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Personalized Medicine and Imaging

Clinical Cancer Research

DNA Topoisomerase I Gene Copy Number and mRNA Expression Assessed as Predictive Biomarkers for Adjuvant Irinotecan in Stage II/III Colon Cancer

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Abstract

Purpose: Prospective–retrospective assessment of the *TOP1* gene copy number and *TOP1* mRNA expression as predictive biomarkers for adjuvant irinotecan in stage II/III colon cancer.

Experimental Design: Formalin-fixed, paraffin-embedded tissue microarrays were obtained from an adjuvant colon cancer trial (PETACC3) where patients were randomized to 5-fluorouracil/folinic acid with or without additional irinotecan. *TOP1* copy number status was analyzed by fluorescence *in situ* hybridization (FISH) using a *TOP1*/CEN20 dual-probe combination. *TOP1* mRNA data were available from previous analyses.

Results: *TOP1* FISH and follow-up data were obtained from 534 patients. *TOP1* gain was identified in 27% using a single-probe enumeration strategy ($\geq$4 *TOP1* signals per cell) and in 31% when defined by a *TOP1*/CEN20 ratio $\geq$ 1.5. The effect of additional irinotecan was not dependent on *TOP1* FISH status. *TOP1* mRNA data were available from 580 patients with stage III disease. Benefit of irinotecan was restricted to patients characterized by *TOP1* mRNA expression $\geq$ third quartile (RFS: $HR_{adjusted}$, 0.59; $P = 0.09$; OS: $HR_{adjusted}$, 0.44; $P = 0.03$). The treatment by *TOP1* mRNA interaction was not statistically significant, but in exploratory multivariable fractional polynomial interaction analysis, increasing *TOP1* mRNA values appeared to be associated with increasing benefit of irinotecan.

Conclusions: In contrast to the *TOP1* copy number, a trend was demonstrated for a predictive property of *TOP1* mRNA expression. On the basis of *TOP1* mRNA, it might be possible to identify a subgroup of patients where an irinotecan doublet is a clinically relevant option in the adjuvant setting of colon cancer. *Clin Cancer Res; 22(7); 1621–31. ©2015 AACR.*

Introduction

Colorectal cancer is one of the leading causes for cancer related mortality in the world (1–3). Tumor stage at diagnosis remains the strongest prognostic factor, and treatment is guided according to the TNM staging system (4–8). Systemic treatment has improved progression-free survival (PFS) and overall survival (OS) for patients with advanced disease, but survival benefit of adjuvant systemic therapy is also evident for patients with high-risk localized disease (high-risk stage II) or regional disease (stage III; ref. 9). A limitation of systemic therapy is the great interpatient variability in drug efficacy and severity of adverse effects (10). In the pursuit of a more personalized treatment approach, it is clinically important to identify tumor characteristics that may serve as biomarkers which will accurately predict the likelihood of benefit in advance of therapy. The discovery and validation of predictive biomarkers are not only relevant in the development of new targeted drugs, but may be equally important for already implemented classic cytotoxic chemotherapy.

The introduction of irinotecan in combination with 5-fluorouracil (5FU)/folinic acid (FA; e.g., FOLFIRI) has improved the clinical outcome of patients with metastatic colorectal cancer, and with efficacy equal to that of the oxaliplatin and 5FU/FA doublets, the FOLFIRI regimen is approved for first and second line therapy (9, 11–13). However, overall objective response rates following FOLFIRI remains below 50% and in combination with noncomplete cross-resistance between FOLFIRI and the oxaliplatin doublets this emphasizes the importance of selecting the right treatment regimen in first line (12–14). Irinotecan is not recommended in the adjuvant setting of colon cancer because superiority of the 5FU/FA + irinotecan combinations over 5FU/FA alone has not been demonstrated in any randomized controlled trials (RCT; refs. 15–18). However, the

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Translational Relevance

Validated predictive biomarkers for irinotecan will have immediate clinical utility in the metastatic setting of colorectal cancer. Additionally, such biomarkers will potentially identify patients with localized and regional colon cancer where an irinotecan doublet may be superior to standard oxaliplatin-based adjuvant therapy. In the present study, we demonstrate a trend for a *TOP1* mRNA-dependent differential treatment effect of irinotecan using a prospective–retrospective study design and prospectively collected tumor samples from the adjuvant colon cancer PETACC3 trial. High *TOP1* mRNA primary tumor expression levels are associated with benefit of additional irinotecan compared with 5-fluorouracil monotherapy, whereas no effect of irinotecan is seen in patients with low or normal *TOP1* mRNA expressing tumors. If these results are confirmed, irinotecan may be an additional option in the adjuvant setting of colon cancer. Furthermore, *TOP1* mRNA expression may prove to be a key predictor of irinotecan efficacy in cancers other than colorectal cancer.

conclusions from these RCTs are based on average efficacy measures that might conceal subpopulations of patients who benefit greatly from additional irinotecan.

Irinotecan is a semi-synthetic derivative of camptothecin, a cytotoxic alkaloid that exerts its activity by transforming DNA topoisomerase 1 (Top1) into a potent cellular toxin (19). Accordingly, irinotecan may be regarded as targeted cytotoxic therapy (20). The biologic function of Top1 is to relax the DNA supercoiling that emerges through DNA transcription and replication. This is achieved through the formation of functional Top1-DNA cleavage complexes (Top1cc) that in a controlled way allow for transient single-stranded DNA breaks. The active form of irinotecan stabilizes the Top1cc by interfacial inhibition, and as a result the rapid moving DNA and RNA polymerases catch up and collide with these stalled complexes whereby irreversible Top1-DNA cross-links are formed (20, 21).

Elevated Top1 protein levels will in theory result in increased cytotoxic activity of irinotecan, and based on *in vitro* cell line studies, Top1 protein has been suggested as a putative predictive biomarker (22–26). Attempts to clinically validate these findings in the setting of advanced colorectal cancer have been carried out in two well-designed retrospective biomarker studies (27, 28). However, the results from these studies are conflicting, and in addition, inconclusive findings have been published for the predictive property of Top1 protein expression in the adjuvant setting (29). This inconsistency may partly be due to inherent analytical limitations of the applied anti-Top1 antibody for immunohistochemistry (IHC), and reports of lack of reproducibility of Top1 staining results have been published recently (30).

The *TOP1* gene copy number and *TOP1* mRNA expression represent potential alternatives to Top1 protein expression as predictive biomarker candidates for irinotecan (31–33). We have developed a gene enumeration *TOP1*/centromere-20 (CEN20) dual-probe for fluorescence *in situ* hybridization (FISH), and in a recently published study we demonstrated that *TOP1* gene gain is associated nonsignificantly with increased odds for response following irinotecan monotherapy in patients with metastatic colorectal cancer (34).

In 2009, Simon and colleagues (35) proposed new guidelines for the use of archived tissue material in the assessment of medical utility of biomarkers. The PETACC3 trial is a large randomized phase III trial (ClinicalTrials.gov NCT00026273) where the study protocol allowed for prospective collection of formalin-fixed, paraffin-embedded (FFPE) tumor material (17). Although failing to generate evidence for superiority of adding irinotecan to 5FU/FA in the adjuvant setting of colon cancer, considering the guidelines of Simon and colleagues (35), the trial design seems ideal for prospective–retrospective assessment of predictive biomarkers for irinotecan. In the setting of the PETACC3 trial, we report the results of a prospective–retrospective analysis of the predictive property of *TOP1* gene copy number status, assessed by FISH, and *TOP1* mRNA expression status in relation to additional irinotecan therapy. We hypothesize that a subpopulation of patients benefit greatly from an irinotecan doublet in early stage colon cancer, and that this subpopulation can be identified by intratumoral *TOP1* gene gain and/or by high *TOP1* mRNA expression levels.

Materials and Methods

Patients and tumor material

A total of 3,278 patients were accrued to the PETACC3 trial (17). Patients were $\geq$18 and $\leq$75 years of age, with completely resected histologically verified stage II or stage III adenocarcinoma of the colon. Patients were stratified by disease stage and participating center and randomly allocated to receive 6 months of either 5FU/FA alone or 5FU/FA in combination with irinotecan. Information on all eligibility criteria, the treatment schedules and follow-up was accounted for in detail in the original publication (17). Written informed consent, including permission for future translational research using biological samples, was obtained from all patients prior to study inclusion. FFPE samples from 1,564 patients were prospectively collected during the PETACC3 study accrual (36, 37), and tissue from 675 of these patients was transferred to tissue micro arrays (TMA; refs. 36, 38). In brief, central and peripheral tumor target areas were identified in hematoxylin-eosin (H&E) stained sections, and for each patient five 0.6-mm tissue cores from the corresponding FFPE donor blocks were transferred to the recipient TMA blocks using a manual tissue arrayer. Approval for the present translational study was obtained from the PETACC3 Translational Research Working Party (PTRW).

TOP1 FISH

A *TOP1*/CEN20 probe combination and the Dako Histology FISH Accessory Kit (Dako Denmark, Glostrup, Denmark) were used as previously reported (32). The FISH dual-probe was developed and analytically validated by Dako Denmark and the University of Copenhagen (Copenhagen, Denmark; ref. 32). In brief, the *TOP1* gene probe is constructed from two bacterial artificial chromosome (BAC) clones, RP11-629I4 and CTD-3193L13. The final 370-kb probe covers the complete *TOP1* genomic sequence of 96 kb at 20q12. Probes made from cloned DNA will inevitably cross-hybridize to the repetitive sequences that are interspersed randomly within coding and noncoding regions. To avoid this cross-hybridization, the probe mixture comprises unlabeled chemically synthesized peptide nucleic acid (PNA) oligonucleotides that block the most frequent repetitive sequences within the Alu repeat DNA sequence family. The

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CEN20 probe is constructed from chromosome 20 centromere specific PNA oligonucleotides. The two probes are labeled by different fluorophores to allow for simultaneous target visualization by fluorescence microscopy when using an appropriate dual-band filter. The fluorophores for this specific probe mixture are composed of Texas Red for *TOP1* and fluorescein isothiocyanate (FITC) for CEN20.

Probe sensitivity and specificity was tested by Dako using metaphase chromosome preparations from normal diploid cells. The probes hybridized to their expected localizations and cross-hybridization to nonrelevant targets was not demonstrated. The probe mixture was optimized for the Dako Histology FISH Accessory Kit (Dako) in the development phase. An additional in-house optimization of the enzymatic digestion time was performed using TMAs composed of normal and neoplastic colon and rectal tissue. This was done in concordance with the quality measures of the Section E9 of the American College of Medical Genetics technical standards and guidelines for FISH.

The hybridized TMA sections were evaluated using a fluorescence microscope (Zeiss AX10). The DAPI counterstain was used to assess nuclear morphology and the *TOP1* and CEN20 fluorescent signals were evaluated separately in relevant single filters and in combination in a double filter. At medium magnification (400x), quality and potential heterogeneity in the signal distribution was assessed in all tissue cores from all patients. Signal enumeration was only performed in tumors showing well defined nuclear morphology and distinct fluorescent signals at medium magnification. The signals were enumerated in 60 nonoverlapping cancer cell nuclei at high magnification (1000x). In case of heterogeneous signal distribution, the tumor areas with the highest number of *TOP1* signals per nuclei were to be used for signal enumeration. The TMA sections were evaluated by a pathologist or a laboratory technician, who was well trained in the FISH technique and the histological appearance of colon cancer. To evaluate interobserver agreement, tumor cores from 42 patients were evaluated by both observers. FISH procedures and scoring were performed blinded to all patient data. To avoid missing a potential *TOP1* gene dosage effect, the average number of *TOP1* signals per cell was used in parallel with the *TOP1*/CEN20 ratio to determine *TOP1* FISH status.

TOP1 mRNA expression

TOP1 mRNA gene expression data were generated previously using the whole set of 1,564 samples (36, 39). In brief, FFPE tumor blocks from 1,404 patients were eligible for RNA extraction. From corresponding micro-dissected tissue sections, RNA of sufficient quantity and quality was successfully extracted from 895 samples. Amplified products were hybridized to the Almac Colorectal Cancer DSA microarray platform (Almac, Craigavon, United Kingdom; ref. 40). Following quality control, *TOP1* mRNA expression data were available for a total of 688 unique samples, including 580 from patients with stage III disease.

Study design and statistical methods

The study design was prospective–retrospective as proposed by Simon and colleagues (35), and the statistical plan and the applied cutoff values were defined prior to FISH analysis. The design included three biomarker study populations which were named: *TOP1* FISH stage II + III, *TOP1* FISH stage III, and *TOP1* mRNA expression stage III. All populations were stratified according to *TOP1* status and treatment randomization. The REMARK guidelines for reporting on biomarker studies were followed as close as possible (41).

In adherence with previous publications addressing biomarkers in the PETACC3 material, the original primary study endpoint, disease-free survival (DFS), was rejected in favor of recurrence-free survival (RFS; ref. 42). In doing so, secondary primary malignancies other than colon cancer were disregarded as events. RFS was defined as time in months from randomization until the occurrence of local, regional, or distant relapse; a second primary colon cancer; or death. OS was retained as secondary endpoint and was defined as time in months from randomization until death.

For initial analyses, the average number of *TOP1* signals per cell, the *TOP1*/CEN20 ratio and the *TOP1* mRNA expression data were treated as continuous variables. To be able to discriminate prognostic and predictive properties, patients were dichotomized in *TOP1* subgroups, categorized as *TOP1* normal and *TOP1* gained/*TOP1* high, which was followed by analyses of treatment effects within these subgroups. *TOP1* gain was defined as an average *TOP1* gene copy number ≥ 4.0 signals per cell or a *TOP1*/CEN20 ratio ≥ 1.5. In relation to the *TOP1* mRNA expression data, the third quartile was chosen to dichotomize the population in *TOP1* normal and *TOP1* high subgroups.

The Kaplan–Meier method was used to estimate RFS and OS rates, and univariate comparisons were made using the log rank test. The effect size of *TOP1* status and treatment arm was estimated in univariate and multivariable analysis using the Cox proportional hazards model. Adjustment variables for multivariable analysis were selected based on significant effects ($P < 0.05$) in univariate analysis. Microsatellite instability (MSI), *KRAS*, and *BRAF* status were available from previous publications (36, 37, 42), and these were tested alongside the clinical and pathological baseline variables: N stage, tumor localization, tumor grade, sex, and age. Formal tests for statistical interaction between dichotomized *TOP1* status and treatment were performed in separate Cox models, including main effects and an interaction term. All results were presented by hazard ratios (HR), estimated 95% confidence intervals (CI) and *P* values from the Wald-test. Based on Schoenfeld residuals no important violations against the assumption of proportional hazards were identified for any of the variables. A secondary exploratory multivariable fractional polynomial interaction (MFPI) approach was performed to decrease the risk of making a type II error (43, 44). Linear, FP1 and FP2 models (flexibility 1) with default parameters and MSI, *KRAS*, *BRAF*, tumor localization, and N stage as adjusting variables were tested in the MFPI analysis.

The χ^2 test was used for testing representativeness of the biomarker study populations in relation to the PETACC3 population as a whole. Likewise, the χ^2 test was used for assessing potential differences in the distribution of the baseline variables between the *TOP1* subgroups. The *P* values from the χ^2 tests were Bonferroni corrected to adjust for multiple comparisons. Pearson correlation coefficients (*r*) were calculated to test for statistical dependence between the *TOP1* variables.

All *P* values were two-sided and the significance level was set at <0.05. The MFPI analysis was performed in STATA 11 (45). All other analyses were performed in R software for statistical computing (R, 2013), version 3.0.2 (46).

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Results

All assays were conducted blinded to the clinical data. A total of 110 patients (16.3%) were excluded following FISH procedures as a result of TMA core loss, weak fluorescent signal intensity, or poorly preserved nuclear morphology. In addition, 31 patients (4.6%) were excluded due to unsuccessful matching of the TMA patient identification numbers with the clinical database. This reduced the *TOP1* FISH stage II + III population to 534 patients and the *TOP1* FISH stage III population to 368 patients. The *TOP1* mRNA stage III population was composed of 580 patients (for CONSORT diagram see Supplementary Fig. S1). The treatment randomization was well preserved in all three biomarker populations, and in relation to the baseline characteristics, the patients were representative of those accrued to the PETACC3 trial as a whole (Supplementary Tables S1 and S2). In adherence with the results of the PETACC3 trial, benefit of additional irinotecan was not identified in any of the biomarker populations (Supplementary Table S3).

The average *TOP1* copy number ranged from 1.4 to 11.6 *TOP1* signals per cell when including all unique samples with acceptable hybridization quality (median = 2.6). Similarly, the range of the *TOP1*/CEN20 ratio was 0.8 to 3.9 (median = 1.3). In the 42 cases where the FISH signals were counted by both observers, the Lin concordance correlation coefficient for interobserver agreement was CCC = 0.99 (95% CI, 0.98–0.999) for the *TOP1* copy number per cell and CCC = 0.96 (95% CI, 0.93–0.98) for the *TOP1*/CEN20 ratio (for Bland-Altman plots see Supplementary Fig. S2).

In the *TOP1* FISH stage II + III population, 142 tumors (27%) had ≥4 *TOP1* signals per cell and 167 tumors (31%) had a *TOP1*/CEN20 ratio of ≥1.5. In the *TOP1* FISH stage III population, 95 tumors (26%) had ≥4 *TOP1* signals per cell while 120 tumors (33%) had a *TOP1*/CEN20 ratio of ≥1.5. Apart from tumor localization, where *TOP1* gain, either as ≥4 *TOP1* signals per cell or a *TOP1*/CEN20 ratio of ≥1.5, was observed more frequently in left-sided tumors than in right-sided tumors, *TOP1* FISH status and *TOP1* mRNA expression status did not associate with any baseline characteristics (Supplementary Tables S4–S8). No statistically significant correlation was observed between the *TOP1*/CEN20 ratio and *TOP1* mRNA expression ($r = 0.25$) or between the *TOP1* gene copy number and *TOP1* mRNA expression ($r = 0.25$; Supplementary Fig. S3).

Prognostic and predictive effects of *TOP1* FISH status

In univariate analyses not stratified by treatment, increasing values of the *TOP1*/CEN20 ratio were associated with prolonged OS in the FISH stage II + III population (HR, 0.74; 95% CI, 0.58–0.95; $P = 0.01$) and in the FISH stage III population (HR, 0.74; 95% CI, 0.56–0.97; $P = 0.02$). Statistical significance was lost when dichotomizing the populations by a *TOP1*/CEN20 ratio of 1.5 (Table 1). The association was not significant between the *TOP1*/CEN20 ratio and RFS (Table 1). When the *TOP1* gene copy number per cell was modeled continuously or dichotomized by ≥4.0 *TOP1* signals per cell, the associations with RFS and OS were nonsignificant both in the FISH stage II + III and the FISH stage III populations (Table 1).

In search of a predictive property of *TOP1* FISH status that was independent of its potential inherent prognostic property, analyses of treatment effects were performed separately in the *TOP1* gained subgroups and the *TOP1* normal subgroups. No significant separation of the Kaplan–Meier survival curves was identified for treatment stratum in any *TOP1* subpopulation (Fig. 1), and the estimated 5-year RFS and OS rates were almost identical within each *TOP1* subgroup (Table 2 and Table 3).

In the exploratory MFPI analyses, *BRAF* status was selected as a prognostic variable for which the models were adjusted. No statistically significant treatment by *TOP1* interaction was demonstrated in any of the linear or flexible models where the *TOP1* gene copy number and the *TOP1*/CEN20 ratio were retained on a continuous scale in separate models (data not shown).

Prognostic and predictive effects of *TOP1* mRNA expression status

In univariate analysis including all available patients, continuous increase in *TOP1* mRNA expression was significantly associated with prolonged OS (HR, 0.74; 95% CI, 0.60–0.92; $P = 0.007$) and nonsignificantly with prolonged RFS (HR, 0.85; 95% CI, 0.7–1.03; $P = 0.10$). When the analysis was repeated using the third quartile as cutoff value, statistical significance was lost for the

Table 1. Univariate combined prognostic and predictive effects of *TOP1* status in relation to recurrence-free survival and OS

		Recurrence-free survival		OS	
	No.	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
TOP1 FISH stage II + III	534				
TOP1 signals per cell					
Continuous		0.96 (0.76–1.22)	0.76	0.84 (0.62–1.12)	0.22
Gain vs. normal		0.86 (0.60–1.22)	0.40	0.75 (0.49–1.16)	0.20
TOP1/CEN20					
Continuous		0.90 (0.75–1.09)	0.28	0.74 (0.58–0.95)	0.01
Gain vs. normal		1.00 (0.72–1.39)	0.99	0.67 (0.44–1.01)	0.06
TOP1 FISH stage III	368				
TOP1 signals per cell					
Continuous		0.96 (0.73–1.27)	0.79	0.81 (0.58–1.13)	0.20
Gain vs. normal		0.83 (0.56–1.23)	0.35	0.69 (0.43–1.12)	0.14
TOP1/CEN20					
Continuous		0.91 (0.74–1.12)	0.35	0.74 (0.56–0.97)	0.02
Gain vs. normal		1.04 (0.73–1.48)	0.82	0.70 (0.45–1.09)	0.11
TOP1 mRNA expression stage III	580				
TOP1 mRNA expression					
Continuous		0.85 (0.70–1.03)	0.10	0.74 (0.60–0.92)	0.007
High vs. normal		0.84 (0.61–1.15)	0.28	0.85 (0.59–1.23)	0.38

NOTE: The analyses are made without stratifying for treatment arm. Cutoff values for *TOP1* gene gain: ≥ 4 *TOP1* signals per cell, *TOP1*/CEN20 ratio ≥ 1.5. Cutoff value for high *TOP1* mRNA expression: ≥ third quartile of the observed *TOP1* expression values. The variables are handled linearly in the continuous models.

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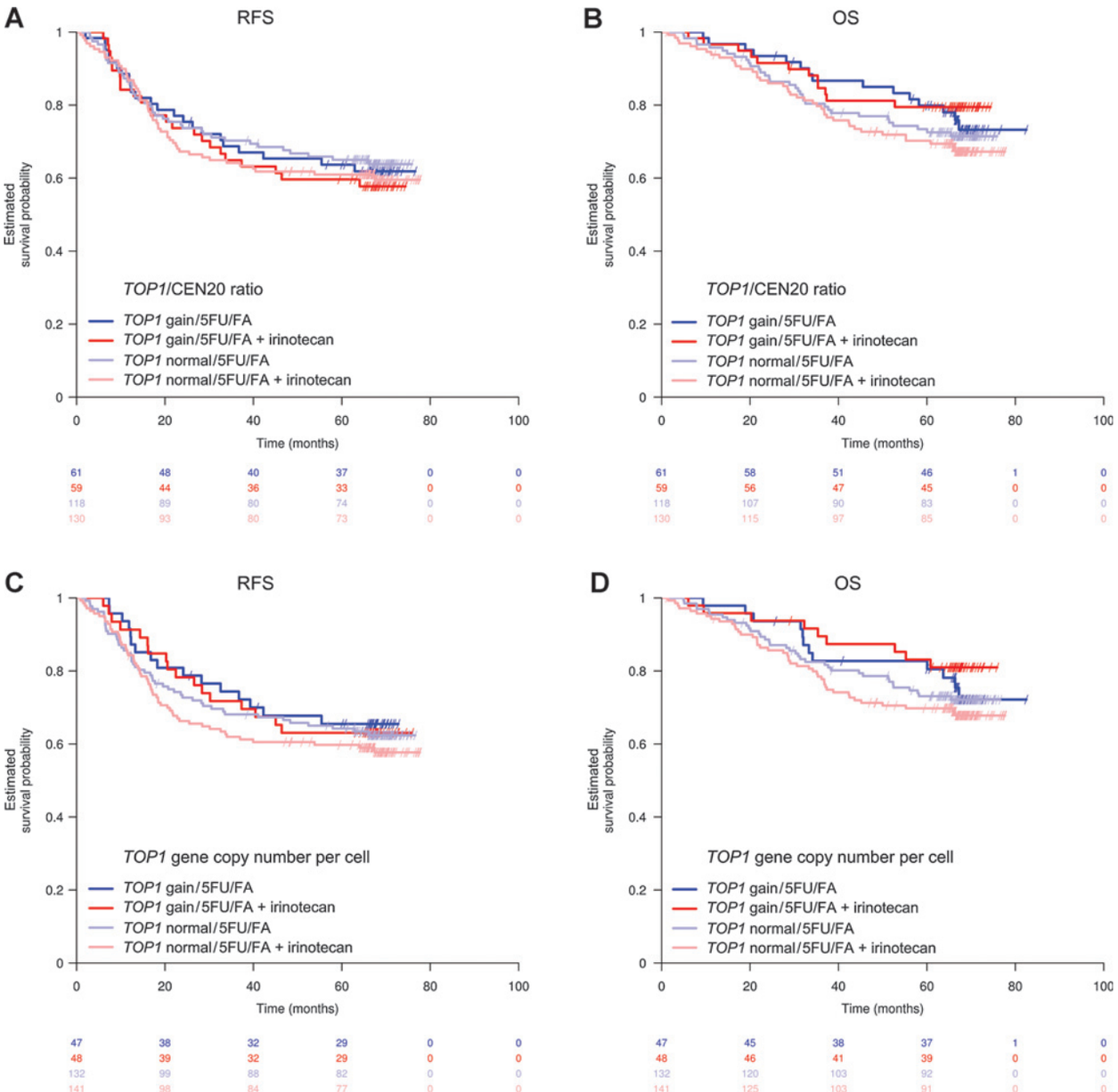

Figure 1.
Kaplan–Meier estimates, *TOP1* FISH stage III. A and B, RFS and OS for treatment group by *TOP1* gene status, stage III. *TOP1* gain: *TOP1*/CEN20 ≥ 1.5; *TOP1* normal: *TOP1*/CEN20 < 1.5. C and D, RFS and OS for treatment group by *TOP1* gene status, stage III. *TOP1* gain: ≥ 4 *TOP1* signals per cell; *TOP1* normal: < 4 *TOP1* signals per cell.

association with OS ($P = 0.38$). However, on a nonsignificant level, the HRs favored high over normal *TOP1* mRNA expression both in relation to RFS and OS (Table 1).

When comparing Kaplan–Meier curves, patients with *TOP1* mRNA high classified tumors showed a trend toward an improvement in RFS and OS when treated with 5FU/FA + irinotecan compared with 5FU/FA alone (Fig. 2). The separation of the survival curves was statistically significant for OS ($P_{\text{log-rank}} = 0.049$), but not for RFS ($P_{\text{log-rank}} = 0.13$). In contrast, the Kaplan–Meier survival curves for treatment stratum in relation to RFS ($P_{\text{log-rank}} = 0.88$) and OS ($P_{\text{log-rank}} = 0.83$) did not separate within the subgroup of patients with tumors classified as *TOP1* mRNA normal (Fig. 2). In the *TOP1* mRNA high subgroup, the relative increase in the survival probabilities at 5 years was 22% (RFS) and 15% (OS) for patients receiving 5FU/FA + irinotecan compared with patients receiving 5FU/FA.

In multivariable models, the benefit of additional irinotecan on OS in the *TOP1* high subgroup was retained when adjusting for tumor localization and *KRAS* status, the only two covariates selected from univariate analysis in this subgroup. The adjusted HR for treatment effect was 0.59 (95% CI, 0.32–1.08; $P_{\text{Wald}} = 0.09$) for RFS and 0.44 (95% CI, 0.21–0.90; $P_{\text{Wald}} = 0.03$) for OS.

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Table 2. Univariate predictive effects of *TOP1* status in relation to recurrence-free survival

		Recurrence-free survival					
	Patients, *n*	**Events, *n***	**5-year rate**	**95% CI**	**HR (95% CI)**	**P_{Wald}**	**$P_{log\ rank}$**
TOP1 FISH stage II + III	534						
TOP1 signals per cell ≥ 4	142						
5FU/FA	70	20	0.73	0.63–0.84			
5FU/FA + irinotecan	72	21	0.70	0.60–0.81	1.07 (0.58–1.97)	0.83	0.83
TOP1 signals per cell < 4	392						
5FU/FA	188	60	0.70	0.63–0.76			
5FU/FA + irinotecan	204	65	0.69	0.63–0.76	1.02 (0.72–1.45)	0.90	0.90
TOP1/CEN20 ≥ 1.5	167						
5FU/FA	83	26	0.70	0.60–0.80			
5FU/FA + irinotecan	84	26	0.69	0.60–0.80	1.03 (0.59–1.77)	0.93	0.93
TOP1/CEN20 < 1.5	367						
5FU/FA	175	54	0.71	0.64–0.78			
5FU/FA + irinotecan	192	60	0.69	0.63–0.76	1.04 (0.72–1.50)	0.84	0.84
TOP1 FISH stage III	368						
TOP1 signals per cell ≥ 4	95						
5FU/FA	47	16	0.65	0.53–0.81			
5FU/FA + irinotecan	48	17	0.63	0.51–0.79	1.08 (0.55–2.14)	0.82	0.82
TOP1 signals per cell < 4	273						
5FU/FA	132	49	0.64	0.57–0.73			
5FU/FA + irinotecan	141	58	0.60	0.52–0.68	1.16 (0.79–1.69)	0.46	0.45
TOP1/CEN20 ≥ 1.5	120						
5FU/FA	61	23	0.65	0.57–0.74			
5FU/FA + irinotecan	59	24	0.61	0.53–0.70	1.13 (0.64–2.01)	0.67	0.67
TOP1/CEN20 < 1.5	248						
5FU/FA	118	42	0.64	0.53–0.77			
5FU/FA + irinotecan	130	51	0.60	0.48–0.74	1.15 (0.76–1.72)	0.51	0.51
TOP1 mRNA expression stage III	580						
TOP1 high	140						
5FU/FA	73	29	0.60	0.49–0.72			
5FU/FA + irinotecan	67	18	0.73	0.63–0.84	0.64 (0.35–1.15)	0.13	0.13
TOP1 normal	440						
5FU/FA	206	80	0.63	0.57–0.70			
5FU/FA + irinotecan	234	92	0.62	0.56–0.68	1.02 (0.76–1.38)	0.88	0.88

NOTE: The relative treatment effects (hazard ratios) are for 5FU/FA + irinotecan vs. 5FU/FA. The *P* values from the Wald test relate to the hazard ratios, whereas the *P* values from the log rank test relate to the estimated Kaplan–Meier functions for the two treatment groups within each *TOP1* subgroup. Cutoff value for high *TOP1* mRNA expression: ≥ third quartile of the observed *TOP1* expression values.

In contrast, the adjusted HRs for treatment effect in the *TOP1* normal subgroup were 1.02 (95% CI, 0.73–1.33; $P_{Wald} = 0.90$) for RFS and 1.02 (95% CI, 0.72–1.44; $P_{Wald} = 0.92$) for OS, thus confirming the lack of benefit of additional irinotecan in this subgroup.

When performing statistical tests for interaction between dichotomized *TOP1* mRNA expression and treatment, the results were nonsignificant both in relation to RFS ($P_{interaction} = 0.16$) and OS ($P_{interaction} = 0.07$). None of the results from the exploratory MFPI models for treatment by *TOP1* mRNA expression were statistically significant. However, the corresponding treatment-effect plots illustrated increasing differential treatment effect in relation to increasing *TOP1* mRNA expression values (Fig. 3). Although the confidence intervals included the log relative hazard of 0 at any *TOP1* mRNA expression value, the trend for benefit of 5FU/FA + irinotecan in patients only with *TOP1* high-expressing tumors was noticeable.

Discussion

Irinotecan is used in the metastatic setting of colorectal cancer, but based on results from several prospective RCTs, irinotecan is not recommended for adjuvant therapy of stage II/III colon cancer (15–18). In the PETACC3 trial, only a nonsignificant trend was observed in favor of additional irinotecan, suggesting that a small subgroup of patients might obtain benefit from the irinotecan doublet. Thus, in order to identify this subpopulation, we have searched for predictive biomarkers for irinotecan both in preclinical models of SN-38 resistant colorectal cancer cell lines and in clinical study cohorts (34, 47). Based on published data and results from our preclinical studies, we raised the hypothesis that Top1, being the sole known target for irinotecan, represents a putative key predictive biomarker for drug efficacy. Because no validated anti-Top1 antibodies for IHC are available (30), we analyzed the predictive property of *TOP1* FISH and *TOP1* mRNA expression status as a proxy for Top1 protein in relation to additional irinotecan in the adjuvant setting of colon cancer.

We applied our validated *TOP1* FISH probe combination to available FFPE material from patients enrolled in the PETACC3 trial (17). The assay has undergone substantial analytical validation also using samples collected and analyzed in real time (refs. 32, 48; EudraCT number 2012-002348-26). According to predefined cutoff values, no significant differential treatment effects were demonstrated in relation to the *TOP1* gene copy number or the *TOP1*/CEN20 ratio in univariate analyses. Although simplifying statistical analyses, disadvantages of dichotomization of continuous variables have been addressed for subgroup analyses in stratified medicine research (41, 43, 49). Among several pitfalls is the increased risk of type II errors as a result of the reduction in the statistical power. To ensure that the negative results were not caused by a biologically nonrelevant subgroup dichotomization and to decrease the risk of type II errors by using

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Table 3. Univariate predictive effects of *TOP1* status in relation to OS

		OS					
	Patients, *n*	**Events, *n***	**5-Year rate**	**95% CI**	**HR (95% CI)**	**P_{Wald}**	**$P_{log\ rank}$**
TOP1 FISH stage II + III	534						
TOP1 signals per cell ≥ 4	142						
5FU/FA	70	15	0.86	0.78–0.94			
5FU/FA + irinotecan	72	12	0.84	0.76–0.93	0.78 (0.63–1.66)	0.51	0.51
TOP1 signals per cell < 4	392						
5FU/FA	188	44	0.77	0.72–0.84			
5FU/FA + irinotecan	204	49	0.77	0.71–0.83	1.06 (0.70–1.59)	0.79	0.79
TOP1/CEN20 ≥ 1.5	167						
5FU/FA	83	15	0.85	0.78–0.93			
5FU/FA + irinotecan	84	14	0.83	0.75–0.91	0.95 (0.46–1.96)	0.88	0.88
TOP1/CEN20 < 1.5	367						
5FU/FA	175	44	0.77	0.71–0.83			
5FU/FA + irinotecan	192	47	0.77	0.71–0.83	0.99 (0.66–1.49)	0.96	0.96
TOP1 FISH stage III	368						
TOP1 signals per cell ≥ 4	95						
5FU/FA	47	12	0.83	0.73–0.94			
5FU/FA + irinotecan	48	9	0.83	0.73–0.94	0.72 (0.30–1.70)	0.45	0.45
TOP1 signals per cell < 4	273						
5FU/FA	132	36	0.73	0.66–0.81			
5FU/FA + irinotecan	141	44	0.70	0.63–0.78	1.20 (0.77–1.86)	0.42	0.42
TOP1/CEN20 ≥ 1.5	120						
5FU/FA	61	15	0.80	0.70–0.91			
5FU/FA + irinotecan	59	12	0.79	0.70–0.91	0.83 (0.39–1.78)	0.63	0.63
TOP1/CEN20 < 1.5	248						
5FU/FA	118	33	0.73	0.66–0.82			
5FU/FA + irinotecan	130	41	0.70	0.63–0.79	1.17 (0.74–1.84)	0.51	0.51
TOP1 mRNA expression stage III	580						
TOP1 high	140						
5FU/FA	73	24	0.72	0.62–0.83			
5FU/FA + irinotecan	67	12	0.83	0.75–0.93	0.51 (0.25–1.02)	0.056	0.049
TOP1 normal	440						
5FU/FA	206	61	0.74	0.68–0.80			
5FU/FA + irinotecan	234	69	0.71	0.66–0.77	1.04 (0.74–1.46)	0.83	0.83

NOTE: The relative treatment effects (hazard ratios) are for 5FU/FA + irinotecan vs. 5FU/FA. The *P* values from the Wald test relate to the hazard ratios, whereas the *P* values from the log rank test relate to the estimated Kaplan–Meier functions for the two treatment groups within each *TOP1* subgroup. Cutoff value for high *TOP1* mRNA expression: ≥ third quartile of the observed *TOP1* expression values.

the full statistical power of the dataset, exploratory MFPI modeling was performed. This allowed for simultaneous testing of both linear and nonlinear interaction models, where the *TOP1* FISH variables were retained on a continuous scale. None of the adjusted best-fit models identified a significant treatment by *TOP1* interaction, which weighs against a predictive property of *TOP1* FISH status in relation to irinotecan. The suggestions of a prognostic effect of *TOP1* FISH status, that is independent of treatment, are interesting and confirmed previous findings (48), but the clinical utility of the information is not apparent.

As stated, the reason why FISH was considered an alternative to IHC was the analytical limitations applying to the most commonly used anti-Top1 antibodies (30). However, limitations apply to *TOP1* FISH analysis as well. *In silico* analysis in the Broad Institute Tumorscape database does not identify *TOP1* as being focally amplified in colorectal cancer, but increased *TOP1* copy numbers are identified frequently as a result of gains spanning large parts of 20q that sometimes include the centromere region (50, 51). This suggests that the *TOP1* FISH probe does not provide exclusive *TOP1* specific information, but rather provides generic information about 20q aberrations that include the *TOP1* genomic sequence. Based on our previous observations such aberrations may still be relevant in relation to prediction of irinotecan response, but we were not able to confirm this in the setting of the PETACC3 trial (48).

In FISH analysis intended for gene enumeration, a gene probe and a same chromosome reference centromere probe is traditionally used in combination to correct for chromosome specific aneusomy. The gene-to-centromere ratio is a surrogate measure of the gene-to-chromosome relationship, and ratios of ≥1.5 suggest the presence of at least one extra gene copy per disomic chromosome set. Because the broad 20q gains may in some cases overlap with the centromere region, CEN20 is not ideal for normalization (52). For this reason we chose to include the uncorrected raw *TOP1* gene copy number data in the statistical analyses. A parallel to this single gene approach may be drawn to *HER2* in situ hybridization (ISH) testing in breast cancer where clinical utility is widely accepted for the identification of patients eligible for trastuzumab therapy (53). However, there is still debate about how to interpret the test results (54). A chromosome 17 centromere probe (CEN17) is used as a reference when evaluating *HER2* status by dual-probe ISH (53, 54). However, gain of CEN17 is more often a result of focal peri-centromeric duplication or 17q gains spanning the centromere region rather than of true polysomy (53). A clinical implication of these observations is the risk of reporting a false negative *HER2* status when relying only on the *HER2*/CEN17 ratio. This concern has contributed to the 2013 revision of the American Society of Clinical Oncology/College of American Pathologists recommendations for *HER2* ISH testing in breast cancer, where a *HER2* gene

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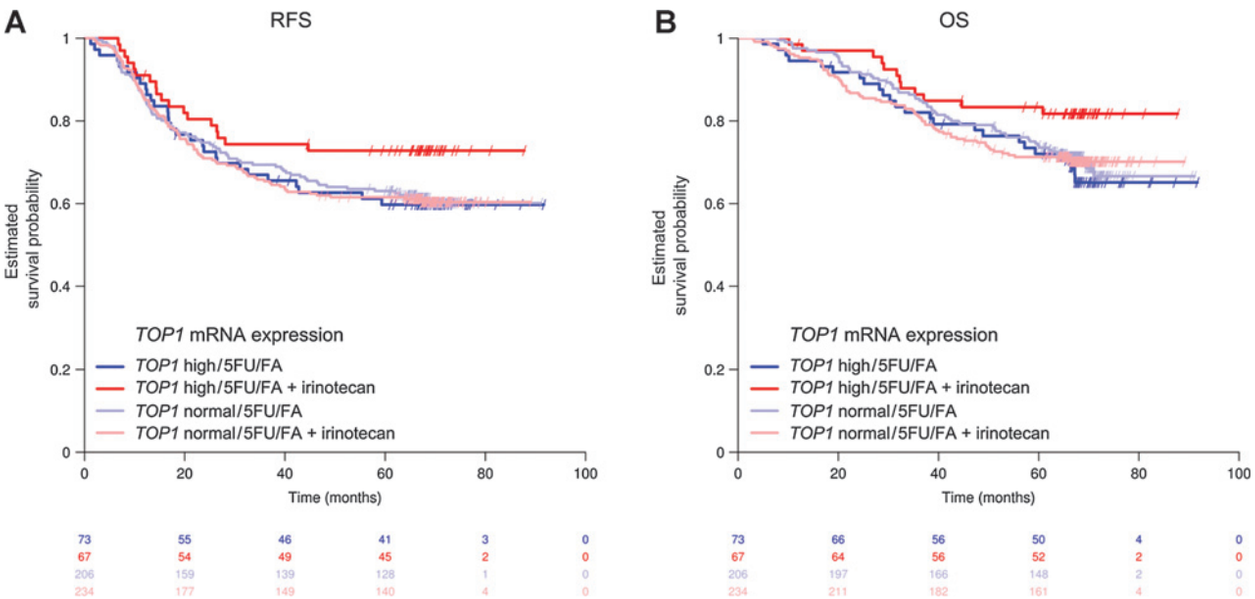

Figure 2.
Kaplan–Meier estimates, *TOP1* mRNA expression stage III. A and B, RFS and OS for treatment group by *TOP1* mRNA status. *TOP1* high: *TOP1* mRNA expression ≥ third quartile of the observed *TOP1* expression values; *TOP1* normal: *TOP1* mRNA expression < third quartile of the observed *TOP1* expression values.

copy number ≥6 signals per cell is now regarded as an unequivocally ISH positive result regardless of the *HER2*/CEN17 ratio. (53). In addition, a *HER2* gene copy number ≥ 4 signals per cell is considered an equivocal result that requires reflex testing (53).

In the present study, we chose ≥4 *TOP1* signals per cell as the cutoff value for *TOP1* gene gain for two reasons. Firstly, this cutoff value identified 24% of stage III colon cancer with *TOP1* gene gain when using data from one of our previously published studies (48). Although a direct comparison cannot be made to the adjuvant setting, this number is close to the 31% overall response rate of FOLFIRI in patients with chemo-naïve advanced colorectal cancer reported by Colucci and colleagues (13). Secondly, this cutoff value is in line with the lowest threshold for equivocal *HER2* ISH results (53). For *TOP1* mRNA expression, the cutoff value was chosen to reflect the expected frequency of tumors with *TOP1* gene gain determined by FISH analysis. This was done to ensure that a similar proportion of patients would have a potential favorable marker status for both methods of analysis.

The available mRNA gene expression data from the PETACC3 material provided us with the opportunity to test for statistical

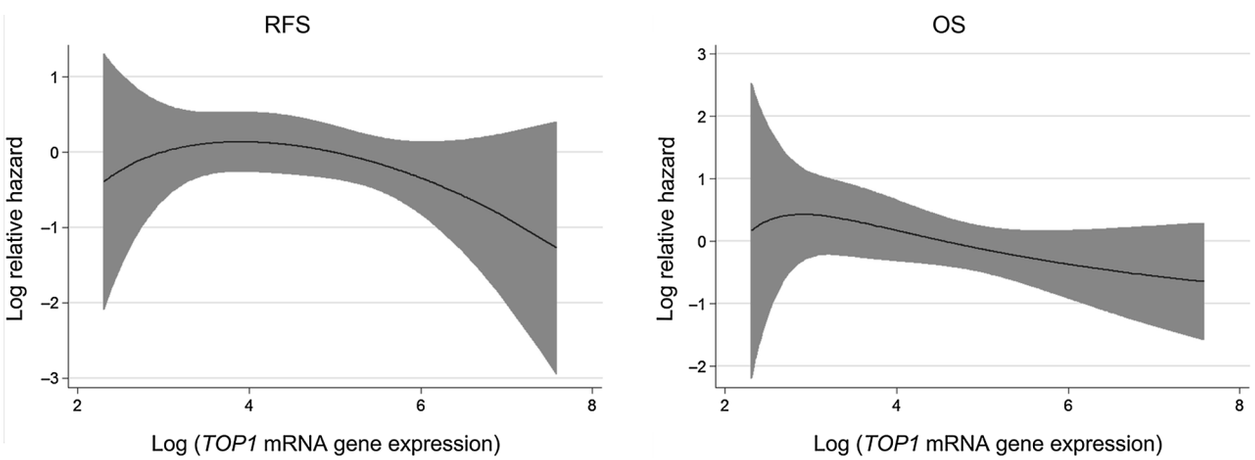

Figure 3.
Treatment-effect plots. Multivariable fractional polynomial interaction (MFPI) treatment-effect plots for treatment effect by *TOP1* mRNA expression status in the *TOP1* mRNA expression stage III population. The curves show the relative hazard (hazard ratio) on a logarithmic scale for 5FU/FA + irinotecan versus 5FU/FA at different values of *TOP1* mRNA gene expression. The shaded areas represent the point-wise 95% confidence intervals. The plots were generated from MFPI second-degree fractional polynomial functions. The RFS model was adjusted for N stage, *KRAS* status, and MSI status ($n = 521$). The OS model was adjusted for N stage, *KRAS* status, and *BRAF* status ($n = 519$). The adjusting covariates were selected based on significant prognostic effects in full models, including: N stage, tumor localization, and status of: *TOP1*, *BRAF*, *KRAS*, and MSI. *P* values for interaction: RFS, $P = 0.34$; OS, $P = 0.26$. RFS: recurrence-free survival.

dependence between *TOP1* gene status, as measured by the two FISH parameters, and *TOP1* mRNA status. No apparent correlations were observed between *TOP1* gene expression and the *TOP1* gene copy number or the *TOP1*/CEN20 ratio. This suggests that *TOP1* mRNA expression is not predominantly dependent on 20q gains involving the *TOP1* locus.

Because *TOP1* mRNA may be a more accurate measure of the amount of target for irinotecan than the gene copy number, the statistical analysis plan was also set up to explore a potential predictive property of *TOP1* mRNA expression. Benefit of 5FU/FA/irinotecan was observed only in the *TOP1* mRNA high subgroup (RFS: $HR_{adjusted}$, 0.59 (95% CI, 0.32–1.08; $P_{Wald} = 0.09$) and OS: $HR_{adjusted}$ (95% CI, 0.21–0.90; $P_{Wald} = 0.03$). The trend for a *TOP1* mRNA-dependent differential treatment effect was supported by the treatment-effect plots from the MFPI models. These results are in line with previous analysis in the neoadjuvant setting of colorectal cancer, where high pretreatment tumor levels of *TOP1* mRNA were associated with improved response from irinotecan-based therapy (33).

The foremost strength of this study was the use of data generated from unique tumor material that was prospectively collected during the accrual of patients for a RCT that included relevant treatment stratification for evaluation of predictive biomarkers for irinotecan. The A + B versus A randomization strategy of the PETACC3 trial fulfilled the design criteria proposed by Simon and colleagues (35) for prospective–retrospective clinical validation of predictive biomarkers for B, in this case irinotecan. Furthermore, all biomarker populations were composed of patients who were representative of the patients accrued to the PETACC3 trial as a whole, i.e., randomization according to baseline patient and tumor characteristics was retained and the treatment stratification was well balanced.

Predictive biomarker studies in the adjuvant setting are inherently limited by the fact that the patients fall into at least three subsets: those who are cured by surgery alone, those who relapse despite of chemotherapy, and those who benefit from chemotherapy. Especially the first group may influence the results of the statistical analyses as the outcome is not dependent on the status of the predictive biomarker in question. Although the biomarker populations were composed of up to 580 patients, the recorded number of RFS and OS events could not ensure enough statistical power to control well for type II errors. Simon and colleagues (35) did not define an exact number of samples necessary for analysis, but access to material from approximately two thirds of the original study population was recommended, a number that we were not able to meet in the present study. In addition, the lack of statistical power limited us to the use of only few adjusting variables in the multivariable models.

In relation to additional irinotecan in the adjuvant setting of stage II and III colon cancer, this study failed to demonstrate a predictive property of *TOP1* gene copy number status, assessed by FISH. We find the trends supporting a predictive property of *TOP1* mRNA expression a positive finding. However, the results can only be viewed as hypothesis generating, but in our opinion further exploration of the differential treatment effect of irinotecan-based therapy in relation to *TOP1* mRNA expression is highly warranted. Based on *TOP1* mRNA expression, it might be possible to identify patients who benefit from irinotecan in the adjuvant setting of colon cancer. However, before drawing definite conclusions on the clinical utility of *TOP1* mRNA expression status, the reported results need to be further studied in multiple independent patient cohorts. In future perspectives, *TOP1* mRNA expression, possibly on a continuous scale, might become a key biomarker in a broader panel of markers that will help clinicians decide between irinotecan- and oxaliplatin-based doublets not only in the adjuvant setting but also in the metastatic setting of colorectal cancer.

Disclosure of Potential Conflicts of Interest

M. Delorenzi has ownership interest (including patents) in Novartis and Roche. N. Brunner is CEO at WntResearch and CSO at Oncology Venture. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: S.B. Nygård, B. Vainer, N. Brünner, E. Budinska
Development of methodology: S.B. Nygård
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.B. Nygård, B. Vainer, F. Bosman, S. Tejpar, A. Roth, M. Delorenzi, N. Brünner
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.B. Nygård, B. Vainer, F. Bosman, N. Brünner, E. Budinska
Writing, review, and/or revision of the manuscript: S.B. Nygård, B. Vainer, F. Bosman, S. Tejpar, A. Roth, M. Delorenzi, N. Brünner, E. Budinska
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.B. Nygård, S.L. Nielsen, N. Brünner
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DNA Topoisomerase I Gene Copy Number and mRNA Expression Assessed as Predictive Biomarkers for Adjuvant Irinotecan in Stage II/III Colon Cancer

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