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Correlation between plasma concentration ratios of SN-38 glucuronide and SN-38 and neutropenia induction in patients with colorectal cancer and wild-type UGT1A1 gene

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Abstract. In irinotecan (CPT-11)-based chemotherapy, neutropenia and diarrhea are often induced. In the present study, the clinical significance of the concentration ratios of 7-ethyl-10-hydroxycamptothecin (SN-38) glucuronide (SN-38G) and SN-38 in the plasma in predicting CPT-11-induced neutropenia was examined. A total of 17 patients with colorectal cancer and wild-type UDP-glucuronosyltransferase (UGT)1A1 gene were enrolled and treated with CPT-11 as part of the FOLFIRI regimen [CPT-11 and fluorouracil (5-FU)]. Blood was taken exactly 15 min following a 2-h continuous infusion of CPT-11. Plasma concentrations of SN-38, SN-38G and CPT-11 were determined by a modified high-performance liquid chromatography (HPLC) method. The median, maximum and minimum values of plasma SN-38G/SN-38 ratios were 4.25, 7.09 and 1.03, respectively, indicating that UGT activities are variable among patients with the wild-type UGT1A1 gene. The plasma SN-38G/SN-38 ratios decreased with an increase in the trial numbers of chemotherapy (r=0.741, p=0.000669), suggesting that CPT-11 treatment suppresses UGT activity, and the low plasma SN-38G/SN-38 ratios resulted in the induction of greater neutropenia. However, in this analysis, 2 clearly separated regression lines were observed between plasma SN-38G/SN-38 ratios and neutropenia induction. In conclusion, UGT activity involved in SN-38 metabolism is variable among patients with the wild-type UGT1A1 gene, and each CPT-11 treatment suppresses UGT activity. One-point determination of the plasma SN-38G/SN-38 ratio may provide indications for the prediction of CPT-11-induced neutropenia and adjustment of the optimal dose, although further studies are required.

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Introduction

The camptothecin derivative irinotecan hydrochloride (CPT-11) is an anticancer agent and is now regarded as the most active drug for the treatment of colorectal cancer patients (1). A small amount of CPT-11 is converted into its active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38) by carboxyesterase in the liver and other tissues, and then into a water-soluble inactive metabolite SN-38 glucuronide (SN-38G) by UDP-glucuronosyltransferases (UGTs). Since SN-38 is 100- to 1000-fold more cytotoxic than CPT-11, plasma levels of SN-38, clearance of SN-38 and/or polymorphism of UGT1A1 are clinically important influencing factors in CPT-11-induced side-effects, including neutropenia and diarrhea (1-4).

However, in clinical practice, the adjustment of the optimal dose of CPT-11 for an individual patient remains unclear, since pharmacokinetic parameters of CPT-11, as well as the incidence of CPT-11-related side effects, are markedly varied among patients (1,3,5,6). The clinical significance of inherited genetic polymorphisms of UGTs involved in SN-38 glucuronidation is well recognized in association with CPT-11-induced side effects (2,7-11). UGTs are classified into 2 families (UGT1 and UGT2) and 3 subfamilies (UGT1A, UGT2A and UGT2B), and UGT1A1 is thought to be the most predominant catalyst in the metabolism of SN-38 among several UGT1A isoforms (1,12). However, it is still considered that the optimal dose of CPT-11 cannot be adjusted solely from the information of genetic polymorphisms of UGT1A1, and we frequently encounter the cases of dose reduction of CPT-11, even in patients with the wild-type UGT1A1 gene.

In the present study, 17 Japanese patients with colorectal cancer and the wild-type UGT1A1 gene were enrolled, and treated with CPT-11 (150 mg/m^2) as a part of the CPT-11 plus infusional 5-fluorouracil/leucovorin (FOLFIRI regimen) in Osaka Rosai Hospital (Japan) (13). To establish pharmacokinetic-based dose adjustment of CPT-11, the clinical significance of plasma SN-38G/SN-38 ratios in predicting

Table I. Serological diagnosis of Japanese colorectal cancer patients with wild-type UGT1A1 gene.

No.	Age (years)	Gender	Body surface area (m^2)	Cr (mg/dl)	BUN (mg/dl)	T-BIL (mg/dl)	AST (U/l)	ALT (U/l)	Neutrophils (μl^{-1})	
									Before	After
1	77	M	1.77	0.7	16	0.5	24	22	1547	1232
2	60	M	1.79	0.7	11	0.3	16	9	3705	1806
3	62	F	1.72	0.8	12	0.4	48	62	3886	2434
4	60	F	1.51	0.7	7	0.6	26	35	1300	530
5	67	M	1.54	0.7	13	0.6	16	10	3154	2751
6	72	M	1.56	1.2	27	0.3	48	26	2796	1716
7	49	M	1.68	1.0	14	0.4	26	20	4537	2300
8	79	M	1.66	0.6	12	1.1	40	31	2639	1141
9	63	M	1.82	0.7	12	0.2	22	21	3813	1005
10	59	M	1.78	0.9	22	0.7	22	14	2646	2305
11	74	M	1.56	0.6	22	0.2	13	8	4219	2862
12	61	F	1.50	0.4	11	0.4	18	14	3426	2268
13	72	F	1.36	1.0	16	0.5	19	21	6313	4312
14	73	F	1.42	0.5	14	0.4	28	21	2780	1916
15	76	M	1.40	0.9	18	0.9	22	16	3788	489
16	65	F	1.49	0.5	11	0.3	19	13	6029	3396
17	68	M	1.62	0.7	8	0.5	19	22	4906	3552

Cr, concentrations of creatinine in plasma; BUN, blood urea nitrogen in plasma; T-BIL, total bilirubin in plasma; AST, aspartate aminotransferase in plasma; ALT, alanine aminotransferase in plasma; neutrophils, the number of neutrophils was determined by a Beckman coulter LH 780 analyzer prior to and following CPT-11 infusion; M, male; F, female; CPT-11, irinotecan hydrochloride.

CPT-11-induced neutropenia was examined. The concentration ratios of SN-38G against SN-38 in the plasma were determined exactly 15 min following a 2-h continuous infusion of CPT-11. Neutropenia was evaluated by counting the number of neutrophils prior to and following CPT-11 infusion. Plasma concentrations of SN-38, SN-38G and CPT-11 were simultaneously determined by a previously reported high-performance liquid chromatography (HPLC) method with a small modification (14).

Materials and methods

Materials. CPT-11, SN-38 and SN-38G were supplied by Yakult Honsha Co., Ltd. (Tokyo, Japan) and used without further purification. Camptothecin, potassium dihydrogen phosphate (KH_2PO_4), zinc sulfate and ethylene glycol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phosphoric acid (1 N), acetonitrile for HPLC and methanol for HPLC were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Sodium 1-decanesulfonate was obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA).

CPT-11 treatment in patients. Genotyping of wild-type UGT1A1 was performed by the Invader UGT1A1 Molecular Assay in the same manner as reported previously (2,10,11). Colorectal cancer patients (11 males and 6 females) with wild-type UGT1A1 gene were treated with CPT-11 as part of the FOLFIRI regimen (Table I) (13). The FOLFIRI regimen consisted of a 2-h continuous infusion of CPT-11 (150 mg/m^2) and leucovorin (200 mg/m^2), immediately followed by bolus injection of 5-FU (400 mg/m^2), and followed by a 46-h continuous infusion of 5-FU (2,400 mg/m^2). Patients received this FOLFIRI regimen every 2 weeks after counting the number of neutrophils prior to and following CPT-11 infusion. The number of neutrophils was counted by a Beckman coulter LH 780 analyzer (Beckman Coulter, Inc. Tokyo, Japan). Informed consent was obtained from all patients. This study was approved by the Institutional Review Board of Osaka Rosai Hospital and conducted in accordance with the Declaration of Helsinki.

Blood (5 ml each) was collected into an ethylenediaminetetraacetic acid (EDTA)-containing blood collection tube to determine plasma concentrations of SN-38, SN-38G and CPT-11 15 min following the completion of a 2-h continuous infusion by measuring the time with a stopwatch. Blood samples were centrifuged for 10 min at 3,000 x g to obtain plasma samples, and the plasma samples were stored at -30˚C until further analysis. Another part of the blood sample was separately subjected to analysis of the concentrations of creatinine (Cr), blood urea nitrogen (BUN), total bilirubin (T-BIL), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and the number of neutrophils in plasma (Table I).

Analytical method of CPT-11-related compounds by HPLC. Stock solutions of CPT-11, SN-38, SN-38G and camptothecin [an internal standard (IS)] were prepared by dissolving stock in dimethylsulfoxide (DMSO) and storing at -30˚C. For the calibration curve, DMSO solution (50 μl) of CPT-11, SN-38 and

SN-38G was diluted with plasma (450 μl) at a concentration range of 500-7,500, 10-100 and 25-200 ng/ml, respectively. A total of 50 μl of DMSO solution of camptothecin was then added to each sample (400 μl) in a test tube, and the mixture solution was deproteinized with 150 μl of deproteinizing agent [a mixture of 1 M zinc sulfate solution, methanol and ethylene glycol (1:1:1)]. Following thorough mixing, the solution was centrifuged at 3,000 x g for 10 min, 100 μl of the supernatant was obtained and 20 μl of 0.5 M KH_2PO_4 was added. After mixing again, the solution was transferred to a HPLC tube. The volume of each sample loaded to the HPLC column was 20 μl. As for the calibration curve, plasma samples obtained from each patient treated with CPT-11 were similarly treated and concentrations of SN-38, SN-38G and CPT-11 in the plasma were determined by the modified HPLC method.

The HPLC system used was composed of a system controller (SCL-10 AVP), an autosampler (SIL-20AC), degasser (DGU-20A3), pump (LC-20ADXL) and a fluorescence detector (RF-10AXL) (Shimadzu, Kyoto, Japan). The column used was Luna® 5 μm C18 100 Å (250x4.60 mm), a reverse-phase column (Shimadzu). The mobile phase was a mixture of solution A [50 mM KH_2PO_4, 4 mM sodium 1-decanesulfonate and 20% acetonitrile (CH_3CN)] and solution B (50 mM KH_2PO_4, 4 mM sodium 1-decanesulfonate and 30% CH_3CN). Solutions A and B were adjusted to pH 3.5 using phosphoric acid. Gradient elution was performed using solutions A and B, with the concentration of solution B being set at 0, 100 and 0% during the first 4 min, from 4 to 30 min, and from 30 min to completion of elution, respectively. The flow rate was 1.5 ml/min, and detection was made at 373 nm for the excitation wavelength and 540 nm for the emission wavelength, respectively.

Results

HPLC chromatograms. HPLC chromatograms of blank plasma and a plasma sample containing CPT-11, SN-38, SN-38G and camptothecin (IS) are shown in Fig. 1A and B, respectively. The retention times of SN-38G, SN-38, IS and CPT-11 were 5.6, 13.2, 14.7 and 24.0 min, respectively. Endogenous substances in plasma did not interfere with the peaks of CPT-11-related compounds at all in the present analytical conditions. A good regression line was obtained as follows: CPT-11 in a concentration range of 500-7,500 ng/ml, y=0.0155x+0.308, r^2=0.9997; SN-38 in a concentration range of 10-100 ng/ml, y=0.0211x-0.0112, r^2=0.9986; SN-38G in a concentration range of 25-200 ng/ml, y=0.0118x+0.0534, r^2=0.9961, where 'y' is the peak area ratio between each compound and IS and 'x' is the concentration (ng/ml) of each compound in the plasma.

Correlation between plasma SN-38 concentrations and neutropenia induction in patients. SN-38 is the principal agent in CPT-11-induced neutropenia due to its potent cytotoxicity. The correlation between plasma SN-38 concentrations obtained 15 min following completion of a 2-h continuous infusion and neutropenia induction (the percentage of decreased neutrophil numbers by CPT-11 treatment) was examined. A higher plasma concentration of SN-38 resulted in a greater decrease in neutrophil numbers as follows: y=1.34x+16.5 (r=0.486, p=0.0481; Fig. 2). This regression line suggested that induction of neutropenia may be related to plasma SN-38 concentrations, although variation was observed among patients.

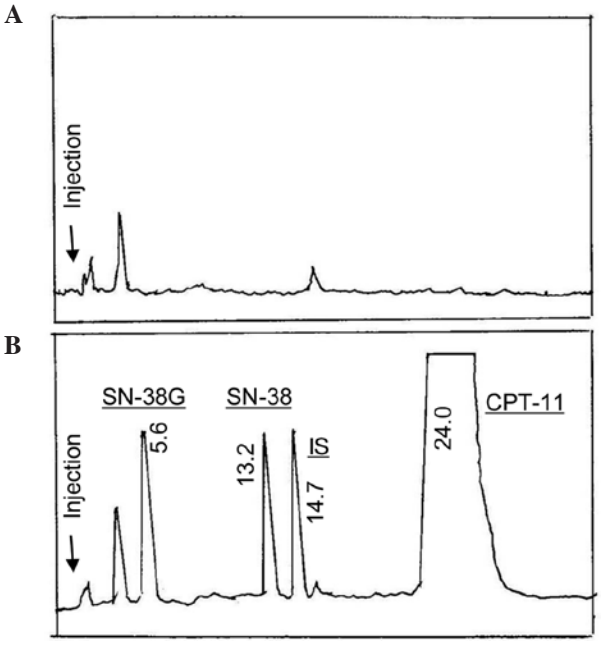

Figure 1. Chromatograms by HPLC. (A) Chromatogram of blank plasma. (B) Chromatogram of plasma sample containing CPT-11 (2,500 ng/ml), SN-38 (50 ng/ml), SN-38G (100 ng/ml) and the IS (500 ng/ml). The numeric value beside the peak represents the retention time of the compound. HPLC, high-performance liquid chromatography; CPT-11, irinotecan hydrochloride; SN-38, 7-ethyl-10-hydroxycamptothecin; SN-38G, SN-38 glucuronide; IS, internal standard.

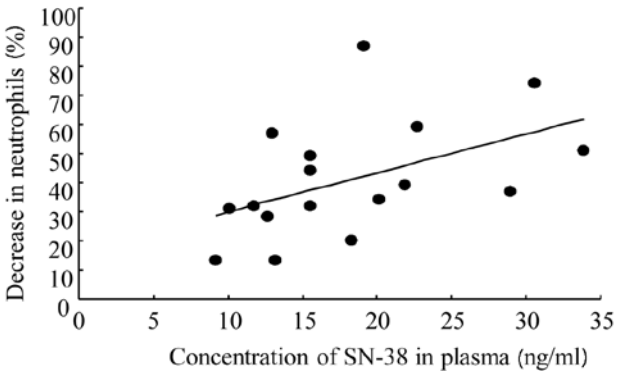

Figure 2. Correlation between plasma SN-38 concentrations and neutropenia induction in 17 patients with wild-type UGT1A1 gene. The regression line: y=1.34x+16.5 (r=0.486, p=0.0481). SN-38, 7-ethyl-10-hydroxycamptothecin.

Relationship between the plasma SN-38G/SN-38 ratio and trial numbers of CPT-11 treatments in patients. It is generally speculated that the cumulative amount [or value of the area under the curve (AUC)] of SN-38 in the plasma, rather than one-point plasma SN-38 concentration, is more directly connected to CPT-11-induced neutropenia, since cytotoxic SN-38 is metabolized to non-toxic SN-38G by UGT1A1 at various rates among patients. To reflect the UGT1A1 activity of each patient, a parameter of plasma SN-38G/SN-38 ratio 15 min following a 2-h continuous infusion of CPT-11 was introduced, and

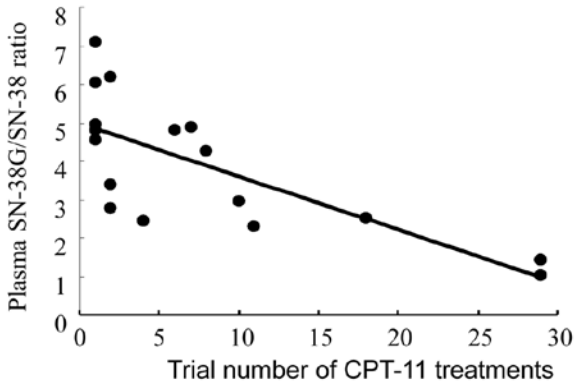

Figure 3. Correlation between the plasma SN-38G/SN-38 ratio and trial numbers of CPT-11 treatments in 17 patients with wild-type UGT1A1 gene. The regression line: y=-0.138x+4.98 (r=0.741, p=0.000669). CPT-11, irinotecan hydrochloride; SN-38, 7-ethyl-10-hydroxycamptothecin; SN-38G, SN-38 glucuronide.

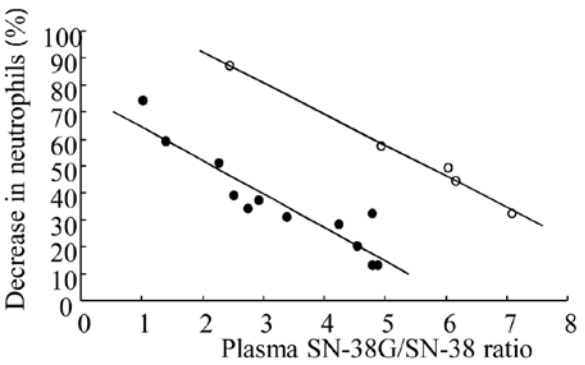

Figure 4. Correlation between the plasma SN-38G/SN-38 ratio and neutropenia induction in 17 patients with wild-type UGT1A1 gene. Upper regression line (n=5): y=-11.5x+115 (r=0.996, p=0.000366); lower regression line (n=12), y=-12.4x+76.8, (r=0.927, p=0.0000147). SN-38, 7-ethyl-10-hydroxycamptothecin; SN-38G, SN-38 glucuronide.

its clinical significance in CPT-11-induced neutropenia was examined. The median, minimum and maximum values of the plasma SN-38G/SN-38 ratio were 4.25, 1.03 and 7.09, respectively, demonstrating a difference of ≥6-fold, even among patients with the wild-type UGT1A1 gene. The effect of trial numbers of CPT-11 treatments on the plasma SN-38G/SN-38 ratio is shown in Fig. 3. The plasma SN-38G/SN-38 ratios significantly decreased with an increase in the trial numbers of chemotherapy (r=0.741, p=0.000669), indicating that CPT-11 treatments decrease UGT activity serially.

Correlation between the plasma SN-38G/SN-38 ratio and neutropenia induction in patients. The correlation between the plasma SN-38G/SN-38 ratio and neutropenia induction in patients was examined (Fig. 4). In this analysis, 2 clearly separated regression lines with high correlation coefficients were obtained as follows: upper regression line (n=5), y=-11.5x+115, (r=0.996, p=0.000366); lower regression line (n=12), y=-12.4x+76.8, (r=0.927, p=0.0000147). These high correlations between the regression lines suggest that low plasma SN-38G/SN-38 ratios, or low plasma clearance of SN-38 due to low UGT activity, induce severe neutropenia. Concurrently, it was suggested that the variation in CPT-11-induced neutropenia among patients was not explained by UGT activity alone in some patients.

Discussion

It is well known that the pharmacokinetics of CPT-11 as well as the incidence of CPT-11-induced side-effects are markedly scattered among patients (1,3,5,6,8,12). In the present study, we aimed to establish a pharmacokinetic-based dose adjustment of CPT-11 and examined the clinical significance of the one-point determination of the plasma SN-38G/SN-38 ratio in predicting CPT-11-induced neutropenia in colorectal Japanese patients with the wild-type UGT1A1 gene. The importance of pharmacokinetic-based dose adjustment of CPT-11 has been noted by numerous investigators, although such a method has not yet been established (3,6,12).

For this purpose, the HPLC analytical method was first investigated to determine SN-38, SN-38G and CPT-11 simultaneously within a relatively short period of time, in order that pharmacokinetic-based dose adjustment of CPT-11 may be applicable even in ambulatory patients. We modified a previously reported HPLC method (14) by using a mixture of 1 M zinc sulfate solution, methanol and ethylene glycol (1:1:1) as a deproteinizing agent, instead of using column extraction. CPT-11 is converted from the lactone to the carboxyl form in the plasma over time following administration, which results in a decrease of antitumor activity. Itoh *et al* (15) reported that it is possible to convert the carboxyl derivatives to lactones by adding 0.5 M KH_2PO_3. However, in the present study, when KH_2PO_3 was added to the plasma sample, the peak of SN-38G overlapped with that of the endogenous substance. To avoid the overlapping of these peaks, the composition of the mobile phase was changed to 20% CH_3CN-KH_2PO_4 buffer from 30% CH_3CN-KH_2PO_4 buffer (14). However, in this case CPT-11 was not eluted until 100 min or more. Thus, following the separation of SN-38G from an endogenous substance by using 20% CH_3CN-KH_2PO_4 buffer, gradient elution was performed to elute CPT-11 with 30% CH_3CN-KH_2PO_4 buffer. As a result, the retention time of CPT-11 was shortened to approximately 20 min. Similarly, a simple HPLC method with a short measurement time and a satisfactory resolution was achieved (Fig. 1).

The contribution of plasma SN-38 concentrations in CPT-11-related side-effects is well recognized (5,6). In the present study, a significant correlation was obtained between plasma SN-38 concentrations and neutropenia induction (Fig. 2). However, the correlation coefficient of the regression line was not very high, indicating that CPT-11-induced neutropenia cannot be predicted correctly from one-point plasma SN-38 concentration alone. Thus, to consider the clearance ability for SN-38 in each patient, the parameter of plasma SN-38G/SN-38 ratio was introduced, since the plasma SN-38G/SN-38 ratio is reported to correlate with the AUC ratio 2 h following the completion of CPT-11 treatment (16). In this study, the plasma SN-38G/SN-38 ratio was scattered by ≥6-fold among patients with the wild-type UGT1A1 gene. This result indicates that the variation in the pharmacokinetics of CPT-11 and CPT-11-induced neutropenia among patients cannot be predicted by genetic polymorphisms of the UGT1A1 gene

alone. Minami *et al* (10) reported that the median and interquartile range (IQR) of the AUC of the SN-38G/SN-38 ratio were 5.55 and 4.13-7.26, respectively. In the present study, the median value of the plasma SN-38G/SN-38 concentration ratio was 4.25 and the IQR was 1.03-7.09. In the present study, the plasma SN-38G/SN-38 ratios significantly decreased with an increase in the trial numbers of CPT-11-based chemotherapy (Fig. 3), indicating that SN-38 may suppress UGT activity on each occasion. In other words, the plasma clearance of SN-38 decreases and the intensity of neutropenia induction increases, with an increase in the trial number of CPT-11 treatments. Finally, the correlation between the plasma SN-38G/SN-38 ratio and neutropenia induction in patients was examined (Fig. 4). In this analysis, 2 clearly separated regression lines with high correlation coefficients were obtained from the two lines. A total of 5 (numbers 7, 10, 11, 15 and 16) out of 17 patients belonged to the upper regression line, indicating that a greater neutropenia was induced in this patient group, even at the same plasma SN-38G/SN-38 ratio. These patients received CPT-11 chemotherapy for the first time (3 out of 5 patients) or the second time (1 patient), suggesting that, in certain cases, neutropenia induction cannot be predicted by UGT activity alone. In the clearances of CPT-11 and SN-38, multiple enzymes and transporters are involved, including carboxyesterases, UGT1A1, CYP3A4, P-glycoprotein, MRP2, BCRP and/or OATP1B1, although the contribution of OATP1B1 remains under discussion (12,17-22). To predict CPT-11-induced neutropenia in all patients systemically (Fig. 4), the contribution of the above enzymes and/or transporters involved in the clearance of SN-38 should be further studied. In addition, the contribution of 5-FU included in the FOLFIRI regimen on neutropenia induction should also be studied carefully. Since standardized dose-reduction criteria are not yet available for CPT-11, even when the risk is considered to be high, excessive dose reduction should be avoided, since it may lead to a decrease of antitumor activity and loss of therapeutic benefits to the patient. By contrast, even if the risk to the patient is considered to be low, serious adverse reactions may occur and specific treatment may be necessary. To determine the most effective and safe doses of CPT-11 based on pharmacokinetic-based dose adjustment, future studies should evaluate the effect of the 'complex hetero', '*28 hetero', '*6 hetero' and '*6 homo' patterns of UGT1A1 on plasma SN-38G/SN-38 ratios in a larger number of Japanese patients (10,18,19,22).

In conclusion, UGT activity involved in SN-38 metabolism is variable among patients, even with the wild-type UGT1A1 gene, and each CPT-11 treatment suppresses UGT activity. One-point determination of the plasma SN-38G/SN-38 ratio may provide evidence for predicting CPT-11-induced neutropenia and adjustment of optimal dose, although further studies are required.

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