>
<th>TS and DPD levels(^a)</th>
<th>No.</th>
<th>In %</th>
<th>CL</th>
<th>4-year disease-specific survival rate</th>
<th>4-year recurrence-free survival rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS &gt; 0.6 and DPD ≤ 0.4</td>
<td>121</td>
<td>84.1</td>
<td>[75.2;92.9]</td>
<td></td>
<td>[61.2;50.7;118.1]</td>
</tr>
<tr>
<td>TS &gt; 0.6 and DPD &gt; 0.4</td>
<td>76</td>
<td>74.8</td>
<td>[62.9;86.8]</td>
<td></td>
<td>[62.5;49.9;75.1]</td>
</tr>
<tr>
<td>TS ≤ 0.6 and DPD ≤ 0.4</td>
<td>79</td>
<td>65.2</td>
<td>[50.7;79.8]</td>
<td></td>
<td>[57.3;43.8;87.6]</td>
</tr>
<tr>
<td>TS ≤ 0.6 and DPD &gt; 0.4</td>
<td>19</td>
<td>53.8</td>
<td>[25.6;82.1]</td>
<td></td>
<td>[29.0;3.0;55.7]</td>
</tr>
</tbody>
</table>

\(^a\) TS and DPD mRNA levels determined from paraffin-embedded tissue sections.

Table 3 Multiple Cox regression for survival in 295 patients receiving adjuvant 5-FU chemotherapy

<table>
<thead>
<tr>
<th>Tumor stage</th>
<th>No. of patients</th>
<th>Risk ratio</th>
<th>CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>UICC II</td>
<td>44</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UICC III</td>
<td>251</td>
<td>4.6</td>
<td>(1.4;14.8)</td>
<td>0.010</td>
</tr>
</tbody>
</table>

Association of TS and DPD Levels with Recurrence-free Survival. Kaplan-Meier curves for age, sex, and localization showed no association with recurrence-free survival. However, patients with lower nodal stage (data not shown) and lower tumor stage (Fig. 5A) had a longer recurrence-free survival. Also, patients with high TS (>0.6) tended to longer disease-free survival (Fig. 5B), whereas the difference in recurrence-free survival was marginal for patients with low DPD (≤0.4) and high DPD (>0.4) levels (Fig. 5C). Comparison of the recurrence-free survival in the TS-DPD subgroups showed that in contrast to the other subgroups, the rate of tumor recurrence was highest in patients with a low TS and a high DPD level (Fig. 5D, Table 2).

Multiple regression with backward elimination for the selection of the prognostic factors, including sex, localization, therapy arm, stage, and the combination of TS and DPD, failed to identify independent important prognostic factors for tumor recurrence.

DISCUSSION

TS and DPD play key roles in 5-FU resistance (7, 10). High intratumoral TS levels are believed to confer 5-FU resistance because of inefficient TS inhibition (7). In tumors with high intratumoral DPD levels, 5-FU seems to be degraded to a large extent before its activation (10). Therefore, TS inhibition may be also inefficient when DPD expression is high. In case of advanced unresectable CRC and other advanced gastrointestinal malignancies, numerous studies have demonstrated the predictive relevance of intratumoral TS and DPD levels for the success of palliative 5-FU treatment and the prognosis of these patients (6, 12, 23–27). However, the role of intratumoral TS and DPD expression for the outcome of adjuvant 5-FU treatment after R0 resection in patients with CRC is still unknown.

To retrospectively examine the prognostic relevance of TS and DPD mRNA expression for disease-specific and recurrence-free survival in patients with stage II and III CRC receiving adjuvant 5-FU chemotherapy, we established a method that enabled us to quantify gene expression from paraffin-embedded tissues. Validation experiments revealed that the determination of gene expression levels with the new method closely correlated with gene expression levels found in fresh-frozen material by a previously published method (12, 21), although paraffin-embedded and frozen specimens were derived from different areas of the tumor. However, the gene expression levels measured from paraffin-embedded specimens were generally lower than the levels seen in corresponding fresh-frozen material. This was probably attributable to partial RNA degradation (data not shown) and modification of the RNA structure by formalin fixation (19). As previously reported (17), we were also able to show that TS mRNA levels correlated with TS protein levels determined by immunoblot and immunohistochemistry in the majority of the samples.

On the basis of this mRNA quantitation method, we demonstrated for the first time that the intratumoral TS mRNA level is a prognostic marker for disease-specific survival in CRC patients receiving adjuvant 5-FU chemotherapy. A high TS level was associated with longer survival in R0-resected CRC patients receiving adjuvant chemotherapy. The opposite correlation between TS and survival has been reported in CRC patients after R0 resection not receiving adjuvant chemotherapy (8, 9, 28, 29). In those studies, untreated patients with high intratumoral TS levels were found to have a poorer prognosis than patients with low TS levels. However, our observations are supported by a subgroup analysis of Johnston’s study and three recent studies of Takenoue et al. (28), Edler (8, 29), and Yamachika (30). Similar to our results, only patients with high TS tumors bene-
fited from adjuvant chemotherapy consisting of MOF or oral or systemic 5-FU, respectively. Thus, patients with tumors expressing low TS had no or only marginal benefits of adjuvant 5-FU-based chemotherapy both with respect to overall and recurrence-free survival (8, 28, 30). In the recently published trial of Edler et al. (29), patients with low TS levels who received adjuvant therapy even had a worse outcome than those who did not, suggesting that 5-FU-based therapy had a deleterious effect on the survival in the low TS population. Compared with surgery alone, adjuvant chemotherapy prolonged the survival of patients with high TS levels, whereas there was no such survival prolongation by adjuvant chemotherapy for patients with low TS levels. There also was an increase in the 5-year survival rate in patients with chemotherapy and high TS expression levels compared with patients with chemotherapy and low TS (8, 28). Our results, together with the subgroup analysis of the four cited studies (8, 28–30), indicate that the observed effect in our trial may be attributable to an increased efficiency of adjuvant, 5-FU-based chemotherapy in tumors with high TS expression and a deleterious effect of 5-FU-based treatment in tumors with low TS expression, suggesting that patients with high TS levels may profit from the presently used adjuvant chemotherapy regimens.

The finding that high TS mRNA level may predict for an increased efficiency of adjuvant, 5-FU-based chemotherapy on the first glance contrasts with reports of an inverse correlation of TS gene expression and response to 5-FU chemotherapy in advanced CRC. However, unlike treatment of advanced disease, the survival benefit of adjuvant chemotherapy is mainly attributed to the eradication of circulating cancer cells before they become established (1). The situation of circulating cells, however, is clearly different from the situation of an established tumor (local recurrence or metastasis) in many respects. Apart from possible differences in accessibility of the tumor for 5-FU in disseminated cells, high TS levels may thus render cells susceptible for 5-FU-induced cell death via other presently unknown mechanisms, being independent of TS inhibition (7, 31, 32).

In accordance with several studies that suggested that DPD is a marker for 5-FU response (12, 33, 34), we found that patients with low DPD levels tended to survive longer, although there was no strong association. However, the combination of the DPD expression levels and TS level highly correlated with survival. By using TS and DPD quantitation, we were able to identify a subgroup of patients with a high recurrence rate and a low disease-specific survival. These patients, who had tumors with low TS and high DPD levels did obviously not benefit from the adjuvant 5-FU chemotherapy because they had worst prognosis. In contrast, patients with tumors that expressed high TS and low DPD had by far the best prognosis. Therefore, in the future, combined TS and DPD mRNA quantitation may be helpful to predict the prognosis of patients receiving adjuvant 5-FU.

In view of the fact that ~50% of the patients will never develop recurrence after resection even without adjuvant chemotherapy and that ~30% do recur regardless of adjuvant therapy, only 20% obviously profit from the present adjuvant treatment (3). Therefore, it would be highly desirable to identify patients that are at risk to develop recurrence to focus treatment on them and to avoid therapy for patients that will never recur. As outlined above, TS and DPD quantitation may provide a tool.
to separate patients who are likely to benefit from the present adjuvant, 5-FU-based chemotherapy (high TS, low DPD) from those who are unlikely to benefit (low TS, high DPD). However, it is too early to conclude from our results that the 5-FU treatment currently used should be targeted preferably to the high TS and low DPD group. Therefore, it seems to be important to conduct randomized controlled studies in R0-resected CRC patients comparing the efficacy of adjuvant 5-FU with other potentially active regimens, e.g., irinotecan (35, 36), for patients with high and low TS and DPD levels. Such studies could eventually result in a recommendation about individualization and optimization of adjuvant chemotherapy of CRC.

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