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Topoisomerase 1 Promoter Variants and Benefit from Irinotecan in Metastatic Colorectal Cancer Patients

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Key Words

Metastatic colorectal cancer · *Topoisomerase 1* · FOLFIRI · Single-nucleotide polymorphism · Transcription factor binding

Abstract

Objective: *Topoisomerase 1* (*topo-1*) is an important target for the treatment of metastatic colorectal cancer (CRC). The aim of the present study was to evaluate the correlation between *topo-1* single-nucleotide polymorphisms (SNPs) and clinical outcome in metastatic CRC (mCRC) patients. **Methods:** With the use of specific software (PROMO 3.0), we performed an in silico analysis of *topo-1* promoter SNPs; the rs6072249 and rs34282819 SNPs were included in the study. DNA was extracted from 105 mCRC patients treated with FOLFIRI ± bevacizumab in the first line. SNP genotyping was performed by real-time PCR. Genotypes were correlated with clinical parameters (objective response rate, progression-free survival, and overall survival). **Results:** No single genotype was significantly associated with clinical variables. The G allelic variant of rs6072249 *topo-1* SNP is responsible

for GC factor and X-box-binding protein transcription factor binding. The same allelic variant showed a nonsignificant trend toward a shorter progression-free survival (GG, 7.5 months; other genotypes, 9.3 months; HR 1.823, 95% CI 0.8904–3.734; $p = 0.1$). **Conclusion:** Further analyses are needed to confirm that the *topo-1* SNP rs6072249 and transcription factor interaction could be a part of tools to predict clinical outcome in mCRC patients treated with irinotecan-based regimens.

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Introduction

Colorectal cancer (CRC) is one of the most common causes of death by cancer in Western countries [1]. Despite recent advances, the prognosis remains poor for metastatic CRC (mCRC) patients, with a median overall survival (OS) of approximately 30 months in most recent studies [1]. Combination chemotherapy with a fluoropyrimidine [5-fluorouracil (5-FU) or capecitabine] and oxaliplatin (LOHP) or irinotecan (CPT-11) is the mainstay

of treatment for mCRC patients. Combining chemotherapy with the anti-vascular endothelial growth factor (VEGF) antibody bevacizumab may further improve outcome in the advanced disease setting [2]. Clinical trials conducted on mCRC patients demonstrated higher activity and efficacy for LOHP- or CPT-11-based doublets with biologics, with response rates (RRs) around 40–50% and a median OS around 24 months [3].

Topoisomerase 1 (topo-1) is the specific target of CPT-11. CPT-11 is a pro-drug that is converted by hydrolysis to SN-38 (active metabolite) by carboxylesterases. SN-38 is inactivated through glucuronidation by uridine diphosphate-glucuronosyltransferase. CPT-11 binds the 'DNA-*topo-1* cleavage complex', leading to irreversible DNA damage and cell death [4]. Some studies showed a correlation between DNA *topo-1* activity and sensitivity to CPT-11 [5]. It is well known that *topo-1* gene copy number may be correlated with the sensitivity to CPT-11 [6] and that a low level of *topo-1* expression is associated with the development of resistance to CPT-11 [7]. Epigenetic regulation of *topo-1* promoter is crucial for determining transcription factor binding (TFB), gene expression, and CPT-11 sensitivity in CRC cells [8]. However, most mCRC patients did not achieve an objective response [9]. Moreover, CPT-11 efficacy is burdened by a potentially severe toxicity. The individualization of cytotoxic therapy in patients with cancer (and in particular in mCRC, where different options are available) is essential for the success of the therapy and the avoidance of futile toxicity for patients with nonsensitive disease.

Based on the identification of molecular markers including *KRAS*, *BRAF*, and *PI3K* mutations and the expression of *topo-1*, ERCC-1, and thymidylate synthase, the efforts of the clinicians and pharmacologists should be directed towards the validation of putative predictive biomarkers, and they should combine them so as to obtain a shared therapeutic algorithm. Indeed, research on personalized treatment of mCRC patients beyond the confirmed negative predictive role of *RAS* mutations for anti-epidermal growth factor receptor (EGFR) antibodies is far from conclusive [10].

Based on the pivotal role of *topo-1* in mCRC treatment, we searched for *topo-1* single-nucleotide polymorphisms (SNPs) located in the promoter region in order to analyze if *topo-1* genetic variants may affect gene expression and, therefore, clinical outcome in mCRC patients treated with CPT-11-based regimens. Moved by this idea, we aimed at studying the role of two *topo-1* SNPs in mCRC patients treated with first-line FOLFIRI ± bevacizumab.

Materials and Methods

Patients and Methods

Patients with histologically confirmed metastatic colorectal adenocarcinoma receiving first-line FOLFIRI (CPT-11 180 mg/sqm i.v. on day 1, leucovorin 200 mg/sqm i.v. on day 1, and 5-FU 400 mg/sqm bolus i.v. on day 1, followed by 5-FU 2,400 mg/sqm 46-hour continuous infusion i.v. on days 1–3 or 5-FU 3,200 mg/sqm 48-hour continuous infusion i.v. on days 1–3) with or without bevacizumab 5 mg/kg i.v. on day 1 (cycles repeated every 2 weeks) were enrolled in the study. Eligibility requirements included: age ≥18 years; Eastern Cooperative Oncology Group performance status 0–2; life expectancy of at least 3 months, and signed informed consent for genetic analyses. Blood samples were anonymously stored at –20°C in laboratories of the Unit of Clinical Pharmacology and Pharmacogenetics, Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy.

Response evaluation was based on investigator-reported assessment by RECIST v.1.0 criteria [11]: carcinoembryonic antigen measurements and contrast-enhanced total-body computed tomography scans were performed every 2 months until disease progression. Progression-free survival (PFS) was considered as the interval ranging from the date of treatment start until the evidence of disease progression or death from any cause, whichever occurred first; OS was considered as the interval ranging from the date of treatment start until death from any cause.

DNA Isolation

Genomic DNA was extracted from peripheral venous blood samples (5 ml) from 105 mCRC patients. DNA was isolated with QIAamp DNA mini kit (Qiagen). The purity and the quantity of DNA were read at 260 and 280 nm with a NanoDrop spectrophotometer (Thermo Scientific). The contamination by proteins was evaluated by the calculation of the 260/280 ratio.

In silico Analysis

Topo-1 SNPs were screened with specific software. We performed an in silico functional characterization based on missense mutations, TFB, and miRNA binding. PROMO 3.0 software (http://algggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) [12, 13]; we considered human factors and human binding sites with a maximum matrix dissimilarity rate of 15), GeneCard (<http://www.genecards.org/>), and MicroSNiPer (<http://epicenter.ie-freiburg.mpg.de/services/microsniper/>) [14] were employed.

SNP Genotyping

The *topo-1* SNPs g.3881A>G (rs6072249) and g.5164C>A (rs34282819) were analyzed with real-time PCR (Applied Biosystems). The PCR reactions were done using 20 ng of genomic DNA diluted in 11.875 µl DNase/RNase-free water, 12.5 µl of TaqMan Universal Master Mix (Applied Biosystems) with AmpliTaq Gold, and 0.625 µl of the SNP genotyping assay mix (specific primers and probe) in 25 µl total volume (protocol: http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042998.pdf). The allelic content of each sample in the plate was determined by reading the generated fluorescence.

Statistical Analysis

A χ^2 test for trend was used to evaluate the association of investigated SNPs with RRs, PFS and OS curves were obtained with the

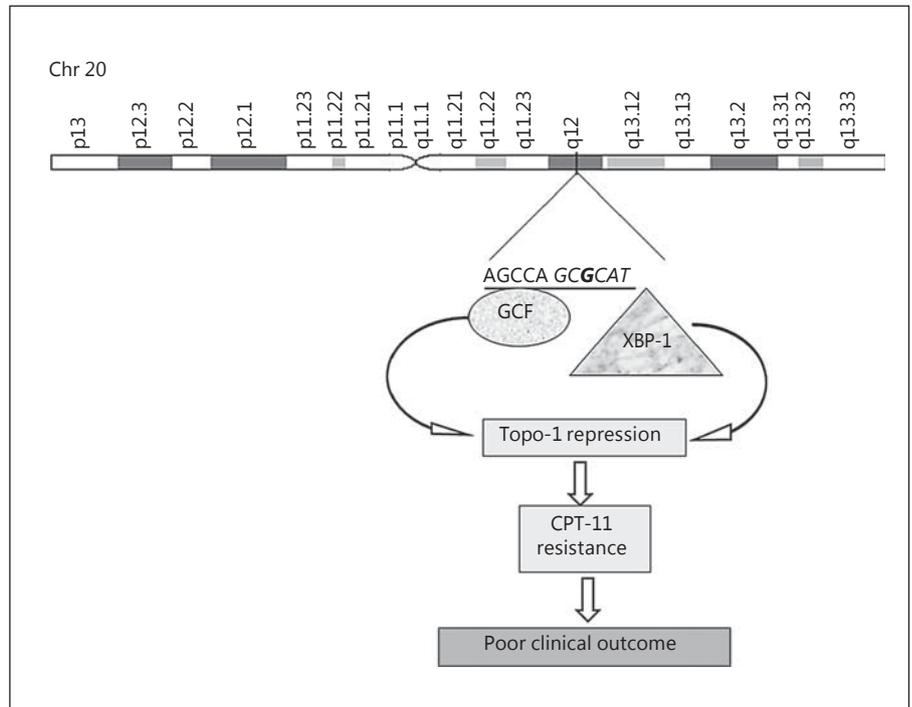


Fig. 1. Hypothesis of interaction between the g.3881A>G *topo-1* SNP and TFB leading to a shorter PFS: patients carrying the g.3881G/G genotype, through GCF and XBP-1 binding, could express lower levels of *topo-1* and be resistant to CPT-11.

Kaplan-Meier method, and we used the log-rank test (significant p value <0.05 for a two-tailed test) to compare the survival distributions between the different genotypes. GraphPad Prism 6 software was used for statistical analyses.

We obtained written informed consent from all patients for genetic polymorphism analysis. The study was approved by the ethical committee of the Pisa University Hospital and was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Results

Patients' Characteristics

A total of 105 patients were enrolled in the study. Patient characteristics are listed in table 1. Overall, 50 patients achieved an objective response (4 complete and 46

disease stabilization as best response and 20 patients (19%) progressed during treatment. Because of the limitation of the small sample size of the bevacizumab cohort, a nonsignificant difference in RR was noted between the FOLFIRI and the FOLFIRI plus bevacizumab groups (45 vs. 78%; p=0.082). At a median follow-up of 38.7 months, all patients experienced disease progres-

Table 1. Patient characteristics

| | Genotype for g.3881A>G | | |
|--------------------------------|------------------------|------------|------------|
| | AA | AG | GG |
| Patients | 46 | 46 | 13 |
| Median age (range), years | 66 (49–78) | 66 (45–80) | 64 (80–35) |
| Gender | | | |
| Male | 30 | 28 | 8 |
| Female | 16 | 18 | 5 |
| ECOG performance status | | | |
| 0 | 37 | 34 | 11 |
| 1 | 8 | 11 | 1 |
| 2 | 1 | 1 | 0 |
| Involved organs | | | |
| 1 | 21 | 25 | 9 |
| 2 | 17 | 15 | 0 |
| ≥3 | 8 | 6 | 4 |
| Previous adjuvant chemotherapy | | | |
| Yes | 12 | 15 | 2 |
| No | 34 | 31 | 11 |
| FOLFIRI | 43 | 40 | 13 |
| FOLFIRI+bevacizumab | 3 | 6 | 0 |

ECOG=Eastern Cooperative Oncology Group.

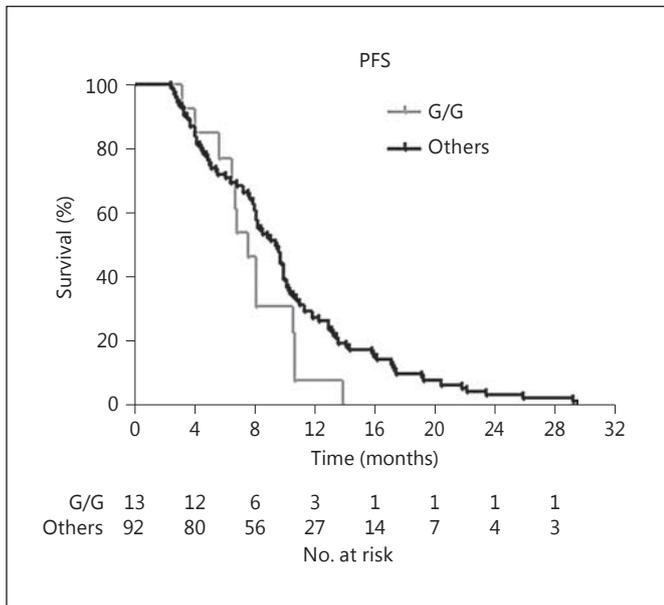


Fig. 2. PFS of g.3881A>G *topo-1* SNP variants: G/G versus other genotypes.

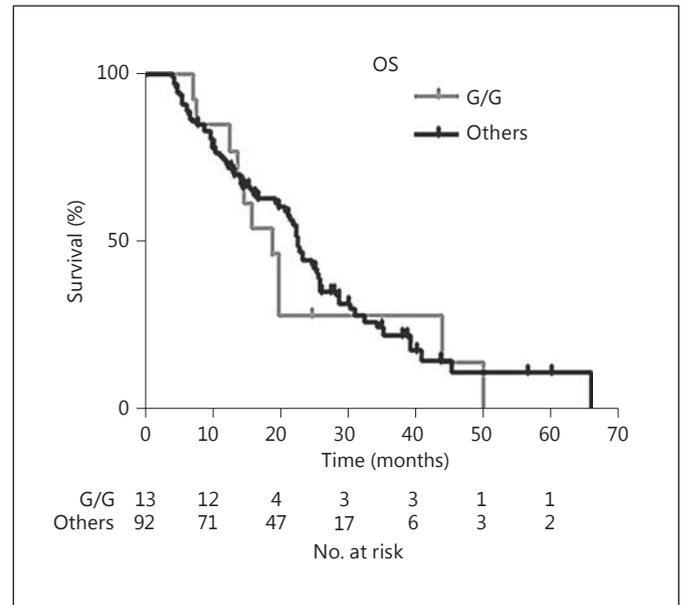


Fig. 3. OS of g.3881A>G *topo-1* SNP variants: G/G versus other genotypes.

Table 2. Distribution of different genotypes for studied SNPs

| | n | % |
|------------------------|----|----|
| Genotype for g.5164C>A | | |
| AA | 90 | 90 |
| AC | 8 | 8 |
| CC | 2 | 2 |
| Genotype for g.3881A>G | | |
| AA | 46 | 44 |
| AG | 46 | 44 |
| GG | 13 | 12 |

sion, and 79 patients died; the median PFS and OS were 8.8 and 21.7 months, respectively.

In silico Characterization of g.3881A>G and g.5164C>A SNPs

With an *in silico* analysis, we selected two *topo-1* SNPs that showed functional relevance in TFB change, and we evaluated the impact of these SNPs in 105 mCRC patients treated with first-line FOLFIRI ± bevacizumab. The selected SNPs are in Hardy-Weinberg equilibrium. We investigated with PROMO 3.0 whether the SNPs located in the *topo-1* promoter may affect TFB. We selected 2 SNP variants, namely g.3881A>G and g.5164C>A.

The g.3881A>G SNP is located on chromosome 20 in the 2-kb upstream variant. The g.3881G allele is responsible for GC factor (GCF) and X-box-binding protein 1 (XBP-1) TFB (fig. 1). The g.5164A allele is responsible for peroxisome proliferator-activated receptor α (PPAR-α) and retinoid X receptor α (RXR-α) TFB (fig. 1).

SNP Genotype Correlation with Clinical Outcome

Prevalences of different genotypes for g.5164C>A and g.3881A>G are listed in table 2. We genotyped 105 mCRC patients for the g.3881A>G SNP and 100 mCRC patients for the g.5164C>A SNP. For the g.5164C>A SNP, we excluded 5 DNA samples whose genotype was unclear. No significant associations were found between g.5164C>A and clinical parameters (RR, PFS, and OS; $p > 0.05$; data not shown). Concerning the g.3881A>G SNP, the median PFS of patients carrying the A/A variant ($n = 46$; events = 46) was 8.9 months; for the A/G variant ($n = 46$; events = 46), it was 9.7 months, and for the G/G variant ($n = 13$; events = 13), it was 7.5 months (log-rank $p = 0.037$). This trend was retained also when patients receiving bevacizumab were excluded from the analysis (the median PFS for A/A, A/G, and G/G patients was 8.2, 8.9, and 7.5 months; log-rank $p = 0.093$). Moreover, we noticed some interesting, although not statistically significant, differences between the three genotypes of g.3881A>G con-

cerning OS. The median OS of patients carrying the A/A genotype (n=46; events=38) was 16.6 months; for the A/G genotype (n=46; events=30), it was 23.1 months, and for the G/G genotype (n=13; events=11), it was 18.7 months (log-rank $p=0.053$). Considering the gene dosage effect, no single genotype was significantly associated with clinical variables. However, when compared to all other genotypes, the g.3881G/G allelic variant showed a nonsignificant trend with a shorter PFS (median for GG, 7.5 months vs. for others, 9.3 months; HR 1.823, 95% CI 0.8904–3.734; $p=0.1$; fig. 2) and OS (median for GG, 18.7 months vs. for others, 22.4 months; HR 0.8348, 95% CI 0.3059–1.364; $p=0.7$; fig. 3). As regards RR, no difference was noted between the three different genotypes of the g.3881A>G SNP (G/G vs. A/G vs. A/A: 54 vs. 50 vs. 43%; $p=0.73$). Even after grouping the A/G and A/A genotypes, the G/G genotype was not associated with RR (G/G vs. others: 54 vs. 47%; odds ratio 1.3295, 95% CI 0.4148–4.2615; $p=0.769$).

Discussion

Since SNP genotyping can be easily performed from blood samples and is more convenient to adopt in clinical practice, we explored the role of *topo-1* SNPs in mCRC patients with regard to clinical outcome. Considering the gene dosage effect, no single genotype was significantly associated with clinical parameters of activity and efficacy. This is consistent with a previous study on *topo-1* pharmacogenetics, where no significant relationship was found between the *topo-1* polymorphisms studied and *topo-1* expression, with an unidentified association with drug cytotoxicity [15]. In our study, these results could be partly due to the small sample size of the population included.

Interestingly, we found that the g.3881G/G genotype showed a trend-like association with shorter PFS. Even though the lack of bevacizumab in the G/G patient cohort could partly explain this result, several additional considerations should be considered. Indeed, the g.3881G allele is located in the binding region of XBP-1 and GCF and could, therefore, modulate this binding. Since binding sites are known to interact frequently with different factors in different contexts, it is possible that XBP-1 and GCF binding could trigger mechanisms related to a different prognosis and response to treatment. The name GCF is correlated with the ability of this transcription factor to bind to GC-rich sequences [16]. GCF is a nuclear phosphoprotein able to repress transcription and to de-

crease mRNA levels of several genes including *transforming growth factor- α* (*TGF- α*), *insulin-like growth factor-II* (*IGF-II*), and *c-met* in gastric tumor cell lines and EGFR [17, 18]. GCF plays a critical role in the regulation of cell growth, even if the molecular mechanism is not completely understood. By increasing GCF binding, the *topo-1* g.3881G allele could contribute to *topo-1* repression. Since GCF is able to repress transcription of *topo-1* and CPT-11 sensitivity is correlated with *topo-1* expression [17, 18], patients carrying the g.3881G/G genotype could express lower levels of *topo-1* and, therefore, be resistant to CPT-11. This mechanism could be at the basis of the shorter PFS observed in mCRC patients treated with the FOLFIRI regimen (fig. 1).

XBP-1 is a transcription factor identified as a basic region-leucine zipper that binds to the X2 box in the promoter region of several proteins regulating its expression [19]. XBP-1 is essential for hepatocyte growth [20] and for plasma differentiation [21], and it is a transcriptional regulator of endoplasmic reticulum stress. Recent studies have shown XBP-1 overexpression in breast carcinoma [22] and in CRC [23]. XBP-1 could play an interesting role in CRC, accelerating the cell growth [23] (fig. 1). Interestingly, also XBP-1 was shown to repress gene expression of target genes. XBP-1 binding of specific DNA sequences may lead to modulation of different genes [24], and its upregulation leads to cell death [25]. It is conceivable that XBP-1 inhibits *topo-1* expression in CRC cells. In patients carrying at least one G allele, increased XBP-1 binding may contribute to *topo-1* downregulation and CPT-11 insensitivity in g.3881GG patients [26]. Indeed, also in this case, we need further mechanistic studies to explain and validate the interaction of transcription factors with the *topo-1* g.3881A>G SNP in mCRC patients.

We are conscious that our study has several limitations. Even if we had identified a trend towards a significant prognostic factor for PFS, this result should be confirmed in larger patient series. Moreover, investigations of biological mechanisms and consequences of the g.3881A>G SNP might contribute to supporting our finding.

In conclusion, we identified a variant of the *topo-1* g.3881A>G SNP showing a trend towards an association with shorter PFS among mCRC patients treated with FOLFIRI±bevacizumab. If these results are confirmed in larger series, the g.3881GG variant could be part of tools predicting the clinical outcome of mCRC patients treated with CPT-11-based therapy.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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