

Topoisomerase I and II α protein expression in primary colorectal cancer and recurrences following 5-fluorouracil-based adjuvant chemotherapy

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Abstract

Purpose Human DNA topoisomerases I and II (topo-I and -II) are essential for vital cellular processes such as DNA replication, transcription, translation, recombination, and repair. In the present study, we correlate topo-I and -II expression and outcome after chemotherapy in primary and relapsed colorectal cancer.

Patients and methods Patients with colorectal cancer that had recurred, following surgery and adjuvant chemotherapy and underwent a second operation were included in the present study. All had undergone surgical resection of the primary tumor and received post-operatively 5-FU-based (5FU + Leucovorin, Mayo Clinic regimen) adjuvant chemotherapy. Tumor tissue was collected at the initial operation from the primary tumor and at the time of recurrence (during the second operation following chemotherapy). All tissue samples were analyzed for levels of expression of both topo-I and topo-II α using standard three-step immunohistochemistry on paraffin sections.

Results Forty patients were included. Levels of expression of topo-I and topo-II were higher in malignant cells from tumor recurrences compared to primary tumors ($P = 0.0001$ for both). There was a statistically significant positive relationship between patients age and levels of topo-I ($P = 0.011$) and topo-II ($P = 0.011$) expression.

Conclusions The study results reported here underscore the role of topoisomerase expression in colorectal cancer and suggest a potential role in tumor recurrence.

Keywords Topoisomerase I · Topoisomerase II α · Colorectal cancer · 5-FU · Chemotherapy

Introduction

The aim of the present study was to investigate whether chemotherapy with 5-FU alters the levels of topoisomerase I (topo-I) and II (topo-II) in neoplastic tissues from patients with colorectal cancer. To this end, we examined the relationship between clinical data and the expression of topo I and II in patients treated with 5-FU post-operatively and who underwent surgery at recurrence.

Human DNA topo-I is an essential nuclear enzyme for vital cellular processes such as DNA replication, transcription, translation, recombination and repair. Topo-I unwinds and uncoils the supercoiled DNA double helix by transiently cleaving one of the two strands and allowing its rotation over the other, following which topo-I reseals the cleaved strand [1–5]. Topo-II works in a similar manner, with the difference that it cleaves both DNA strands, allowing the passage of an intact double helix through the break. The entire reaction takes place at the expense of ATP hydrolysis [6]. In contrast to topo-I, which is monomeric, two homologous but distinct isoforms of type II human topoisomerases

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have been identified, DNA topo-II α and II β [6, 7]. The α isoform is the type II topoisomerase that was originally described and characterized in mammalian species [6].

Several studies have shown topo-II α to be a reliable marker of cell proliferation in tumors [8, 9]. In contrast to topo-II, topo-I levels are not cycle-specific and remain stable throughout the cell cycle [10]. Topo-II α plays important roles in DNA synthesis and transcription, as well as chromosomal segregation during mitosis. Beyond its physiological functions, topo-II α is reported to be a sensitive and specific marker of actively proliferating cells (in the late S, G₂ and M-phases of the cell cycle) and has been used as a proliferation marker in studies of colorectal cancer [7, 8, 10]. In addition to the well described role of the three MDR-related proteins, topo-II α has been implicated in drug resistance of tumor cells [9].

Topo-I catalytic activity has been evaluated in various studies and has been detected in all normal tissues at fairly constant levels [10]. Additional parameters were further investigated: topo-I immunoreactive protein levels were estimated by Western blot analysis, and topo-I gene expression (topo-I mRNA) was evaluated by Northern blot analysis. With regard to colorectal tumors, they were found to exhibit 5- to 35-fold increases in topo-I levels compared to the adjacent normal colonic mucosa [11].

Topo-I expression has also been evaluated by immunohistochemistry in paraffin-embedded human tissues [12], and its expression has been demonstrated in numerous neoplastic tissues. Increased topo-I has been shown in ovarian carcinomas (elevated topo-I expression in 43% of tumors) [12]; colorectal carcinomas (ranging from 43–86% in various studies) [13–15]; testicular tumors (30–38% in seminomas, 30% in embryonal carcinomas, and 100% in teratomas and yolk sac tumors) [16]; transitional carcinoma of the bladder (overexpression in 77%) [17], and renal cell carcinomas (ranging from 36–100% according to grade) [18]; malignant melanomas (overexpression in 41.6%) [19]; gastric carcinomas (overexpression in 68%) [20]; sarcomas (high levels of topo-I in 13%) [21]; breast carcinomas (overexpression in 41%) [22], oral dysplasias, and squamous cell carcinomas (overexpression in 79 and 92%, respectively) [23, 24]. In normal tissues, topo-I expression appeared to be higher in the germinal centers of the tonsil and in the mucosal lymphocytes of the colon, while topo I positivity was also detected in the glandular epithelium of the colon [12].

Patients and methods

Patients

Forty patients with colorectal cancer that had recurred following surgery and adjuvant chemotherapy who underwent

Table 1 Patients characteristics

Patients (<i>n</i>)	40
Gender	
Men	25
Women	15
Median age at diagnosis (years)	58 (35–75)
Performance status (WHO), 0–1	40
Location of primary tumor	
Right colon	6
Left colon	28
Rectum	6
Stage (Dukes')	
B2	13
C	27
Relapse	
Local	17
Distant	23
Differentiation	
Well	2
Moderate	31
Poor	7
Disease-free survival (months)	18 (range, 6–79)

a second operation were included in this study. All had undergone surgical resection for their primary tumor and received post-operatively 5-FU-based [5FU and Leucovorin (LV), Mayo Clinic regimen] adjuvant chemotherapy [25]. Patients characteristics are described in Table 1.

Study plan

Tumor tissue was collected during the initial operation from the primary tumor, before the administration of any adjuvant chemotherapy and also at the time of recurrence (during the second operation following chemotherapy). All these tissues were analyzed for expression of both topo-I and topo-II α .

Determination of topo-I and topo-II α

The expression of topo-I and topo-II α was studied on paraffin sections by a standard three-step immunohistochemistry. Topogen, an anti-topo-I monoclonal antibody (mAb) (2012-3) was applied. Topogen is a mouse mAb (IgG2b isotype); its epitope has not been mapped [26–28]. The primary mAb JH2.7 for topo-II α (Biocare Medical, CA, USA) is a mouse mAb (IgG₁ isotype) that recognizes a 170-kDa protein, the epitope of which maps between aminoacid 854–1447 of topo-II α [27–29]. Counterstaining with hematoxylin was applied in the end of the classical immunohistochemistry procedure.

Positive and negative controls

For the expression of topo-I and -II α , normal human tonsil tissues immunostaining served as positive controls. Non-specific, isotype-matched monoclonal Abs worked well as substitute negative controls [26–29].

Determination of topo-I and topo-II α expression

First step Sections were examined for quantified immunoreactivity by two independent investigators blinded to any relevant patient clinical data. They evaluated more than 1,000 neoplastic cells in consecutive areas of neoplastic tissue. The numbers of positive cells were then expressed as a percentage of labeled tumor cells with respect to the total number of tumor cells that were evaluated. Immunostaining for topo-I and -II α was graded according to the percentage of tumor cells staining positive (and \pm for <5%, + for 5–50% and ++ for 50 to 75% of cells with positive staining) and intensity of staining (weakly positive, moderately positive, or strongly positive). To simplify the scoring, we graded them as 0 (negative cells), 1 (\pm and/or <5%), 2 (+ and/or 5–50%), 3 (++ and/or 50–75%). Then, the sections were evaluated classified in pairs (one section from the first surgery and one section from the second surgery for each patient).

Second step The percentages of immunoreactive malignant cells were calculated using an image analysis system with an appropriate software package [Sigma Scan Pro, Version 5.0 (SPSS Science, Erkrath, Germany)]. The ratio was calculated as a percentage of immunohistochemically positive neoplastic cells over the total number (stained and unstained) of neoplastic cells. The membrane, cytoplasmic or nuclear intensity of the specific immunohistochemical stain, was also assessed. All the positively stained cells were classified in two groups (weakly stained and strongly stained).

DNA ploidy of neoplastic cells was evaluated by image DNA cytometry performed on Feulgen-stained sections. Images were acquired using a Zeiss Axiolab microscope (Carl Zeiss Jena GmbH, Jena, Germany) with a mechanical stage, fitted with a SONY-iris CCD video camera (SONY Corporation, Tokyo, Japan). The latter was connected to a Pentium II personal computer which included the relevant software. Slides were examined at low power magnification (40 \times) to identify the areas with the highest cellularity. In each case, a total number of ≥ 200 Feulgen-stained nuclei was selected at high power magnification (400 \times) and stored as JPEG file [1,550 \times 1,070 pixels, 16.7 million colors (24-bit)]. Then the images were converted to gray scale and the staining intensity of the Feulgen-stained nuclei was measured semi-automatically. A classification of the nuclei in pairs according to their staining intensity followed.

Finally, the graphic presentation of the nuclei demonstrating their distribution according to their DNA content was also performed.

Statistical analysis

All analyses were performed with SPSS version 10.1 (Statistical Product and Service Solutions; SPSS Inc., Chicago, IL, USA). We used McNemar's paired Chi-square test to assess the possible alteration of the levels of topoisomerases after chemotherapy with 5-FU. Fisher's exact test was performed in order to assess the possible relationship of topoisomerase increase with gender, Dukes' stage, tumor grade and localization. Mann–Whitney *U* Test was performed to assess a possible relationship between patient age and alteration in topoisomerases levels.

Results

Patients

Forty patients were included in the present study. Of these, 25 were men and 15 were women. The median age was 58 years (range, 35–75). All patients had a performance status (PS) of 0–1. Tumor localization at the time of diagnosis was: right colon 6, left colon 28, and rectum 6. According to pathological classification, 13 patients had Dukes B2 tumors, and the remaining 27 Dukes C. With regard to differentiation, 2 tumors were well differentiated, 31 moderately and, 7 were poorly differentiated. Of all these patients, 17 relapsed locally and 23 with distant metastases, with a median relapse-free interval of 18 (range, 6–79) months (Table 1). All patients who entered the trial were evaluable for analysis.

Ploidy

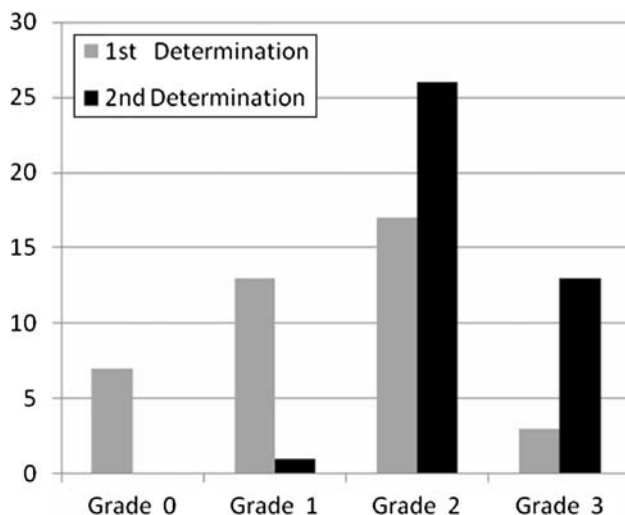
Out of 40 primary tumors, 12 were highly aneuploid and the remaining 28 were moderately aneuploid. There was no association between the degree of DNA aneuploidy and the expression of any of the analyzed markers.

Topoisomerase I

Immunohistochemical analysis revealed that levels of topo-I expression were higher in malignant cells from tumor recurrences compared to cells from the primary tumors ($P = 0.0001$) (Table 2; Fig. 1); decreased in grade 1 ($P = 0.007$) and increased in grade 3 ($P = 0.003$) (Table 2). In image analysis evaluation, there was a significant increase in malignant cells from the tumor recurrences ($P = 0.0001$) (Table 3); low expression was noticed in

Table 2 Photomicroscopic evaluation

Examined parameters	Photo, microscopic evaluation			<i>P</i>
	Grade	First evaluation	Second evaluation	
Topoisomerase I	0	7	0	0.0117
	1	13	1	0.0007
	2	17	26	0.0722
	3	3	13	0.0103
Increased: 25, decreased: 0, no change: 15, <i>P</i> = 0.0001				
Topoisomerase II α	0	0	0	1
	1	10	2	0.0252
	2	30	28	0.8027
	3	0	10	0.0010
Increased: 18, decreased: 0, no change: 22, <i>P</i> = 0.0001				

**Fig. 1** Topoisomerase I (Topo-I) expression in relation to tumor grade according to immunohistochemical evaluation

range 1–5 ($P = 0.0001$) and 26–30 ($P = 0.0143$) (Table 3; Fig. 3). The increase in topo-I levels was not significantly correlated with gender, performance status (WHO), location of the primary tumor, Dukes' stage, grade of differentiation, and localization of relapse. There was a statistically significant relationship between the age of patients and the expression of topo-I ($P = 0.011$). Patients with an increased expression of topo-I levels were older in age (median = 62.5) than patients without increased expression (median = 50).

Topoisomerase II α

Malignant cells from tumor recurrences showed statistically significantly increased levels of topo-II, compared to those of the primary tumors ($P = 0.0001$) (Table 2; Fig. 2); while topo-II levels were decreased in grade 3 lesions ($P = 0.0001$) (Table 2; Fig. 4). In image analysis, there was

a significant increase in malignant cells from tumor recurrences ($P = 0.0001$) (Table 3); low expression was noticed in the range of 16–20 ($P = 0.0143$) (Table 3). There was a statistically significant relationship between the age of patients and increased levels of expression of topo-II α ($P = 0.011$). Levels of topo-II α expression were higher in malignant cells from tumor recurrences compared to cells from primary tumors ($P = 0.0001$). There was a statistically significant positive correlation between the age of patients and increased levels of expression topo-II α ($P = 0.011$). The increase in topo-II α levels did not demonstrate any significant correlations with gender, age, PS (WHO), localization of primary tumor, Dukes' stage, tumor grade, and location of relapse when compared to topo-II α levels in the primary tumors ($P = 0.0001$).

Discussion

In our study, 33 out of 40 (83%) samples of colorectal cancer stained positive for topoisomerase (Table 2; Fig. 3). This finding is in close agreement with the results of Staley et al. [14], who reported 86% of positive staining in 29 samples, but in contrast with the results from Boonsong et al. [13], who demonstrated a lower percentage of cells expressing topo-I; 51% of the samples, including 24.4% with >50% positive tumor cells [13]. Topo-I immunoreactivity was confined to the nucleus in all samples, being consistent with the role of topo-I as a nuclear protein. For topo-II α , 21 out of 40 (52%) samples of colorectal cancer stained positive (Table 2; Fig. 4), including 10/40 (25%) with weak positive staining (grade 1). This finding was confirmed by Image Analysis (Table 3). These data are consistent with those reported by Burden and Osheroff [6].

In this study, we present significantly increased topo-I and topo-II α expression in recurrent colorectal tumors, reinforcing the view that their expression is likely part of

Table 3 Image analysis evaluation (%)

Range	First sample (no.)	Second sample (no.)	<i>P</i>	Range	First sample (no.)	Second sample (no.)	<i>P</i>
<i>Topoisomerase I</i>							
0	0	0	1	36–40	3	0	0.2405
1–5	21	1	0.0001	41–45	1	1	1.000
6–10	4	0	0.1156	46–50	1	1	1.000
11–15	2	4	0.6752	51–55	2	4	0.6752
16–20	3	5	0.7119	56–60	0	4	0.1156
21–25	2	3	1	61–65	0	5	0.0547
26–30	1	9	0.0143	66–70	0	0	
31–35	0	3	0.2405	71–80	0	0	
<i>Topoisomerase IIa</i>							
0	0	0	1	36–40	2	3	1
1–5	10	4	0.1395	41–45	5	1	0.2007
6–10	6	5	1	46–50	0	3	0.2405
11–15	5	2	0.4315	51–55	0	2	0.4937
16–20	9	1	0.0143	56–60	0	0	1
21–25	0	5	0.0547	61–65	0	1	1
26–30	3	3	1	66–70	0	2	0.4937
31–35	0	3	0.2405	71–80	0	5	0.0547
Total	First determination			Second determination			<i>P</i>
	Mean	Median	Range	Mean	Median	Range	
Topoisomerase I	13.875	4.5	1–54	35.375	29.5	4–65	0.0001
Topoisomerase IIa	16.775	15	1–45	34.575	31	2–79	0.0001

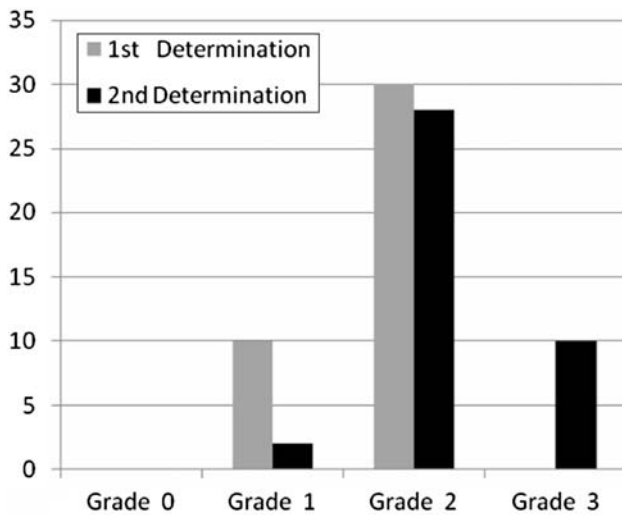


Fig. 2 Topoisomerase II α (Topo-II α) expression in relation to tumor grade according to immunohistochemical evaluation

the malignant cells phenotype in recurrent colorectal carcinomas. This observation is in agreement with previous studies [15, 30], which demonstrated a direct correlation between thymidylate synthase (TS) and topo-I tumor expression and hypothesized, similar to TS [31], that high topo-I expression is related to a more aggressive biological

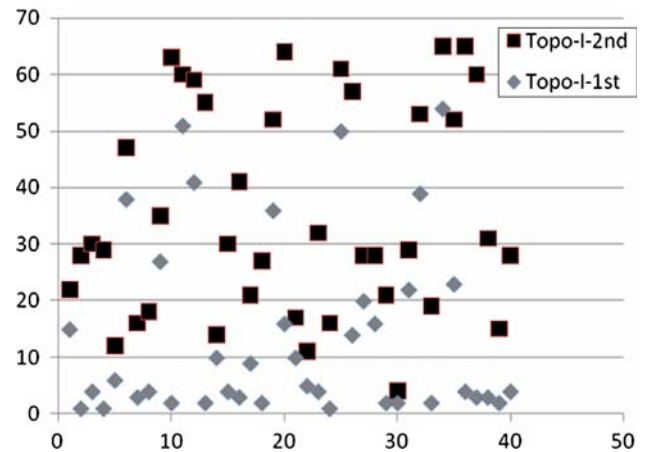


Fig. 3 Dispersion diagram of topoisomerase I (Topo-I) expression as assessed by immunohistochemistry

phenotype. Of note, increased topo-II α expression characterizes rapidly proliferating cells [6] and represents an accepted marker of aggressive tumor behavior [32].

Since the increase of topo-I and -II α expression was demonstrated following 5-FU-based treatment, it is tempting to attribute this change to 5-FU. High levels of topo-I correlate with sensitivity to camptothecins (irinotecan and topotecan) [17], whereas high levels of topo-II α correlate

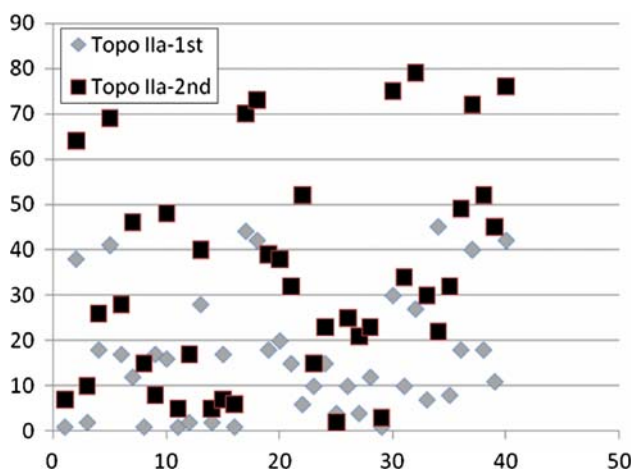


Fig. 4 Dispersion diagram of topoisomerase II α Topo-IIA expression as assessed by immunohistochemistry

with sensitivity to etoposide, a known topo-II α -targeting agent [32]. Thus, it is reasonable to consider applying these agents sequentially for the treatment of colorectal cancer; i.e. 5-FU \rightarrow irinotecan and 5-FU \rightarrow etoposide.

Irinotecan (CPT-11), a water soluble camptothecin derivative, is a specific potent inhibitor of topo-I [33]. In a European phase II study, irinotecan achieved response rates of 19% in chemotherapy-naïve patients and 18% in pre-treated patients with advanced disease [34].

Drugs that target topo-II, such as etoposide (VP-16), doxorubicin, and mitoxantrone, are among the most effective anticancer drugs in clinical use. Etoposide destroys cells by inhibiting the ability of topo-II to ligate nucleic acids cleaved during the double-stranded DNA passage reaction [35].

Irinotecan yielded response rates of 15–25% in 5-FU-refractory patients [36–44], not dissimilar to those observed in first-line treatment of colorectal cancer (18–32%) [45–47] and in contrast to the expected decreasing response rate from the application of irinotecan in second-line treatment. Treating patients with irinotecan upon progression to 5-FU/LV seems to be yielding more significant results compared to the opposite sequence, based in our previous experience, where the best results with sequential monotherapies were obtained when 5-FU/LV was followed by irinotecan (5-FU/LV \rightarrow CPT11) at disease progression or relapse [41].

With regard to irinotecan efficacy, *in vitro* data suggest that topo-I expression could be regarded as an important cellular sensitivity determinant [16]. In particular, decreased DNA topo-I expression was correlated with camptothecin-resistant cell lines [27], while Jansen et al. [36] reported a positive correlation between irinotecan sensitivity and topo-I activity.

Topo-II α is the primary cytotoxic target of some of the most effective chemotherapeutic drugs, such as the epipod-

ophylotoxins, etoposide (VP-16) and teniposide (VM-26) [32]. Studies in previously untreated patients with advanced colorectal carcinoma evaluating the combination of etoposide with cisplatin or 5-FU demonstrated minimal activity in metastatic colorectal cancer [48–51]; there have been no clinical data supporting the *in vitro* synergy observed between these cytotoxic agents [52, 53]. Other studies failed to prove any benefit with the combination of etoposide with 5-FU or cisplatin/carboplatin [54]. However, the combination of etoposide with 5-FU + LV had demonstrated some activity when administered as second-line treatment after failure of weekly 5-FU + LV in patients with metastatic colorectal cancer [55, 56].

The results reported herein underscore the role of topoisomerase (topo-I and topo-II α) expression in colorectal cancer. It is believed that translational studies of molecular targets for currently applied cytotoxic and biological agents (like bevacizumab and cetuximab) might form the basis of current and future drug combinations, in rationalizing the optimal chemotherapeutic drug schedule and sequence that, will eventually translate in improved tumor eradication and survival prolongation.

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