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Close Association of *UGT1A9* IVS1+399C>T with *UGT1A1*28, *6* or **60* Haplotype and its Apparent Influence on SN-38 Glucuronidation in Japanese

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Short title: Role of *UGT1A9* IVS1+399C>T on SN-38 glucuronidation

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Abstract

The anti-cancer prodrug, irinotecan, is converted to its active form SN-38 by carboxylesterases, and SN-38 is inactivated by UGT1A1-mediated glucuronidation. UGT1A9 also mediates this reaction. Recently, it was reported that the *UGT1A9* IVS1+399 (I399)C>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 *UGT1A9*1b* (-126_-118T_9>T_{10}, |D'|=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$(TA)_6$TAA>A$(TA)_7$TAA, 0.25). 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 glucuronide/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 by *UGT1A1*6*, **28* or **60* haplotype. Thus, at least in Japanese, influence of I399C>T on SN-38 glucuronidation is attributable to its close association with either *UGT1A1*6*, **28* or **60*.

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Introduction

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 SN-38 by carboxylestrases, 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 (Gagne et al., 2002; Saito et al., 2007).

The UGT1A gene complex consists of 9 active first exons including *UGT1A10*, *1A9*, *1A7* and *1A1* (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 UGT1A 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$(TA)_6$TAA>A$(TA)_7$TAA (*UGT1A1*28* allele) , 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, **6* allele), found mainly in East Asians, causes reduced protein expression levels and SN-38 glucuronidation activity (Gagne et al., 2002; Jinno et al., 2003). Another SNP in the enhancer region of *UGT1A1*, -3279T>G (**60* 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) and **60* (0.44-0.55) in Caucasians (Saito et al., 2007). Previously 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 **60* allele (and without the **28* or **6*

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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 glucuronide (SN-38G)/SN-38 area under concentration-time curve (AUC) ratios, an *in vivo* parameter for UGT1A activity (Minami et al., 2007).

Recently, an intronic SNP of *UGT1A9*, IVS1+399 (I399)C>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). Elevation of SN-38 glucuronidation activity by this SNP is significant among subjects without *UGT1A1*28*. Sandanaraj *et al*. (2008) also have reported that I399C/C patients showed higher SN-38 AUC than C/T and T/T patients. With the same *UGT1A1* diplotypes, patients with I399T/T (and *UGT1A9* -126_-118T_{10}/T_{10}) have shown higher SN-38G Cmax than I399C/T (and T_9/T_{10}) patients. *UGT1A9*1b* (*UGT1A9* -126_-118T_9>T_{10}) has been shown to have no impact 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)

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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). Briefly, patients consisted of 135 males and 42 females with a mean age of 60.5 (26 to 78), and their performance status was 0 (84 patients), 1 (89) or 2 (4). Irinotecan administrations were conducted according to the standard protocols in Japan as follows: intravenous 90-minute infusion at a dose of 100 mg/m^2 weekly or 150 mg/m^2 biweekly in irinotecan monotherapy; and 60 mg/m^2 weekly with cisplatin in most combination therapies.

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)$_6$TAA>A(TA)$_7$TAA), *UGT1A1*6* allele [211G>A (Gly71Arg)], *UGT1A1*60* allele (-3279T>G), *UGT1A7*2* haplotype [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 Trp208Arg)] and *UGT1A9*1b* allele (-126_-118T$_9$>T$_{10}$) were previously determined (Saeki et al., 2006). Hardy-Weinberg equilibrium analysis of I399C>T, linkage disequilibrium (LD) analysis of the *UGT1A9*, *UGT1A7* and *UGT1A1* polymorphisms, and haplotype estimation with an expectation-maximization algorithm were performed using SNPAlyze ver. 7.0 software (Dynacom, Chiba, Japan).

Pharmacokinetics

Pharmacokinetic data for the 176 irinotecan-treated patients (data for one patient was unavailable) were previously described (Minami et al., 2007). Briefly, heparinized blood was collected before irinotecan administration, and at 0, 0.33, 1, 2, 4, 8, and 24 hours after termination of the first infusion of irinotecan. SN-38 and SN-38G plasma concentrations were determined by HPLC, and AUC was calculated using the trapezoidal method in WinNonlin ver. 4.01 (Pharsight Corporation, 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 (I399)C>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), 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 (Saeki et al., 2006). When assessed by the |D'| value, I399C>T was in complete LD with *UGT1A7* 387T>G, 391C>A and 392G>A (*UGT1A7*2*, |D'|=1.000); in strong LD with *UGT1A9* -126_-118T_9>T_{10} (*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*60*, 0.554), but weakly associated with *UGT1A1* -54_-39A$(TA)_6$TAA>A$(TA)_7$TAA (*UGT1A1*28*, 0.252). In r^2 values, the I399C>T was in strong LD with *UGT1A7*2* (r^2=0.976), *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* -126_-118T_9 alleles, and 100%

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(225/225) of the T alleles were linked with the T_{10} alleles (*UGT1A9*1b*). The I399C alleles were completely (129/129) linked with the *UGT1A7* 387G, 391A and 392A alleles, and most T alleles (223/225) were linked with the 387T, 391C and 392G 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$(TA)_6$TAA>A$(TA)_7$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), **28b* (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 *UGT1A9* IVS1+399C>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 *UGT1A1* haplotypes, *UGT1A1*28* (**28b* and **28d*), **6* (**6a*) and **60* (**60a*) (combined and shown as *UGT1A1* "+").

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

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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-dependency 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 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 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 Caucasians. In contrast, using human liver microsomes from 46 Caucasian subjects, Ramirez *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 Cmax) 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 impact on UGT1A9 enzymatic activity. Note that at least for Japanese, 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). Also, 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 Cmax has been observed even after stratified 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. In our hands, increase in SN-38G/SN-38 AUC ratio (*i.e.*, increased glucuronidation activity) was also found with I399C>T, but after stratified by the "*UGT1A1*6, *28* or **60*" haplotypes (haplotype +) showing reduced SN-38 glucuronidation activity (Sai et al., 2004; Minami et al., 2007), any significant effect of the I399C>T was no longer observed. Thus, no direct effect of I399C>T on SN-38 glucuronidation was shown in the current study in Japanese. The discrepancy between our study and others might be derived from ethnic and/or population differences in haplotype distribution. In fact, in our Japanese population, 98% of the I399C alleles were linked with either *UGT1A1 *6, *28* or **60*, while 85% of the T alleles were linked with *UGT1A1*1*. On the other hand, in Sandanaraj's report (in Chinese + Malay), 84% of the I399C alleles were linked with *UGT1A1 *6, *28* or **60*, while only 67% of the T alleles were linked with *UGT1A1*1* (Sandanaraj et al., 2008).

In irinotecan therapies, genetic polymorphisms leading to increases in SN-38 AUC, which closely correlates with increased risk of severe neutropenia (Minami et al., 2007), are clinically important. The current study also demonstrated no significant influence of I399C>T on SN-38 AUC/dose after stratified by *UGT1A1* haplotypes. Consistent with this finding, no influence of this SNP was observed on the

incidence of grade 3 or 4 neutropenia after irinotecan therapy in our population (data not shown). Recently, genetic testing of *UGT1A1*6* and **28*, which are related to severe neutropenia in Japanese, has been approved for clinical application in Japan. This study indicates that there is no clinical necessity for additional genotyping of I399C>T, at least in Japanese.

In conclusion of this study, the apparent influence of I399 (*UGT1A9* IVS1+399)C>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 appears to be clinically irrelevant for Japanese.

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Footnotes

Yoshiro Saito and Kimie Sai contributed equally to this work.

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Figure Legends

Fig. 1: Haplotypes assigned by using common *UGT1A9, UGT1A7* and *UGT1A1* polymorphisms. [1]Haplotypes were shown as *UGT1A9* haplotypes - *UGT1A7* haplotypes - *UGT1A1* haplotypes. Major allele, white; minor allele, gray. **1C*, T_9 and I399C; **1bC*, T_{10} and I399C; **1bT*, T_{10} and I399T in *UGT1A9*. [2]*UGT1A7*2* and **3* are the haplotypes harboring the three and four *UGT1A7* alleles, respectively. [3]*UGT1A1* $(TA)_6$>$(TA)_7$ indicates -54_-39A$(TA)_6$TAA>A$(TA)_7$TAA.

Fig. 2: Association analysis of *UGT1A9* IVS1+399 (I399)C>T with SN-38G/SN-38 AUC ratio (A, B) and SN-38 AUC/dose (C, D). (A, C) I399 C/C, C/T and T/T patients were further divided by the presence of *UGT1A1*28*, **6* or **60* haplotypes: -/-, no *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, 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.

Gene		*UGT1A9*		*UGT1A7*[2]				*UGT1A1*[3]			Number	Frequency
Nucleotide change		-126_-118 T_9>T_{10}	IVS1+399 C>T	387 T>G	391 C>A	392 G>A	622 T>C	-3279 T>G	$(TA)_6$>$(TA)_7$	211 G>A		
Allele name		**1b*		**2, *3*	**2, *3*	**2, *3*	**3*	**60, *28*	**28*	**6*		
Haplotypes[1]	**1C-*3-*6a*			■	■	■	■			■	47	0.133
	**1C-*2-*60a*			■	■	■		■			44	0.124
	**1C-*3-*28b*			■	■	■	■	■	■		21	0.059
	**1C-*2-*28b*			■	■	■		■	■		4	0.011
	**1C-*3-*60a*			■	■	■	■	■			2	0.006
	**1C-*3-*28d*			■	■	■	■		■		1	0.003
	**1C-*2-*6a*			■	■	■				■	1	0.003
	**1bC-*3-*6a*	■		■	■	■	■			■	6	0.017
	**1C-*2-*1*			■	■	■					2	0.006
	**1C-*3-*1*			■	■	■	■				1	0.003
	**1bT-*1-*1*	■	■								190	0.537
	**1bT-*3-*1*	■	■	■	■	■	■				1	0.003
	**1bT-*1-*28b*	■	■					■	■		22	0.062
	**1bT-*1-*60a*	■	■					■			5	0.014
	**1bT-*1-*6a*	■	■							■	5	0.014
	**1bT-*1-*28d*	■	■						■		1	0.003
	**1bT-*2-*60a*	■	■	■	■	■		■			1	0.003
Alelle frequency		0.653	0.636	0.370	0.370	0.370	0.223	0.280	0.138	0.167	354	1.000

Fig. 1

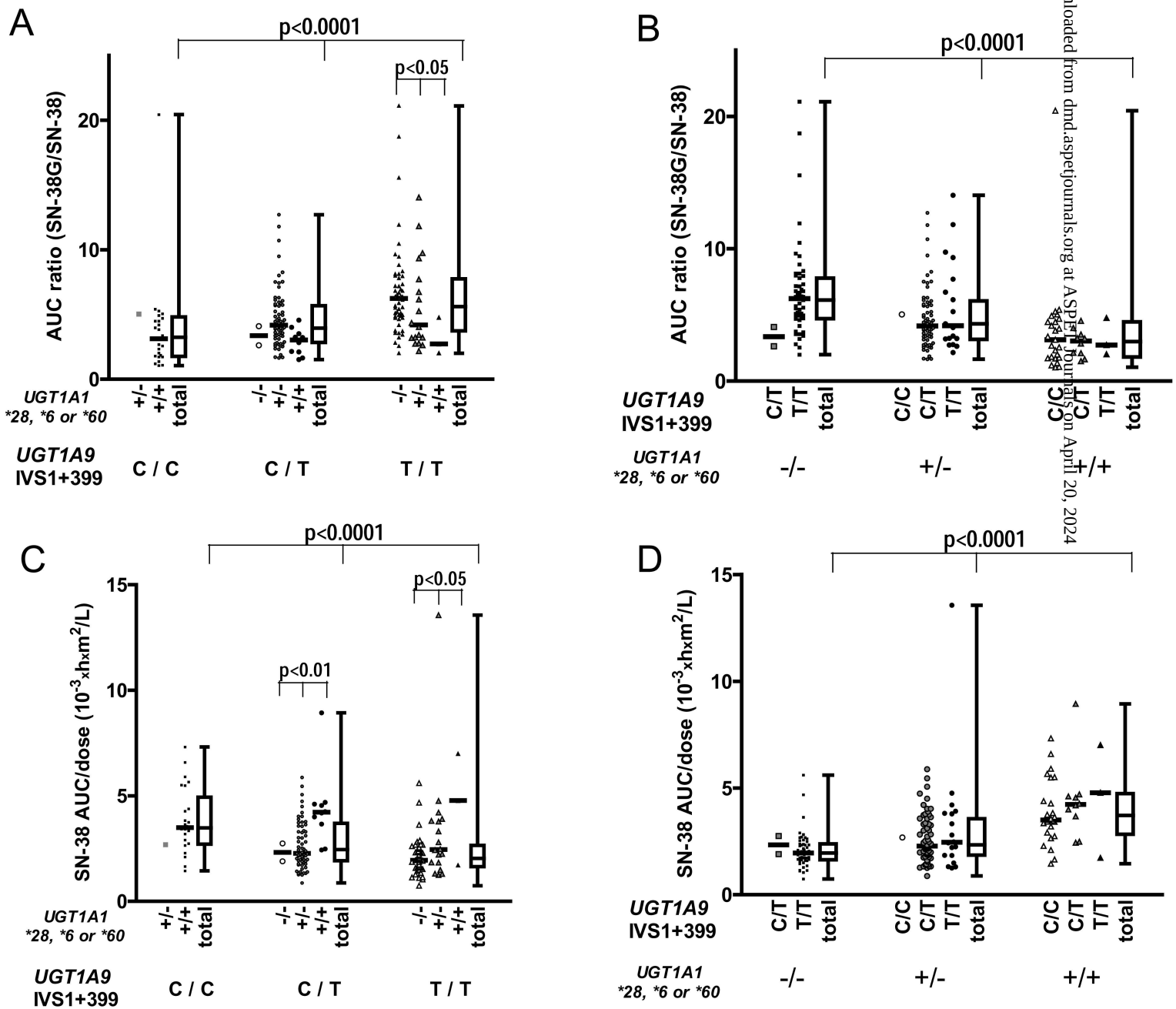

Fig. 2