Short Communication

Close Association of UGT1A9 IVS1+399C>T with UGT1A1*28, *6, or *60 Haplotype and Its Apparent Influence on 7-Ethyl-10-hydroxycamptothecin (SN-38) Glucuronidation in Japanese

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ABSTRACT:
The anticancer prodrg, irinotecan, is converted to its active form 7-ethyl-10-hydroxycamptothecin (SN-38) by carboxylesterases, and SN-38 is inactivated by UDP-glucuronosyltransferase (UGT)1A1-mediated glucuronidation. UGT1A9 also mediates this reaction. In a recent study, it was reported that the UGT1A9 IVS1+399 (I399C>T) polymorphism is associated with increased SN-38 glucuronidation both in vitro and in vivo. However, its role in UGT1A9 expression levels and activity is controversial. Thus, we evaluated the role of I399C>T in SN-38 glucuronidation using 177 Japanese cancer patients administered irinotecan. I399C>T was detected at a 0.636 allele frequency. This polymorphism was in strong linkage disequilibrium (LD) with UGT1A1*T10 (>3279T, 0.99) and UGT1A1*6 (211G>A, 0.86), in moderate LD with UGT1A1*60 (>3279T>G, 0.55), but weakly associated with UGT1A1*28 (<54, >39A>TAA>A(TA)G, 0.08). Haplotype analysis showed that 98% of the I399C alleles were linked with low-activity haplotypes, either UGT1A1*6, *28, or *60. On the other hand, 85% of the T alleles were linked with the UGT1A1 wild-type haplotype *1. Although I399T-dependent increases in SN-38 glucuronidation/SN-38 area under concentration-time curve (AUC) ratio (an in vivo marker for UGT1A activity) and decreases in SN-38 AUC/dose were apparent (P < 0.0001), these effects were no longer observed after stratified patients by UGT1A1*6, *28, or *60 haplotype. Thus, at least in Japanese populations, influence of I399C>T on SN-38 glucuronidation is attributable to its close association with either UGT1A1*6, *28, or *60.

Irinotecan is an important drug for treatment of various tumors including lung, colon, and gastric (Smith et al., 2006). The infused drug is metabolized to its active form 7-ethyl-10-hydroxycamptothecin (SN-38) by carboxylesterases, and SN-38 is inactivated by glucuronidation. At least four UDP-glucuronosyltransferase (UGT) isoforms, namely UGT1A1, UGT1A7, UGT1A9, and UGT1A10, are known to glucuronidate SN-38 (Gagné et al., 2002; Saito et al., 2007).

The UGT1A gene complex consists of 9 active first exons including UGT1A10, IA9, IA7, and IA1 (in this order) and common exons 2 to 5. One of the 9 first exons can be used in conjunction with the common exons (Tukey and Strassburg, 2000). The UGT1A1 N-terminal domains (encoded by the first exons) determine substrate-binding specificity, and the C-terminal domain (encoded by exons 2 to 5) is important for binding to UDP-glucuronic acid. The 5’- or 3’-flanking region of each exon 1 is presumably involved in regulation of its expression. Substantial interindividual differences have been detected in mRNA and protein levels and enzymatic activity of the UGT1A isoforms (Fisher et al., 2000; Saito et al., 2007).

SN-38 glucuronidation is thought to be mediated mainly by UGT1A1, and its genetic polymorphisms affecting irinotecan pharmacokinetics and adverse reactions have been already identified. The TA-repeat polymorphism, <54, >39A>TAA>A(TA)G, is associated with lower promoter activity, resulting in reduced SN-38 glucuronidation (Beutler et al., 1998; Iyer et al., 1999). The single nucleotide polymorphism (SNP) 211G>A (Gly71Arg, * allele), found mainly in East Asians, causes reduced protein expression levels and SN-38 glucuronidation activity (Gagné et al., 2002; Jinnno et al., 2003). Another SNP in the enhancer region of UGT1A1, <3279T>G (* allele), is also a causative factor for reduced expression (Sugatani et al., 2002). Allele frequencies have been reported for *28 (0.09–0.13), *6 (0.15–0.19), and *60 (0.26–0.32) in Japanese and Chinese populations and for *28 (0.30–0.39), *6 (0.44–0.55) in whites (Saito et al., 2007). In a previous study, in the Japanese population, we defined haplotype *28 as the haplotype harboring the *28 allele, haplotype *6 as that harboring the*6 allele, and haplotype *60 as that harboring the *6 allele (and without the *28 or *6 allele) (Sai et al., 2004; Saeki et al., 2006). Note that most of the *28 haplotypes concurrently harbored the *60 alleles, and that the *28 and *6 alleles were exclusively present on the different chromosomes (Sai et al., 2004; Saeki et al., 2006). We have also revealed that the haplotype *28, *6, or *60 was associated with reduced SN-38 glucuronidation (SN-38G/SN-38 area under concentration-time curve (AUC) ratios, an in vivo marker for UGT1A activity) (Minami et al., 2007).

In a recent study, an intronic SNP of UGT1A9, IVS1+399 (I399C>T), has been shown to be associated with increased UGT1A9 protein levels and glucuronidation activities toward SN-38 and the UGT1A9 probe drug propofol (Girard et al., 2006).
SN-38 glucuronidation activity by this SNP is significant among subjects without UGT1A1*28. Sandanaraj et al. (2008) have also reported that I399C/C patients showed higher SN-38 AUC than C/T and T/T patients. With the same UGT1A1 diplotype, patients with I399T/T (and UGT1A9 126C, 118T9, 391C > A) show higher SN-38G Cmax than I399C/T (and T/T/T9) patients. UGT1A9*1b (UGT1A9 126C, 118T9, 391C > A) has been shown to have no effect on UGT1A9 expression levels (Girard et al., 2006; Ramirez et al., 2007; Sandanaraj et al., 2008). Thus, two groups did suggest that I399T allele was associated with higher glucuronidation activity. However, using human liver microsomes, Ramirez et al. (2007) showed that I399C/T had no significant effect on both UGT1A9 mRNA levels and glucuronidation activities for two UGT1A9 substrates. Therefore, the roles of I399C/T in UGT1A9 activities as well as SN-38 glucuronidation remain inconclusive.

In the present report, we reveal the linkage of I399C/T with UGT1A1, UGT1A7, and UGT1A9 polymorphisms and analyze its association with the SN-38G/SN-38 AUC ratio and SN-38 AUC/dose (per dose) to clarify its role in SN-38 glucuronidation.

**Materials and Methods**

**Patients.** One hundred and seventy-seven patients (81 lung, 63 colon, 19 stomach, and 14 other cancer patients) administered irinotecan at the National Cancer Center were enrolled in this study as described previously (Minami et al., 2007). This study was approved by the ethics committees of the National Cancer Center and the National Institute of Health Sciences, and written informed consent was obtained from all participants. Eligibility criteria, patient profiles, and irinotecan regimens are summarized in our previous report (Minami et al., 2007). In brief, patients consisted of 135 males and 42 females with a mean age of 60.5 (26–78 years old), and their performance status was 0 (84 patients), 1 (89 patients), or 2 (4 patients). Irinotecan administrations were conducted according to the standard protocols in Japan as follows: i.v. 90-min infusion at a dose of 100 mg/m2 weekly or 150 mg/m2 biweekly in irinotecan monotherapy; and 60 mg/m2 weekly with cisplatin in most combinations.

**Genotyping and Haplotype Analysis.** Genomic DNA was extracted from whole blood of 177 irinotecan-administered patients (Saeki et al., 2006). UGT1A9 IVS1 + 399C>T (rs2741049) was genotyped using the TaqMan SNP Genotyping Assay kit (C_9096281_10) according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). The UGT1A1*28 allele [−54, −39A(TA)TAA>A(TA)TAA], UGT1A6* allele [211G>A (Gly71Arg)], UGT1A6*6 allele [−3279T>G], UGT1A7*2 [387T>G, 391C>A and 392G>A (Asn129Lys and Arg131Lys)], UGT1A7*3 haplotype [387T>G, 391C>A, 392G>A, and 622T>C (Asn129Lys, Arg131Lys, and Thr208Arg)], and UGT1A9*1b allele [−126C, −118T9, 391C > A] were determined previously (Saeki et al., 2006). Hardy-Weinberg equilibration analysis of I399C>T, linkage disequilibrium (LD) analysis of the UGT1A9, UGT1A9, and UGT1A9 polymorphisms, and haplotype estimation with an expectation-maximization algorithm were performed using SNPAlayze version 7.0 software (Dynacom, Chiba, Japan).

**Pharmacokinetics.** Pharmacokinetic data for the 176 irinotecan-treated patients (data for one patient was unavailable) were described previously (Minami et al., 2007). In brief, heparinized blood was collected before irinotecan administration and at 0, 0.33, 1, 2, 4, 8, and 24 h after termination of the first infusion of irinotecan. SN-38 and SN-38G plasma concentrations were calculated by high-performance liquid chromatography, and AUC was calculated using the trapezoidal method in WinNonlin version 4.01 (Pharsight, Mountain View, CA).

**Statistical Analysis.** Gene dose effects of I399C>T and UGT1A1 haplotypes (*28, *6, or *60) were assessed by the Jonckheere-Terpstra test using StatExact version 6.0 (Cytel Inc., Cambridge, MA). Multiplicity adjustment was conducted with the false discovery rate. The significant difference was set at \( p = 0.05 \) (two-tailed).

**Results**

**Linkages of UGT1A9 IVS1+399 (I399C)T with Other Polymorphisms.** In our patients, I399C>T was detected at a 0.636 allele frequency, which is almost the same as those in the HapMap data (rs2741049) for Japanese (0.663) and Han Chinese (0.633) populations, but higher than those for Europeans (0.383) and Sub-Saharan Africans (Yoruba) (0.417). Genotype distribution for this SNP was in Hardy-Weinberg equilibrium \((p = 0.418)\). LD analysis was performed between I399C>T and the previously determined genotypes, UGT1A9*1b, UGT1A7*2 and *3, and UGT1A1*28, *6, and *60, which were detected at >0.1 frequencies in Japanese populations (Saeki et al., 2006). When assessed by the ID1 value, I399C>T was in complete LD with UGT1A7 387T>G, 391C>A and 392G>A (UGT1A7*2, ID1 = 1.00); in strong LD with UGT1A9 126C, −118T9, 391C > A (UGT1A9*1b, 0.987), UGT1A7 622T>C (UGT1A7*3, 0.977), and UGT1A1 211G>A (UGT1A1*6, 0.864); and in moderate LD with UGT1A1 3279T>G (UGT1A1*6, 0.554), but weakly associated with UGT1A1 −54, −39A(TA)TAA>A(TA)TAA, and −60 (−3279T>G). In \( r^2 \) values, the I399C>T was in strong LD with UGT1A7*2 \((r^2 = 0.976)\) and UGT1A9*1b (0.916), in moderate LD with UGT1A7*3 (0.478), but in weak LD with UGT1A1*6 (0.261) and UGT1A1*60 (0.208), and in little LD with UGT1A1*28 (0.018).

**Haplotype Analysis.** Haplotype analysis was performed using the 9 polymorphisms including I399C>T. As shown in Fig. 1, 95% (123/129) of the I399C alleles were linked with the UGT1A9 126C, −118T9 alleles, and 100% (225/225) of the T alleles were linked with the UGT1A7*2 alleles (UGT1A9*1b). The I399C alleles were completely (129/129) linked with the UGT1A7 387T>G, 391A, and 392A alleles. The 40% (51/129) and 60% (78/129) of the I399C alleles were linked with UGT1A7*2 and UGT1A7*3 haplotypes, respectively. We also found that 98% (126/129) of the I399C alleles were linked with the UGT1A1*6 (211G>A), *28 [−54, −39A(TA)TAA>A(TA)TAA], or −60 (−3279T>G). According to the UGT1A1 haplotype definition by Sai et al. (2004), 42% (54/129), 36% (46/129), 19% (25/129), and 1% (1/129) of the I399C alleles were linked with the UGT1A1 haplotypes *6a (harboring *6 allele), *60a (harboring *60 allele), *28h (harboring *60 and *28 alleles), and *28d (harboring *28 allele), respectively. On the other hand, 85% (191/225) of the T alleles were linked with the UGT1A1 wild-type haplotype *1.

**Association Analysis.** The associations of I399C>T with irinotecan pharmacokinetic parameters were then analyzed using the estimated haplotypes. First, association with SN-38G/SN-38 AUC ratio, an in vivo parameter of UGT1A activity (Sai et al., 2004; Minami et al., 2007; Sandanaraj et al., 2008), was analyzed. UGT1A7*2 had unchanged activity for SN-38 glucuronidation (Gagne et al., 2002), and neither UGT1A9*1b nor UGT1A7*3 had significant effects on the SN-38G/SN-38 AUC ratio in our previous study (Minami et al., 2007). On the other hand, the UGT1A1*6, *28, and *60 haplotypes were associated with the reduced SN-38G/SN-38 AUC ratios (Minami et al., 2007). Although effects of the haplotype *28 and *6 were more striking, haplotype UGT1A1*60, harboring only the *60 allele without the *28 allele, was weakly associated with the reduced ratio. To remove even this weak effect and clarify the real effect of I399C>T, UGT1A1*60 was also considered as low-activity haplotype in this analysis. Namely, we analyzed the associations of I399C>T with the AUC ratio within the groups stratified by the UGT1A9 haplotypes, UGT1A9*28 (*28h and *28d), *6 (*6a), and *60 (*60a) (combined and shown as UGT1A1*4+)

When stratified by the I399C>T genotype, a T allele-dependent
increase in the SN-38G/SN-38 AUC ratio was observed (p < 0.0001, Jonckheere-Terpstra test) (Fig. 2A). However, this trend was obviously dependent on biased distributions of UGT1A1 haplotypes; e.g., 96% of the I399C/C patients were homozygotes for UGT1A1*28, *6, or *60; and “UGT1A1*28, *6, or *60”-dependent reduction of SN-38G/SN-38 AUC ratio was found within the I399T/T genotypes (p < 0.05). As shown in Fig. 2B, UGT1A1*28, *6, or *60 (UGT1A1+) dependent reduction in the SN-38G/SN-38 ratio was observed when patients were stratified by these three haplotypes. However, no significant effect of I399C>T was found within the stratified patients (p > 0.05 within the −/−, −/+ , or +/+ patient group in Fig. 2B). As for SN-38 AUC/dose (SN-38 AUC values adjusted by the doses used), a similar UGT1A1 haplotype dependence was observed. Although the I399T-dependent reduction of SN-38 AUC/dose was detected (p < 0.0001), biased distributions of the UGT1A1*28, *6, or *60 were again evident, and the UGT1A1 + haplotypes-dependent increase was significant within the I399 C/T and T/T patients (p < 0.01 and p < 0.05, respectively) (Fig. 2C). Moreover, no significant effect of I399C>T on SN-38 AUC/dose was found when stratified by the UGT1A1 haplotypes (p > 0.05 within the −/−, −/+ , or +/+ patient group in Fig. 2D).

Discussion

In the present study, LD between I399C>T and UGT1A1, UGT1A7, or UGT1A9 polymorphisms in Japanese populations was shown for the first time. Moreover, the apparent effect of I399C>T on SN-38 glucuronidation in Japanese cancer patients was suggested to result from its close association with UGT1A1*28, *6, or *60.

As for the influence of I399C>T on UGT1A9 activity, conflicting results have been reported. Girard et al. (2006) have shown that I399C>T was associated with increased UGT1A9 protein levels and enzyme activity toward an UGT1A9 probe drug propofol using 48 human liver microsomes derived mainly from whites. In contrast, using human liver microsomes from 46 white subjects, Ramírez et al. (2007) have revealed that the I399C>T had no significant effects on UGT1A9 mRNA levels and in vitro glucuronidation activities toward the two UGT1A9 substrates, flavopiridol and mycophenolic acid. Furthermore, another report has demonstrated that I399C>T had no influence on the pharmacokinetic parameters (such as AUC and C_{max}) of mycophenolic acid in 80 Japanese renal transplant recipients (Inoue et al., 2007). Thus, these latter two studies did suggest that the I399C>T polymorphism has no effect on UGT1A9 enzymatic activity. Note that, at least for Japanese populations, no study has reported that I399C>T affects UGT1A9 activity.

As for the influence of I399C>T on SN-38 glucuronidation, a possible enhancing effect has been suggested. Girard et al. (2006) have shown an increasing effect of I399C>T on SN-38 glucuronidation, and that this SNP did not show any close linkages with the UGT1A1*28 or *60 allele (r^2 < 0.06). In addition, Sandanaraj et al. (2008) have reported that in 45 Asians consisting of Chinese (80%), Malay (18%), and others (2%), I399C/C patients had higher SN-38 AUC than C/T and T/T patients. Again, this SNP was not in LD with the UGT1A1*28, *6, or *60 allele (r^2 were <0.09). Furthermore, association of I399T with increased SN-38G C_{max} has been observed even after stratified patients by UGT1A1 genotypes, although the study sample size was small. These findings suggest that the I399T
allele was associated with increased glucuronidation activity for SN-38 without linkages with the UGT1A1 polymorphisms. Our data demonstrate that an increase in SN-38G/SN-38 AUC ratio (i.e., increased glucuronidation activity) was also found with I399C/H11022; however, after stratified patients by the UGT1A1*28, *6, or *60 haplotypes: /-/-, 0 UGT1A1*28, *6, or *60; /-/+ heterozygotes for either UGT1A1*28, *6, or *60; +/-, homozygotes or compound heterozygotes for either UGT1A1*28, *6, or *60. B and D, UGT1A1 /-/-, /-/+ and +/- patients were further divided by I399 C/C, C/T, and T/T genotypes. Gene dose effects of I399C>T and the UGT1A1 haplotype were assessed by the Jonckheere-Terpstra test.

In conclusion of this study, the apparent influence of I399 (UGT1A9 IVS1+399C>T on SN-38 glucuronidation is attributable to its close association with UGT1A1*6, *28, or *60 in the Japanese population. Furthermore, additional genotyping of I399C>T for personalized irinotecan therapy seems to be clinically irrelevant for Japanese populations.

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