Carboxylesterase Isoform 2 mRNA Expression in Peripheral Blood Mononuclear Cells Is a Predictive Marker of the Irinotecan to SN38 Activation Step in Colorectal Cancer Patients

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Abstract

Purpose: Irinotecan (CPT11) is a prodrug activated in humans mainly by carboxylesterase 2 (CES2) generating the SN38 metabolite responsible for the drug efficacy and toxicity. The inter-patients variability in CPT11 activation step could cause unpredictable toxicity. To identify a predictive molecular marker for CPT11 activation in cancer patients, we investigated the CES2 mRNA expression in peripheral blood mononuclear cells (PBMC) and correlated it to CPT11 activation rate, toxic effects, and response.

Experimental Design: Forty-five colorectal cancer patients were treated with a CPT11-including regimen (FOLFIRI). CES2 mRNA expression in PBMC was quantified by reverse transcription-PCR in real time. Plasma concentrations of CPT11, SN38, and SN38-glucuronide were determined by high-performance liquid chromatography and the pharmacokinetic variables calculated adopting the noncompartmental model. Toxicity was evaluated by the National Cancer Institute Common Toxicity Criteria scale and response by the WHO criteria.

Results: A high interindividual variability in CES2 mRNA relative expression was observed (median, 1.45; range, 0.01-28.21). CES2 mRNA expression level was significantly associated with CPT11 activation ratio \([\frac{\text{AUC}_{\text{SN38}} + \text{AUC}_{\text{SN38G}}}{\text{AUC}_{\text{CPT11}}}]\). Patients with CES2 mRNA expression above the median cutoff value presented an activation ratio higher (median, 0.25; range, 0.15-0.42) than those with CES2 mRNA below the median (median, 0.20; range, 0.10-0.40; \(P = 0.013\)). This was associated with a nonsignificant trend of 1.34-fold increase of SN38 AUC in the group of patients with high CES2 mRNA expression (mean, 1.03±0.62 versus 0.77±0.32 \(\mu\)mol/L hour). Eight of 23 high CES2 mRNA expressing patients (34.8%) developed grade 3 to 4 neutropenia or diarrhea compared with 2 of 22 (9.1%) in the low CES2-expressing group (\(P = 0.071\)).

Conclusion: Our data support a predictive power of CES2 mRNA expression in PBMC for the activation rate of CPT11.

Irinotecan \{7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, CPT11\} is a topoisomerase I inhibitor, active in cancer chemotherapy. It is used as a first-line treatment for metastatic colorectal cancer in association with 5-fluorouracil and leucovorin and is active in ovarian, lung, breast, pancreatic, and cervix cancer (1). A great interpatient variability has been reported in terms of toxicity to CPT11, especially diarrhea and neutropenia, possibly related to differential plasma levels of the active metabolite SN38 (2). This depends on several factors such as the activation of CPT11 to SN38, the glucuronidation of SN38 to the inactive SN38 glucuronide (SN38G), or to the biliary excretion levels of SN38 (3).

Presently, an impaired glucuronidation activity of the uridine diphosphoglucuronosyltransferase isoform 1 \{UGT1A1\} enzyme, possibly due to the genetic polymorphism of the UGT1A1 gene, has been considered to explain toxicity variability. In particular, UGT1A1*28 polymorphism, characterized by a variation in the number of TA repeats in the promoter region of the gene, is thought to be associated with interpatient differential glucuronidation of SN38 possibly leading to CPT11 pharmacokinetics and pharmacodynamics variability (4–6). The possibility of using a genetic marker for toxicity or response allows a simple methodologic approach of analysis that can be easily done in peripheral blood mononuclear cells (PBMC) allowing CPT11 dosage on the individual genetic profile. Nonetheless, data reported until now (7, 8) seem to suggest that UGT1A1 polymorphisms are not by themselves sufficient and should be enclosed in a more complete set of markers involved in drug metabolism, to increase their predictive power.

A limiting step in CPT11 activation to SN38 in vivo is the CPT11 dipiperidino side chain hydrolysis mainly mediated by
the human carboxylesterase isofoms (CES2) exhibiting a higher affinity and velocity for CPT11 conversion compared with its isofom CES1 (9). It shows a quite variable activity among individuals. Recent articles report the existence of several genetic polymorphisms for CES2 gene; but presently, their functional meaning is not completely validated thus not directly applicable as pharmacokinetic and pharmacodynamic markers in patients (10–12). The attempt to describe the interindividual variation in the efficiency of the CES2-mediated activation step of CPT11 should therefore exploit the analysis of a phenotypic marker of the enzyme efficiency as its mRNA expression or protein activity. However, this implies a higher complexity of performance than the genetic analysis. CES2 is mainly a liver and intestinal enzyme, and phenotypic CES2 analysis would require a hepatic or intestinal biopsy difficult to be enclosed in the clinical practice because of its high invasiveness (13).

Nonetheless, besides liver, CES2 enzyme is expressed also in other tissues (13), and CPT11 hydrolysis activity has been detected in the tumor (14) and in the plasma (15). It seemed also to be expressed at very low but detectable level in PBMC and, as shown for other hepatic enzymes, such as dihydrolipoyldehydrogenase (16) and several cytochrome isofoms (17, 18) PBMC mRNA expression could be representative of systemic drug metabolism.

In this study, we investigated the predictive value of CES2 mRNA expression level in PBMC of patients treated with a CPT11-including regimen on the efficiency of the CPT11 to SN38 conversion and on the toxicity and response to therapy.

### Materials and Methods

**Patient enrollment.** Forty-five colorectal cancer patients, afferent to the Centro di Riferimento Oncologico, National Cancer Institute of Aviano, Italy, were enrolled in the study. The subjects were treated with FOLFIRI regimen simplified by De Gramont (ref. 19; CPT11 180 mg/m², 2-hour infusion, day 1; 5-fluorouracil 400 mg/m² i.v. bolus + 2.4 g/m², 46-hour infusion, days 1 and 14; leucovorin 200 mg/m², 2-hour infusion, days 1 and 14) every 2 weeks. One cycle of treatment consisted of a 2-week therapy. Eligible cases were patients with diagnosis of metastatic colorectal adenocarcinoma, ages between 18 and 75 years, and of Caucasian ethnicity. The cases were treated with FOLFIRI regimen as first-line metastatic colorectal cancer with unresectable metastases; absolute neutrophil count of ≥10,000/μL, platelets ≥100,000 μL, performance status (WHO), 0 to 2; life expectancy of ≥3 months; at least one measurable cancer lesion; normal renal function (creatinine clearance > 65 ml/min by Cockcroft; ref. 20); and alanine aminotransferase, aspartate aminotransferase <1.25× normal value or <2× for Gilbert’s syndrome. All cases recruited in the study signed a written informed consent approved by the local Ethical Committee.

**Evaluation of CES2 mRNA expression by reverse transcription-PCR in real time.** A relative quantitation of mRNA expression value was done. The PBMC fraction was extracted from 20 mL of whole, peripheral blood collected before the beginning of drug infusion to exclude possible drug effects on the enzyme expression by using Fycol as separator medium. Total RNA was extracted from PBMC pellets stored at −80°C using RNA-fast kit (M-Medical S.r.l, San Diego, CA) based on guanidine salts and urea method. Total RNA was quantified by spectrophotometric analysis and reverse transcription was done on 2.5 μg of total RNA. A reaction volume of 20 μL in 50 mmol/L Tris-HCl buffer (pH 8.3) was used with 50 mmol/L MgCl₂, 0.5 mmol/L spermidine, 10 mmol/L DTT, 200 mmol/L deoxynucleotide triphosphate mixture, 1 μmol/L poly-dT oligonucleotides,10 units of RNase inhibitor, and 20 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). cDNA samples were diluted five times and 3 μL were used for each real-time PCR reaction. Sybr Green technology was employed in a reaction volume of 50 μL of SYBR Green PCR Master Mix (AB Applied Biosystems, Warrington, United Kingdom) with 50 mmol/L of each oligonucleotide primer. Following a hot start (10 minutes at 95°C) there were 40 cycles of denaturation (15 seconds at 95°C) and annealing/extension steps (1 minute at 60°C for CES2 and 65°C for β-actin) followed by a dissociation step to check for nonspecific double-strand DNA, in a thermocycler (ABI PRISM 7900HT, Foster City, CA). The primer sequences were chosen to avoid nonspecific amplification of genomic DNA. Therefore, sequences overlapping the junction between two different exons were selected (reference sequence: NT_010478). For CES2, isofom-specific sequences were chosen checking the alignment with the other CES isofoms mRNA sequences. For CES2, a product of 151 bp was obtained (forward primer, 5'-GTAGCAGATTCAGTGTTC3-'; reverse primer, 5'-GTAGTGGCCCCAAAAGAA-3'). For β-actin, used as housekeeping gene, a fragment of 456 bp was amplified (forward primer, 5'-GACCCAGATCATGTTTGGAGA3-'; reverse primer, 5'-GACTCCATGCCAGGAA3'). The fluorescence generated at each amplification cycle by Sybr Green interaction with double strand DNA was detected by a CCD camera. Serial dilutions of a reference cDNA sample (produced from a colon tumor cell line, DLD) were used to build up a standard curve for each experiment to deduce CES2 and β-actin relative expression values. Values of expression of CES2 were normalized on β-actin expression value for each sample. Each measure was repeated four times.

**CPT11 and metabolites assay.** For CPT11 and metabolite analyses, serial blood samples were collected into heparinized tubes predose and at 0.5, 1.0, 1.5, 2.0, 2.25, 2.50, 2.75, 3.0, 3.50, 4.0, 6.0, 8.0, 10.0, 14.0, 26.0, 50.0 hours from the CPT11 infusion start. Plasma was harvested immediately by centrifugation at 3,000 × g for 15 minutes at 4°C and stored at −80°C until analysis. The total plasma concentration (lactone plus carboxylate) of CPT11 and metabolites SN38 and SN38G were determined using a high-performance liquid chromatography with fluorescence detection as described in previously reported methods (21, 22) with few modifications. In brief, 100 μL of plasma sample were deproteinized by treatment with 1,000 μL of acidified ice-cold acetonitrile/methanol (50:50 by volume) solution containing 100 ng of camptothecin as internal standard. The samples were mixed and spun at 24,000 × g for 15 minutes, and the clear supernatant was collected and dried in a speed vacuum. The residual was dissolved with 200 μL of 0.5 mol/L ammonium acetate (pH 3.5) and 20 μL of this solution were injected in the HPLC System-Gold (Beckman Coulter, Fullerton CA). Chromatographic separation of CPT11 and SN38 metabolite were achieved using a 4 × 250 mm C18 novapack 4 μm (Waters, Milford MA) by a isocratic elution with 50 mmol/L potassium dihydrogen phosphate, 25% acetonitrile with 0.1% TEA at 3.5 pH. The mobile phase was delivered at 1 mL/min and the column effluent monitored at excitation and emission wavelength of 380 and 532 nm, respectively. Under these conditions, retention times were 5.9, 9.9, and 12.2 minutes for CPT11, SN38, and the internal standard, respectively. The plasma concentration of SN38G metabolite was indirectly determined as the increase of SN38 concentration obtained following 30 minutes of incubation of the same sample with 1,000 units of β-glucuronidase (Sigma-Aldrich Milan, Italy). The intra- and inter-day variability was <10% in a concentration range between 1.5 and 1,500 nmol/L.

**Pharmacokinetics analysis.** Noncompartmental analysis was used to determine the pharmacokinetic variables for CPT11, SN38, and SN38G. The observed plasma concentration-time data for each species were plotted on semi-log coordinates to graphically estimate the duration of the terminal elimination phase. The apparent terminal, elimination rate constant (k) were determined by log-linear regression analysis of the terminal phase of the plasma concentration-time curve. The terminal half-life was calculated as 0.693/k. A linear-log trapezoidal numerical integration method was used to calculate the area under the drug concentration-time curve (AUC0-last) from time 0 to the last sampling.

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time ($C_{\text{last}}$). Area under the CPT11, SN38, and SN38G plasma concentration-time curves to infinite time (AUC) were calculated by adding $C_{\text{last}}/k$ to AUC$_{\text{CO}, \text{last}}$. Total body clearance from plasma (CL) of CPT11 was calculated by dividing the total given drug dose by its AUC (dose/AUC), whereas the apparent volume of distribution at a steady state ($V_{\text{ss}}$) was calculated according to the statistical moment theory.

The extent of CPT11 to SN38 metabolism was expressed by the metabolic activation ratio as (AUC$_{\text{SN38}}$ + AUC$_{\text{SN38G}}$)/AUC$_{\text{CPT11}}$.

Collection of clinical data. Objective clinical evaluation, blood counts, hepatic, and renal function tests were done within 48 hours before each cycle. Patients were questioned specifically about nausea and vomiting, mucositis, diarrhea, malaise, and appetite at every cycle. Computed tomography, scans of measurable lesions, was assessed at baseline and repeated every four cycles. A single chemotherapy administration was considered sufficient to evaluate acute toxicity, whereas response to treatment was evaluated only for patients who had received at least four cycles of chemotherapy. Toxicity data were registered after each chemotherapy cycle and classified according to the National Cancer Institute Common Toxicity Criteria scale. The maximum toxicity levels developed during the entire course of therapy were considered. Data on response to the therapy were evaluated by the WHO criteria.

Statistical analysis. Nonparametric tests were used for statistical analysis. CES2 mRNA expression, variable with continuous data was classified into two groups based upon the median. This methodology precludes strong assumptions about the relationship between expression and pharmacokinetics while avoiding the bias of searching for “optimal cutoff points” (23). Categorical data were associated with the pharmacokinetic, pharmacodynamic, and clinicopathologic features of the patients, using the two-tailed Fisher’s exact test or the $\chi^2$ test. Mann-Whitney $U$ test was employed to estimate the association between the continuous variables represented by the pharmacokinetic variables (AUC$_{\text{SN38}}$, AUC$_{\text{SN38G}}$, AUC$_{\text{CPT11}}$, and activation ratio) and CES2 mRNA levels grouped according to the median cutoff value. The Mann-Whitney $U$ test was used also to evaluate association between toxicity (grouped as severe, grade 3-4, and mild or no toxicity, G0-1-2) and CES2 mRNA expression when considered as a continuous variable.

Linear correlation of CES2 mRNA expression with pharmacokinetic variables was tested by the Spearman’s correlation coefficient ($\rho$).

Results

**CES2 mRNA expression and pharmacokinetic variables.** Forty-five metastatic colorectal cancer patients were treated with FOLFIRI regimen. Patients’ characteristics are reported in Table 1.

$\beta$-Actin normalized amounts of CES2 mRNA expression, in PBMC, are unitless numbers that can be used to compare the target gene mRNA quantity in different patients. The patients exhibited a large spreading of mRNA expression ranging from 0.01 to 28.21, median of 1.45, and mean of 2.89 ± 4.63. In particular, 10.5% of patients with extreme expression values brought the percentage of coefficient of variation to 160%.

In the investigated patients’ population, the mean AUC of CPT11 was >30-fold higher than the AUC of SN38; the AUC of SN38G was about 4-fold higher than that of SN38 (Table 2). The late phase of elimination rate of CPT11 was higher for CPT11 than SN38 and SN38G and the late half-life for CPT11 was 9.73 ± 1.67 hours, whereas for SN38 and SN38G it was 18.13 ± 4.96 and 16.35 ± 3.37 hours, respectively. The CPT11 clearance was 17.18 ± 4.77 L/h/m², whereas the volume of distribution at steady state was 110.67 ± 27.64 L/m². A substantial interpatient variability was observed for the parent drug and its metabolites according to previous studies (24).

Patients were classified into two subgroups based on the median value of CES2 mRNA expression to identify a discriminative factor of high and low CES2 metabolizers and to possibly highlight a cutoff value with clinical implications. No differences between the high and low CES2 expression groups were found with respect to the age, gender, location, and stage of the tumor (Table 1).

When patients were stratified as function of the high and low mRNA CES2 expression, a substantial difference in the metabolic activation ratio was observed. The results of this analysis are summarized in Table 2. Patients with the CES2 mRNA expression above the median value exhibited a significantly higher activation ratio (median, 0.25; range, 0.15-0.42) compared with the patients belonging to the low expression group (median, 0.20; range, 0.10-0.40), indicating a higher efficiency of the CPT11 to SN38 conversion step ($P = 0.013$). In Fig. 1, the plot of the CES2 mRNA expression against CPT11 activation ratio is reported; a moderate Spearman’s linear correlation ($P = 0.052$, $\rho = 0.295$) was found between these variables. AUC of SN38 was also increased in the high expression group (median, 0.87 mol/L hour; range, 0.40-2.73) in comparison with the low expression group (median, 0.75 mol/L hour; range, 0.31-1.46). However, such a trend did not reach statistical significance ($P = 0.181$; Fig. 2). Other pharmacokinetic variables (i.e., CPT11 AUC and SN38G AUC) resulted not significantly related to CES2 mRNA expression (Table 2; Fig. 2).

**CES2 mRNA relative expression, SN38 AUC, activation ratio, and clinical data.** All 45 patients enrolled in the study were valuable for toxicity, whereas 38 patients were valuable for response (Table 3). The median number of cycles administered was 8 (range, 2-14). Maximum hematologic toxicity developed

| Table 1. Clinical-pathologic features of the patients according to CES2 mRNA expression |
|---------------------------------------------|-----------------------------|
| **No. patients** | **P** | **All patients, N = 45 (%)** |
| Low CES2*, $n = 22$ (%) | High CES2*, $n = 23$ (%) | |
| **Gender** | | |
| Male | 11 (45.8) | 13 (54.2) | NS | 24 (100.0) |
| Female | 11 (52.4) | 10 (47.6) | | 21 (100.0) |
| **Age (y)** | | | | |
| <51 | 12 (60.0) | 8 (40.0) | NS | 20 (100.0) |
| ≥51 | 10 (40.0) | 15 (60.0) | | 25 (100.0) |
| **Tumor location** | | | | |
| Colon | 16 (55.2) | 13 (44.8) | NS | 29 (100.0) |
| Rectum | 6 (37.5) | 10 (62.5) | | 16 (100.0) |
| **Stage** | | | | |
| II | 2 (50.0) | 2 (50.0) | NS | 4 (100.0) |
| III | 5 (45.4) | 6 (54.5) | | 11 (100.0) |
| IV | 15 (50.0) | 15 (50.0) | | 30 (100.0) |

Abbreviation: NS, not significant.

* Corresponding to patients with mRNA CES2 expression higher or lower than the median expression value.

1 $P$ by Fisher’s exact test.

2 $P$ by $\chi^2$ test.

3 $P = 0.052$, $\rho = 0.295$.

4 $P = 0.181$.

5 $P = 0.013$.
during the entire course of therapy, graded 3 to 4 by the National Cancer Institute Common Toxicity Criteria scale, was registered in nine patients (20.0%; six patients with grade 4 neutropenia, one with grade 4 neutropenia, one with grade 3 neutropenia and grade 3 anemia, and one with grade 3 anemia). Nonhematologic toxicity was grade 3 in 11 patients (24.4%; four patients with grade 3 diarrhea, two with grade 3 vomit, three with grade 3 stomatitis, and two with grade 3 alopecia). In Table 3, relationships between the maximum hematologic and nonhematologic toxicity, developed during the entire course of therapy, and CES2 mRNA expression are reported. Toxicity known to be induced mainly by CPT11 (i.e., neutropenia and diarrhea) was also considered by itself for association with CES2 mRNA expression. Eight patients of 23 (34.8%) in the high expression group presented CPT11-related toxicity (two patients with grade 3 diarrhea, two with grade 3 neutropenia, and three with grade 3 neutropenia, and one with grade 4 neutropenia) compared with 2 of 22 (9.1%) in the low expression group (both grade 3 neutropenia; \( P = 0.071 \), Fisher’s exact test; Table 3). The Mann-Whitney \( U \) test showed an increase of CES2 mRNA expression among patients with grade 3 to 4 CPT11-related toxicity (median, 3.41; range, 0.28-12.15) compared with those with G0-1-2 toxicity (median, 1.04; range, 0.01-28.21; \( P = 0.064 \); Fig. 3).

CPT11-related toxicity resulted not significantly associated with CPT11 activation ratio or SN38 AUC. Among patients developing grade 3 to 4 diarrhea/neutropenia, median (range) of activation ratio and SN38 AUC were 0.21 (0.15-0.40) and 0.86 (0.40-1.87), respectively; whereas among patients with G0-1-2 diarrhea/neutropenia, they were 0.22 (0.10-0.42) and 0.78 (0.24-2.73), respectively.

Objective responses were observed in 16 of 38 patients (42.1%) and included three (7.9%) complete responses and 13 (34.2%) partial responses. Stable disease was observed in 13 (34.2%) patients. Nine (23.7%) patients experienced disease progression. No correlation between the CES2 mRNA expression levels and response was found (Table 3; \( P = 0.324 \)).

### Discussion

An interplay of different enzymes affects CPT11 metabolism and the interindividual differences in protein activity/expression could be responsible for interpatient variability in terms of side effects (especially diarrhea and neutropenia) or tumor response. CES2-mediated activation of CPT11 to SN38 is a limiting step in CPT11 metabolism and it is one of the factors possibly responsible for the differential pharmacologic effect of CPT11 among patients. It has been reported that differential levels of CES2 enzyme expression are correlated
with differential CPT11 activation to SN38 in in vitro experiments on liver cellular extracts (25).

Surrogate markers of the liver CES activity have been explored for validation as predictive factors for the in vivo CPT11 activation efficiency. Guemei et al. (15) highlighted an association between the specific plasma esterase activity and SN38 AUC. Conversely, Shingoji et al. (26) reported no association between the activity of CPT11 hydrolase in plasma (mediated by CES) and the AUC of SN38 in cancer patients.

Because CES2 is an intracellular metabolic enzyme present in the microsomal fraction of the cells (27), we thought that its intracellular levels of CES2 in PBMC could be more representative than those in the plasma for correlation with systemic activation of CPT11. On these grounds, we investigated the

| Table 3. Response to therapy and maximum toxicity observed during six cycles of treatment and association with CES2 mRNA relative expression |
|---------------------------------|-----------------|-----------------|-----------------|
| Toxicity                        | Low CES2*, n = 22 (%) | High CES2*, n = 23 (%) | All patients, N = 45 (%) |
|                                 |                  |                  |                  |
| Hematologic                     |                  |                  |                  |
| Grade 0-2                       | 19 (52.8)        | 17 (47.2)        | 36 (100.0)       |
| Grade 3-4                       | 3 (13.3)         | 6 (66.7)         | 9 (100.0)        |
| P                               | 0.459*           |                  |                  |
| Nonhematologic                  |                  |                  |                  |
| Grade 0-2                       | 19 (56.9)        | 15 (44.1)        | 34 (100.0)       |
| Grade 3                         | 3 (27.3)         | 8 (72.7)         | 11 (100.0)       |
| P                               | 0.165*           |                  |                  |
| Diarrhea/neutropenia            |                  |                  |                  |
| Grade 0-2                       | 20 (57.1)        | 15 (42.9)        | 35 (100.0)       |
| Grade 3-4                       | 2 (20.0)         | 8 (60.0)         | 10 (100.0)       |
| P                               | 0.071*           |                  |                  |
| Response                        |                  |                  |                  |
| Complete/partial response       | 6 (37.5)         | 10 (62.5)        | 16 (100.0)       |
| Stable/progressive disease      | 13 (58.1)        | 9 (40.9)         | 22 (100.0)       |
| P                               | 0.324*           |                  |                  |

*Corresponding to patients with mRNA CES2 expression higher or lower than the median expression value.

*P by two-tailed Fisher’s exact test.

† Patients with diarrhea and/or neutropenia developed during the entire course of therapy.

*Seven patients were not valuable for response since they underwent less than four cycles of chemotherapy. Causes of discontinuation of therapy were death (one case), refusal of the patient to continue the treatment (one case), and toxicity development (two cases). Three patients were still under therapy.
predictive role of the cellular PBMC levels of CES2, evaluated as mRNA expression, on the CPT11 pharmacokinetics and pharmacodynamics in a population of patients treated with FOLFIRI regimen. This approach is supported by other studies using the enzymatic expression/activity measured in PBMC as surrogate marker of systemic drug metabolism. In particular, a correlation was detected between mRNA expression levels, in liver and PBMC, of cytochrome P450 isoforms, typically hepatic enzymes (17, 18). In addition, the enzymatic activity of another hepatic enzyme, dihydropyrimidine dehydrogenase, in PBMC, was found to be predictive of the systemic 5-fluorouracil pharmacokinetics and was subsequently linked to the enzymatic activity in the liver (16). This approach has practical and ethical consequences, overcoming the need of hepatic or intestinal biopsy (18). In our study, the analysis of the CES2 mRNA expression by real-time reverse transcription-PCR in PBMC resulted in a more sensitive and reproducible method compared with the analysis of the enzymatic activity in PBMC microsomes. In fact, according to Zhang et al. (13), reporting low CES2 protein expression level in PBMC, we found that CES2 activity was very low and not accurately measurable in PBMC derived from common blood samples, making the estimate of the interpatient variability difficult (data not shown). On the contrary, we observed a scattered distribution of mRNA CES2 expression in PBMC among the patients we analyzed, with some patients exhibiting very high or low values of expression. The mRNA expression variability was similar to that previously reported for the hepatic tissue (11, 28).

In an attempt to discriminate patients with high or low CPT11 activation ratio, evaluated as (AUC<sub>SN38</sub> + AUC<sub>SN38G</sub>)/AUC<sub>CPT11</sub>, we used the median CES2 mRNA expression value as possible cutoff of high and low expression. This method has been previously used (29, 30) to identify useful prognostic or therapeutic molecular markers. High CES2 mRNA-expressing group exhibited significantly higher (P = 0.013) efficiency of CPT11-to-SN38 activation ratio than the low-expressing group, highlighting that CES2 mRNA expression can be predictive of the efficiency of the first metabolic step of CPT11 metabolism in the patient. We subsequently confirmed our results considering CES2 mRNA expression as a continuous variable and found a moderate (P = 0.052, ρ = 0.295) correlation with CPT11 activation ratio, by a linear correlation model, with higher activation ratio for patients expressing higher quantity of CES2 mRNA. This suggestion is further supported by the trend of increased SN38 AUC in the high CES2 mRNA–expressing group (P = 0.181; Fig. 1). This could indicate how the efficiency of the metabolic step considered is able to influence the disposition of the active CPT11 metabolite, although the complexity of the mechanisms determining plasma levels of SN38, including other steps such as the SN38 glucuronidation and biliary elimination of the drug, could prevent statistical significance to be reached.

The increase of CES2 mRNA expression was associated with an increased incidence of the grade 3 to 4 diarrhea/neutropenia with 34.8% of patients developing grade 3 to 4 toxicity in the high CES2-expressing group versus 9.1% in the low-expressing group (P = 0.071, Fisher’s exact test). Moreover CES2 mRNA expression levels among patients developing severe (grade 3-4) diarrhea/neutropenia resulted higher than those of patients with mild/no toxicity (G0-1-2; P = 0.06, Mann-Whitney U test). These data, even if requiring larger confirmatory trials, suggest a role of the intracellular efficiency in CPT11 activation, indicated as CES2 mRNA expression, as a phenotypic variable on the development of toxicity to FOLFIRI regimen. Response resulted not significantly related to CES2 mRNA expression.

Relationship between pharmacokinetic variables and pharmacodynamic effect (toxicity or response) remains an intriguing and controversial issue in the CPT11-based treatments leaving it as an open question (2). Herben et al. (31) reported no correlation between SN38 plasmatic levels and CPT11-related toxicity (either diarrhea or neutropenia), whereas de Forni et al. (32) reported an association between CPT11-related toxicity and the SN38 AUC. Gupta et al. (33) reported that the SN38 glucuronidation ratio and the biliary index of excretion of the drug are important for the active metabolite bioavailability and for the pharmacodynamic effect. Toxicity is a polyfactorial event probably influenced by the interplay of several factors not only including SN38 plasma levels but also the dose intensity, the age, the liver functionality, and the fecal β-glucuronidase activity. In our study, we did not find any association between SN38 AUC and toxicity to the treatment as well as we did not find any association between activation ratio, calculated as (AUC<sub>SN38</sub> + AUC<sub>SN38G</sub>)/AUC<sub>CPT11</sub> and toxicity. On this ground, CES2 mRNA expression seems a stronger prognostic marker for toxicity than the activation ratio. It could be that mRNA expression is a real representation of what is happening intracellularly, whereas activation ratio takes into account other systemic phenomena not directly linked to the development of intracellular toxicity.

In conclusion, our data showed that CES2 mRNA expression level in PBMC could be a marker of the systemic activation reaction of the CPT11 and, although just in a trend fashion, of CPT11-related toxicity. Therefore, data reported in this study seem to support the use of CES2 mRNA expression in PBMC as a low-invasive and easy-to-perform test that can contribute to the definition of a comprehensive set of markers useful to predict interpatient variability of the pharmacologic effect of CPT11.
References


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