Topoisomerase I expression correlates to response to neoadjuvant irinotecan-based chemoradiation in rectal cancer

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Biomarkers may help predict the efficacy of neoadjuvant chemoradiation in patients with rectal cancer. We hypothesized that the expression of topoisomerase I (Topo I) and thymidylate synthase (TS) may help predict the treatment response in patients undergoing irinotecan and capecitabine-based chemoradiation. Patients with rectal cancer (cT3/4Nx or Tx/N+) received neoadjuvant chemoradiotherapy within clinical studies with irinotecan and capecitabine. Samples of normal and tumour tissues were collected before the start of the treatment and during surgical resection. Topo I and TS were measured using real-time PCR. The results of gene expression levels were compared between responders (defined as ypT0–2 ypN0) and nonresponders (ypT3–4 or ypN1/2). A total of 38 patients were analysed, 18 of them were responders. The biopsies of the untreated tumour tissue of responding patients showed a significant higher expression of Topo I compared with nonresponding patients ($P=0.015$). Normal tissue did not show this difference ($P=0.126$). During chemoradiation, the Topo I expression in tumour tissue of responders decreased significantly. TS did not show any differences between responders and nonresponders before treatment, but a significant decrease in the tumour tissue of responders was noted at the end of the treatment. Our data suggest that Topo I expression in rectal tumour mucosa might serve as a predictor of response to the neoadjuvant irinotecan-based chemoradiation, and hence might be a factor contributing to the development of individualized treatment. *Anti-Cancer Drugs* **20:519–524**

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Introduction

Neoadjuvant combined modality treatment is used as a standard regime for locally advanced rectal cancer. This change of the treatment strategy is mainly based on the German rectal cancer trial demonstrating the superiority of this approach over postoperative treatment concerning local tumour control and adverse events [1]. Furthermore, only preoperative chemoradiation is able to downstage and downsize a tumour before surgical resection. In a subgroup analysis of the German rectal cancer trial, patients with good tumour regressions showed an improved disease-free survival [2,3]. Consequently, intensified chemoradiotherapy regimes are currently being evaluated to increase the rate of downstaging or downsizing [4]. Several trials have evaluated a combination of neoadjuvant irinotecan-based and 5-fluorouracil (5-FU)-based chemoradiation in patients with locally advanced rectal cancer [4–6]. In these trials, the tumour regression rates reported seem to be promising with approximately 40% of the patients exhibiting complete or near-complete tumour remissions [4,6]. However, reliable markers that could predict the likelihood of the response of an individual patient are warranted to perform a tailored therapy sparing unnecessary toxicity from non-responding patients.

Identification of predictive markers in pretreatment tissue biopsies allowing clinicians to predict tumour response to neoadjuvant chemoradiation remains a major challenge of rectal cancer research [7]. So far, little is known about biological and biochemical markers of colorectal tumour responsiveness and differential sensitivity to chemoradiation.

Capecitabine is an oral fluoropyrimidine that mimics the pharmacokinetics of continuous 5-FU infusion [8]. It is

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preferentially converted within the tumour tissue by a three-step enzymatic process. Thymidine phosphorylase, an enzyme that is preferentially expressed in tumour tissue, finally converts the prodrug into 5-FU. Thus, higher intratumoral 5-FU concentrations may be achieved [9].

Thymidylate synthase (TS) is a key enzyme in nucleotide biosynthesis, and is the main intracellular target of the active metabolite of 5-FU, fluorodeoxyuridylate, which forms a ternary complex with TS and 5,10-methylenetetrahydrofolate. This complex prevents methylation of dUMP to dTMP, the sole de-novo intracellular source of thymidylate [10]. Thus, 5-FU limits the DNA synthesis as a folate inhibitor.

Irinotecan, a semisynthetic derivative of campthotecin is a topoisomerase I (Topo I) inhibitor and was introduced several years ago in the treatment of metastatic colorectal cancer [11].

Topoisomerase I enzymatic reaction involves the binding of Topo I to DNA, the cleavage of one strand of DNA, the passage of the intact strand through Topo I DNA complex and the resealing of the cleaved strand, without modification of the DNA sequence [12]. Irinotecan leads to the conversion of single-strand breaks into irreversible double-strand breaks, resulting in cell death. Owing to its interference with DNA replication, irinotecan also showed radio-sensitizing properties in preclinical studies [13].

On the premise of limited or controversial data concerning TS and Topo I as predictors of treatment efficacy, we investigated tissues of patients with rectal cancer included in phase I and phase II studies on neoadjuvant treatment with irinotecan-based and capecitabine-based chemoradiotherapy [14–17]. We were mainly interested in the potential predictive value of Topo I and TS with respect to the histopathological downstaging in these patients. Besides this, we investigated the changes of mRNA expression levels of Topo I and TS in normal and rectal cancer tumour tissue under radiochemotherapy.

Patients and methods

Eligibility criteria

Patients with histologically confirmed, locally advanced nonmetastatic rectal adenocarcinoma (endorectal ultrasound stage cT3-4, any N and cT2, N+ distal rectum) were included in prospective clinical trials on intensified neoadjuvant chemoradiotherapy using weekly irinotecan (40–50 mg/m^2), daily capecitabine (500 mg/m^2 twice daily, days 1–38) and weekly cetuximab (initial dose 400 mg/m^2 day 1; then weekly 250 mg/m^2) as well as pelvic radiotherapy (45+5.4 Gy boost). The pretherapeutical staging was performed by rectal endosonography and MRI scan. Study protocols were reviewed and approved by the local institutional review board and the studies were conducted according to the Declaration of Helsinki. All patients provided written informed consent for the participation in the study as well as the collection of tumour and normal tissue. Toxicity data, dose modifications as well as preliminary results on the antitumor activity of the phase I study were published earlier [18].

Pretreatment evaluation

Before study admission, all patients underwent physical examination, biopsy with confirmation of adenocarcinoma, digital rectal examination, rigid rectoscopy, transrectal ultrasonography, rectal manometry, pelvic and abdominal computed tomographic scans, pelvic MRI, colonoscopy and chest radiographs. Ten biopsies of each normal and tumour tissue were taken at rigid rectoscopy and stored immediately in RNAlater (Qiagen, Hilden, Germany) at −80°C.

Surgery and histopathologic examination of resected specimen

Total mesorectal excision was performed in all the patients according to a standardized technique 4–6 weeks after the completion of the chemoradiation. After surgical resection of the rectum, samples of normal and tumour tissue were ascertained and stored immediately in RNAlater (Qiagen) in liquid nitrogen, followed by long-term storage at −80°C. No tumour samples were obtained in seven patients exhibiting a complete clinical remission. Two pathologists, blinded to the clinical data, performed the histopathological examinations.

RNA extraction

RNA extraction of all samples and reverse transcription to cDNA was performed within 3 months. The tissue samples were first homogenized by using an Ultra Turrax Tube Drive (Ika, Staufen, Germany). RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, California, USA) following the standard procedures. The extracted RNA was resuspended in 30 µl of RNAse/DNAse-free water and immediately cooled on ice and stored at −80°C after separating an amount of 5 µl for testing quantity and quality of the extracted RNA. The amount of extracted RNA was quantified by ultraviolet absorbance at 260 and 280 nm GeneQuant spectrophotometer (Amersham Pharmacia Biotech, Freiburg, Germany); integrity control was assessed using the Bioanalyzer 2100 (Agilent, Palo Alto, California, USA). Following the results of the Bioanalyzer 2100 (Agilent), high-quality RNA was extracted from each sample that was used for real-time polymerase-chain reaction (PCR). If signs of RNA degradation were observed its extraction was repeated.

Real-time polymerase-chain reaction

Purified RNA (500 ng) was then subjected to reverse transcription using random hexamer priming and MMLV reverse transcriptase (Invitrogen) following the manufacturer's protocol. Quantification standards were prepared

by the cloning of amplified cDNA fragments of Topo I or TS after reverse transcription of RNA extracted from the SW480 colon carcinoma cell line using the Expand High Fidelity Plus PCR system (Roche Diagnostics, Mannheim, Germany). Amplification reactions were undertaken for 32 cycles at 60°C annealing temperature. PCR products were cloned into the PCR2.1-TOPO vector (Invitrogen) and introduced into chemical competent *Escherichia coli* TOP10F′ according to the manufacturer's instructions. Plasmid DNA containing the desired construct was isolated using the Plasmid Maxi KitTM (Qiagen). The resulting plasmid was linearized by the restriction enzyme XbaI (Roche Diagnostics) at 37°C for 2 h followed by heat inactivation at 65°C for 20 min. Beta-glucuronidase (GUS) mRNA transcripts were measured as internal control using a standard plasmid (pME-2) [19]. Dilutions of the linearized plasmid were prepared in 10 mmol/l Tris–HCl pH 8.0; 1 mmol/l EDTA containing 20 µg/ml tRNA (Roche Diagnostics).

The transcripts were amplified with primers derived from Topo I exon 17 and exon 19 and TS between exon 4 and exon 6; hybridization probes were localized in exon 18 and exon 5, respectively.

The quantitative PCR reactions were performed using the universal LightCycler Mastermix containing buffer, dNTP, Taq polymerase and 4 mmol/l MgCl (LightCycler Faststart plus set hybridization probes; Roche Diagnostics). Each reaction was performed in a final volume of 20 µl using 2 µl cDNA or plasmid dilutions. The PCR mix contains 2 µl of Mastermix, 0.5 µmol/l of each primer, 0.25 µmol/l of 3′ and 5′ hybridization probes (TIB Molbiol, Berlin, Germany) and 1 U heat labile uracil DNA glycosylase (Roche Diagnostics) to prevent reamplification of carryover PCR products.

Amplification and detection were performed using the LightCycler instrument 1.5 (Roche Diagnostics). Cycler conditions were the following: 10 min denaturation at 95°C, 45 cycles of 10 s at 60°C annealing and 26 s at 72°C elongation. A 5 log series of plasmid dilutions of the standard plasmid of the respective gene was amplified within every PCR run.

Definition of tumour response

We used the histopathological downstaging as surrogate parameter of tumour response. A T-level downstaging of at least one T-level was considered to be a sign of response. Generally, only patients exhibiting ypT0–2 ypN0 were regarded as responders. The normalized RNA expression of Topo I and TS between the groups of responders and nonresponders was compared.

Statistical analysis

The statistical analysis was performed with the SPSS software (version 15.0, SPSS Inc., Chicago, Illinois, USA) using the nonparametric Mann–Whitney *U* test. Significance was set at a *P* value of less than 0.05.

Results

Tumour as well as normal tissue of 38 study participants was available for analysis. R_0 resection was performed in all but one of these patients. The assessment of tumour downstaging revealed 18 responders and 20 nonresponders along the ypT and ypN stages (Table 1).

Comparison of topoisomerase I in pretreatment biopsies

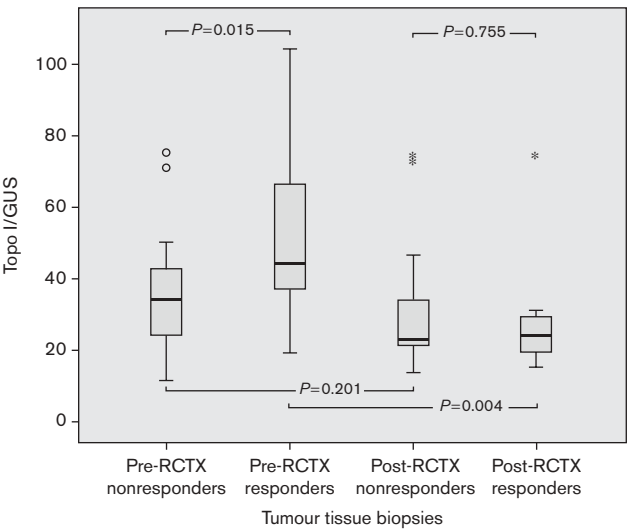
The median levels of pretreatment Topo I expression compared with the standard enzyme GUS (β-GUS) of all patients showed a lower level in normal than in tumour tissue without reaching the level of significance (30.88 vs. 39.87 Topo I/GUS; $P = 0.072$). The biopsies of the untreated tumour tissue of responding patients showed a statistically significant higher expression of Topo I compared with nonresponding patients (44.68 vs. 34.55;

Table 1 Comparison of pretherapeutic and histopathological stages

	Histopathology[a]				
Clinical stage	ypT0	ypT1	ypT2	ypT3	ypT4
uT2	3	0	3	1	0
uT3	4	0	8	16	1
uT4	0	0	1	0	1

[a]ypN1 stage counted as nonresponse. Therefore the number of responders differs in one patient from the ypT stages.

Fig. 1



Topoisomerase I (Topo I) mRNA expression of responders and nonresponders in tumour tissue before and after chemoradiotherapy. GUS, glucuronidase; RCTX, chemoradiotherapy.

$P = 0.015$; Fig. 1). However, in the normal tissue biopsies, the Topo I expression could not differentiate between responders and nonresponders (34.1 vs. 24.34; $P = 0.126$).

Comparison of topoisomerase I in surgical resection specimens

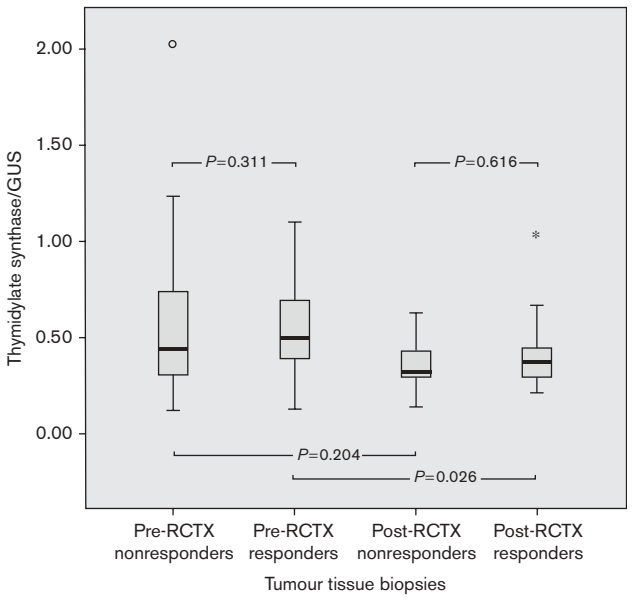
Topo I expression in normal tissue after chemoradiation was unrelated to treatment response (21.83 vs. 22.13 in responders and nonresponders, respectively; $P = 0.615$). In tumour tissue as well, Topo I expression did not show a different expression between responding and nonresponding patients (24.02 vs. 23.40; $P = 0.755$).

In nonresponders, there was no significant difference in the level of Topo I expression in the tumour tissue before chemoradiation and after surgical resection ($P = 0.201$). In contrast, it was significantly different in responding patients ($P = 0.004$). The difference in downregulation between responding and nonresponding patients was significant as well ($P = 0.013$).

Role of thymidylate synthase

The median levels of TS expression in the untreated tissue of all patients were statistically significant different between normal and tumour tissue (0.31 vs. 0.39; $P = 0.05$). However, TS expression in pretherapeutically obtained normal tissue could not differentiate between responders and nonresponders (0.32 vs. 0.31; $P = 0.196$) as well as in tumour tissue biopsies (0.43 vs. 0.35; $P = 0.311$). After chemoradiation, the TS expression in the normal tissue did not differ between responding and nonresponding patients (0.23 vs. 0.24; $P = 0.445$) as well as in the tumour tissue (0.28 vs. 0.25; $P = 0.616$; Fig. 2).

Fig. 2



Thymidylate synthase mRNA expression of responders and nonresponders in tumour tissue before and after chemoradiotherapy. GUS, glucuronidase; RCTX, chemoradiotherapy.

In nonresponders, there was no significant difference in the level of TS expression in the tumour tissue before chemoradiation and after surgical resection ($P = 0.204$). In responding patients, a significant decrease after surgery compared with the level before chemoradiation was noted ($P = 0.026$). However, the difference of downregulation between responders and nonresponders was not statistically significant ($P = 0.244$).

Discussion

In this analysis in patients with locally advanced rectal cancer, we found that the Topo I mRNA expression level may be a useful marker for the prediction of response to neoadjuvant irinotecan-based chemoradiotherapy. Our data suggest that patients with high RNA expression levels of Topo I in rectal tumour tissue derive a benefit from irinotecan-based chemoradiation. Similarly, in a recently published analysis of the FOCUS trial, Braun *et al.* [20] found that the level of Topo I may be a predictive and prognostic marker in patients with metastatic colorectal cancer undergoing irinotecan-based therapy. Our analysis suggests that Topo I expression may be also used as a predictive factor concerning downstaging in patients with locally advanced rectal tumours receiving neoadjuvant irinotecan-based chemoradiation. This finding has not been described before [17,21].

Topo I expression is reduced after chemoradiation in tumour and in normal tissue. This observation may be explained by therapy-induced downregulation of Topo I. It has been reported that Topo I protein expression in colon cancer cells decreases under exposure to SN-38, the active metabolite of irinotecan [22]. Particularly noteworthy is the extent of Topo I downregulation being distinctly greater in the tumour tissue of responding patients than in that of nonresponders. This is in line with former results showing that the extent of downregulation of Topo I activity after radiation correlated with enhanced survival in human cells [23].

Irinotecan arrests the cleavage complexes between Topo I and DNA by colliding replication forks. That leads to genome fragmentation and cell death [24]. It has been shown in xenograft models that complexes of Topo I and DNA are significantly higher in better responsive tumours to treatment with CPT-11 [25]. In cells, Topo I activity is shown to be important for surviving ultraviolet-induced DNA damage [26]. Topo I activity may be required for DNA damage processing. In our collective, patients with a significant decrease of Topo I expression levels exhibited a better response. It is speculative but the Topo I/DNA complexes might be cleared or repaired in cells that are resistant to treatment

with irinotecan. Maybe the higher Topo I levels before the start of the treatment are the first condition for irinotecan efficacy in terms of having a point of contact; the second possibility for resistance might be an alteration of a cellular process downstream from the formation of Topo I/DNA cleavage complexes [27]. To answer this question, one would have to examine whether patients with less response do have fewer complexes of Topo I/DNA irrespective of a higher Topo I level before beginning of the treatment.

In contrast to the Topo I findings, TS levels in the analysed samples did not show any relevant differences between responding and nonresponding patients before or after treatment in this study. Earlier studies have shown that tumoural expression of TS may be a prognostic factor in both primary and metastatic colorectal cancer undergoing 5-FU-based chemotherapy [28,29]. However, other investigators report contradictory results [17,30].

Generally, biopsies taken from resected tumours should be interpreted with caution, as surgically induced tumour ischaemia may change the expression of some genes. The decrease is notable as has been shown especially for TS [31]. In our collective, the decrease of TS in the tumour tissue of well-responding patients is significant, and may be caused as a surgically induced side effect. As it is the only significant result for TS, its informative value may be limited. Admittedly, it remains unclear whether the Topo I diminution might also be partially caused by ischaemia. However, this would not be in accordance with the data from the literature [22,23].

So far, there is no consensus on what early histopathological parameters may be used to best define responders and to estimate their long-term prognosis [32–34]. According to a retrospective analysis of the EORTC trial 22921 patients exhibiting ypT0–2 stages after preoperative chemoradiotherapy had an improved prognosis (when 5-FU treatment was continued in the postoperative treatment as well) [33]. The nodal negative status should be included in the patient's response anyway as it is an important prognostic factor [35]. Histopathological regression seems to be a predictor of disease-free survival as it correlates with the ypT and ypN stages [3,34]. However, the correlation is not linear and prognosis is still most accurately assessed by the final histopathological stage [36].

In our study, the correlation between Topo I expression levels and tumour downstaging is assessed. Clearly, tumour downstaging represents an early marker for the treatment efficacy and a longer follow-up is needed to answer whether an improvement in long-term prognosis occurs as well. Moreover, we used a multidrug regimen in this trial (capecitabine, irinotecan and cetuximab). Clearly, it remains to be shown whether or to what extent other factors may influence treatment response (such as KRAS or BRAF mutations, and the PTEN expression status for cetuximab) [37,38].

Conclusion

We found a significant higher level of pretreatment Topo I expression in the tumour tissue of well-responding patients by using real-time PCR for quantification of Topo I. These data suggest that Topo I in tumour tissue may serve as a predictive factor in irinotecan-based chemoradiation for rectal cancer.

Both the heterogeneity of the therapies and the individual differences between the tumours as well require the evaluation of separate markers. Consequently, further investigations in larger patient groups will be necessary to elucidate, if markers identified as predictive for a particular drug (such as Topo I in irinotecan-based chemoradiotherapy) retain their predictive relevance when using multidrug regimens.

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