RACIAL VARIABILITY IN HAPLOTYPE FREQUENCIES OF UGT1A1 AND GLUCURONIDATION ACTIVITY OF A NOVEL SINGLE NUCLEOTIDE POLYMORPHISM 686C>T (P229L) FOUND IN AN AFRICAN-AMERICAN

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ABSTRACT:

Ethnic differences in genetic polymorphisms in UDP-glucuronosyltransferase 1A1 (UGT1A1) were investigated among African-Americans, Caucasians, and Japanese using samples obtained from 150 individuals for each population. Genotyping of −3279T>G in the phenobarbital-responsive enhancer module, TA repeats in the TATA box, 211G>A (G71R) and 686C>A (P229Q) in exon 1, and three single nucleotide polymorphisms (SNPs) (1813C>T, 1941C>G, and 2042C>G) in the 3′-untranslated region in exon 5 was performed. Eight haplotypes of block 1 (exon 1 and its 5′-flanking region) harboring the first four variations were assigned to each individual. The dominant haplotype for African-Americans was *28b (−3279G;TA6;211G;686C) (0.446), whereas that for the Japanese was *1a (−3279T;TA5;211G;686C) (0.610). Frequencies of the two haplotypes *1a and *28b were comparable in Caucasians. Haplotype *6a (−3279T;TA6;211A;686C) was characteristic of the Japanese, whereas haplotypes *36b and *37b (−3279T;TA6 and TA5;211G;686C) were found mostly in African-Americans. Although the three SNPs in block 2 (exons 2–5) were in complete linkage in the Japanese, they were not completely linked in African-Americans or Caucasians. These differences in haplotype distribution patterns among the three populations suggest the possibility of ethnic differences in toxicity profiles of drugs detoxicated by UGT1A1. A novel SNP, 686C>T (P229L), was found in an African-American. The intrinsic clearance of 7-ethyl-10-hydroxycamptothecin (SN-38) by P229L UGT1A1 expressed in COS-1 cells was about 3% of the wild type. The results of Western blotting and real-time reverse transcription-polymerase chain reaction suggest that the low glucuronidation activity of the variant was partly due to its low stability. The variation 686C>T may cause high toxicity during 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) therapy or hyperbilirubinemia in patients.

UDP-glucuronosyltransferases catalyze the glucuronidation of various lipophilic endogenous and exogenous substances including drugs and environmental toxicants to produce glucuronides. UDP-glucuronosyltransferase 1A1 (UGT1A1) is a member of the UGT1A family and is exclusively involved in the metabolism of bilirubin (Tukey and Strassburg, 2000). It is also known to glucuronidate 7-ethyl-10-hydroxycamptothecin (SN-38), the active metabolite of an anticancer drug, irinotecan (CPT-11; 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin), to form inactive 7-ethyl-10-hydroxycamptothecin glucuronide (SN-38G) (Iyer et al., 1998; Hanioka et al., 2001). Since SN-38 is associated with severe diarrhea due to administration of CPT-11 (Araki et al., 1993), detoxication of SN-38 by UGT1A1 could play a significant role in protecting against the severe side effects caused by CPT-11.

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ABBREVIATIONS: SN-38, 7-ethyl-10-hydroxycamptothecin; CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; SN-38G, 7-ethyl-10-hydroxycamptothecin glucuronide; CN, Crigler-Najjar; SNP, single nucleotide polymorphism; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction.
African-American individual were done by dideoxy sequencing as reported in the /H11022 G for African-American (Saeki et al., 2003). Genotypes were determined using the PSQ 96MA (Bioan and annealing of the sequencing primers have been described previously (Saeki et al., 2003). The single nucleotide polymorphism (SNP) –3279T>G is located in the phenobarbital-responsive enhancer module, which is involved in activation of UGT1A1 transcription by the constitutive androstane receptor (Suganagi et al., 2002).

A recent pharmacogenetic study has suggested that there are advantages in the use of haplotypes rather than individual SNPs to investigate the association between genotypes and phenotypes (Judson et al., 2000). Racial variability in haplotype frequencies of UGT1A1 may contribute to an interpretation of the racial diversity of pharmacokinetics and toxicity of drugs metabolized by the enzyme. Recently, Sai et al. (2004) revealed the haplotype structure of UGT1A1 in Japanese cancer patients using –3279T>G, TA repeats in the TATA box, 211G>A, 686C>A, and three SNPs (1813C>T, 1941C>G, and 2042C>G) in the 3’-untranslated region in exon 5 as markers. Therefore, we studied in this report the differences in the haplotype frequencies of UGT1A1 among African-American, Caucasian, and Japanese populations using genomic DNA obtained from 150 individuals in each population, assessing the genotypes of these markers with dideoxy sequencing and pyrosequencing methods previously reported by Saeki et al. (2003). The function of a novel SNP 686C>T (P229L) that was found in an African-American individual during this study was also investigated by utilizing a heterologous expression system with COS-1 cells.

Materials and Methods

Materials

SN-38 (lot 970507R) and SN-38G (lot 970507R) were kindly supplied by Yakult Honsha Co. Ltd. (Tokyo, Japan). COS-1 cells were obtained from healthy Japanese volunteers, 100 of which were kindly provided by Professor Ichiro Ieiri from the Tottori University Hospital with permission of the ethics committee of the Tottori University Faculty of Medicine. Written informed consent was obtained from all participants. Peripheral blood samples of healthy Caucasian and African-American volunteers (150 each) were purchased from the Tennessee Blood Service Corporation (Memphis, TN). The ethics committee of the National Institute of Health Sciences approved this study.

Peripheral Blood Samples.

One hundred fifty peripheral blood samples were obtained from healthy Japanese volunteers, 100 of which were kindly provided by Professor Ichiro Ieiri from the Tottori University Hospital with permission of the ethics committees of the Tottori University Faculty of Medicine. Written informed consent was obtained from all participants. Peripheral blood samples of healthy Caucasian and African-American volunteers (150 each) were purchased from the Tennessee Blood Service Corporation (Memphis, TN). The ethics committee of the National Institute of Health Sciences approved this study.

UGT1A1 Genotyping by Pyrosequencing and Dideoxy Sequencing.

Genotyping of UGT1A1 using Pyrosequence methods was performed as previously reported (Saeki et al., 2003). Briefly, genomic DNA (10–15 ng) was amplified by Ex-Taq (1 U; Takara Shuzo, Otsu, Japan) with a specific primer pair in which one of the primers was biotinylated. The primers for amplification of the single-stranded fragment from pDONR201 into pcDNA-DEST40, a mammalian expression vector, was performed by the Gateway LR reaction.

Expression levels of UGT1A1 mRNA and protein were determined as described previously (Jinno et al., 2003b). SN-38 glucuronidation activity of the wild-type and P229L variant UGT1A1 was assayed according to the method of Hanioka et al. (2001).

Data Analysis.

The χ2 test in the SAS Preclinical Package (SAS Institute Japan Ltd., Tokyo, Japan) was applied to s × r contingency tables for comparison of haplotype frequencies and analysis of haplotype distribution patterns among the three ethnic groups. In vitro Michaelis-Menten kinetic parameters were estimated by nonlinear regression analysis using Prism version 3.0 (GraphPad Software Inc., San Diego, CA). The average values of the kinetic parameters were calculated using results from three independent preparations. The t test supplied in the software version 3.0 was applied to the comparison of the average values of protein expression and mRNA levels between wild-type and variant UGT1A1 at a significance level of 0.05.

Results

Differences in Allele Frequencies among Three Ethnic Groups.

Allele frequencies of polymorphisms in UGT1A1 tested in this study are summarized in Table 1. The estimated allele frequency of –3279T>G (UGT1A1*60; for UGT1A1 allele nomenclature refer to Mackenzie et al., 1997 and http://som.flanders.edu/uFUSA/ClinPharm/UGT1A1alleles.htm) was 0.847, 0.550, and 0.257 in African-Americans, Caucasians, and Japanese, respectively, and they were significantly different (p < 0.0001 by the χ2 test). The variation in the TATA box, the allele TA5, was most common in the Japanese population, whereas frequencies of TA5 and TA6 (UGT1A1*28) were equally frequent in African-Americans. The allele frequencies of TA6 (UGT1A1*36) or TA5 (UGT1A1*37) in African-Americans were about 5%, but no carriers with TA5 or TA6 were found in the Japanese. The allele distribution patterns for the wild-type and three variants in the TATA box of the three ethnic groups were significantly different (p < 0.0001 by the χ2 test). The variant 211G>A (G71R) (UGT1A1*6) was frequently found in the Caucasian population, but not in the Caucasians population (only two heterozygous subject), and none were found in African-Americans. The variant 686C>A (P229Q) (UGT1A1*27) was very rare in all ethnic groups, and only one heterozygous carrier was found in the Japanese group. A novel variation in the same position as the variant 686C>A, but with a different nucleotide change (686C>T), was found in an African-American. This novel variant led to an amino acid change from proline to leucine (P229L). Pyrograms for 686C>C, 686C>A, and 686C>T are compared in Fig. 1A and the nucleotide change of the novel variant was confirmed by the dideoxy sequencing method (Fig. 1B). This is the first report detailing allele frequencies of three SNPs in the 3’-untranslated region in exon 5, 1813C>T, 1941C>G and
<table>
<thead>
<tr>
<th>Position</th>
<th>Change</th>
<th>African-American</th>
<th>Caucasian</th>
<th>Japanese</th>
<th>p (χ² test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number of Heterozygotes/ Homozygotes</td>
<td>Allele Frequency (lower limit, upper limit)</td>
<td>Number of Heterozygotes/ Homozygotes</td>
<td>Allele Frequency (lower limit, upper limit)</td>
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<td>Number of Heterozygotes/ Homozygotes</td>
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<td></td>
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<td>(lower limit, upper limit)</td>
<td>(lower limit, upper limit)</td>
<td>(lower limit, upper limit)</td>
<td>(lower limit, upper limit)</td>
</tr>
<tr>
<td>−3279</td>
<td>T &gt; G</td>
<td>36/109</td>
<td>0.847 (0.806, 0.888)</td>
<td>65/90</td>
<td>0.550 (0.494, 0.606)</td>
</tr>
<tr>
<td></td>
<td>TA box</td>
<td>TAα</td>
<td>0.044 (0.021, 0.067)</td>
<td>0.017 (0.002, 0.032)</td>
<td>0.007 (0.000, 0.017)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>69/32</td>
<td>0.446 (0.390, 0.502)</td>
<td>77/48</td>
<td>0.588 (0.532, 0.644)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>65/34</td>
<td>0.446 (0.390, 0.502)</td>
<td>78/18</td>
<td>0.388 (0.332, 0.444)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17/1</td>
<td>0.064 (0.000, 0.092)</td>
<td>2.0</td>
<td>0.000 (0.000, 0.016)</td>
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<td>211</td>
<td>G &gt; A</td>
<td>G71R</td>
<td>0/0</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>686</td>
<td>C &gt; A</td>
<td>P229Q</td>
<td>0/0</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>C &gt; T</td>
<td>P229L</td>
<td>0/1</td>
<td>0.003 (0.000, 0.009)</td>
<td>0.000</td>
</tr>
<tr>
<td>1813</td>
<td>C &gt; T</td>
<td></td>
<td>61/22</td>
<td>0.350 (0.296, 0.404)</td>
<td>64/6</td>
</tr>
<tr>
<td>1941</td>
<td>C &gt; G</td>
<td></td>
<td>43/6</td>
<td>0.183 (0.139, 0.227)</td>
<td>47/1</td>
</tr>
<tr>
<td>2042</td>
<td>C &gt; G</td>
<td></td>
<td>68/23</td>
<td>0.380 (0.325, 0.435)</td>
<td>60/6</td>
</tr>
</tbody>
</table>

a Values in parentheses are numbers of individuals determined.
b Assumed to be equal to the frequency for 1941 C > G.
2042C→G in African-Americans and Caucasians. The frequencies of
1813C→T and 2042C→G in the Japanese were not determined in this
study, because these alleles were reported to be in complete associa-
tion with 1941C→G in a Japanese population without exception (Sai et
al., 2004; unpublished data). Therefore, allele frequencies of
1813C→T and 2042C→G in Japanese were assumed to be equal to
that of 1941C→G. On the other hand, we found that 1813C→T and
2042C→G were not always in association with 1941C→G in African-
Americans and Caucasians, as shown in Table 1. The allele frequen-
cies of the three SNPs were highest in African-Americans and lowest
in the Japanese. Significant differences in the allele frequencies were
detected (p < 0.0001, p = 0.0074, and p < 0.0001 for 1813C→T,
1941C→G, and 2042C→G, respectively, by the χ² test).

Differences in Haplotype Frequencies. Sai et al. (2004) previously
reported that UGT1A1 could be divided into two blocks, with the transcription-regulating and promoter regions and exon 1 in block
1, and exons 2 to 5 in block 2, according to linkage disequilibrium
analysis. Therefore, haplotype/diplotype analysis was performed us-
ing four marker variations in block 1 (−3279T→G, 211G→A, and
686C→A, and the TATA box), and three marker variations in block 2
(1813C→T, 1941C→G, and 2042C→G). The diplotype configurations
(combinations of haplotypes) were estimated for each subject by
PHASE software (Stephens et al., 2001; Stephens et al., 2003).

Concerning block 1, the diplotype configurations were inferred with greater than 0.99 certainties for 145 Japanese, 144 Caucasians,
and 147 African-Americans. The haplotypes identified are summa-
rized in Table 2 along with their frequencies for each population,
where the 14 subjects with ambiguous diplotype sequences were excluded. We
calculated the haplotype nomenclature previously reported (Sai et al.,
2004). One Japanese subject carrying heterozygous 686C→A also
was confirmed in this study, as previously reported by Huang et al. (2000) and Sai et al. (2004). A new haplotype, designated
*6d, having variations in both positions −3279 and 211, was identi-
ﬁed that was not found in the previous study (Sai et al., 2004). We
could not determine whether the novel variation 686T and the *28
allele were on the same chromosome, because the subject with 686T
was also found in Caucasians (0.06). Thus, the haplotype distri-
bution patterns in block 1 for the individual populations were signiﬁcantly different (p < 0.0001 by the χ² test).

In block 2, the diplotype configurations using the three SNPs were
inferred with certainties greater than 0.98 for all Caucasians and
African-Americans (150 each). The haplotypes and their frequen-
cies are summarized in Table 3. Four novel haplotypes, *IC, *ID, *IE, and
*IF, were identiﬁed in the Caucasian and African-American popula-
tions, although *IE was not found in the latter. These haplotypes were
not found in the Japanese, as shown in a previous study (Sai et al.,
2004). Although the three SNPS were reported to be completely
associated with each other in the Japanese (Sai et al., 2004), they were
not always linked with each other in the rest of the populations.
However, it is noteworthy that 1941G allele was always associated
with 1813T and 2042G alleles except for the haplotype *IE, found
only in two Caucasians. Haplotype *IA was predominant for all ethnic
groups. The second major haplotype was *IB in both the Japanese and
Caucasians. However, the frequencies of *IB and *IC were similar in
African-Americans (0.183 and 0.163, respectively). The haplotype
*IC was also found in Caucasians (0.06). Thus, the haplotype distri-
bution patterns in block 2 for the three populations were also signiﬁcantly different (p < 0.0001 by the χ² test).

Expression and SN-38 Glucuronidation of a Novel Variant
P229L Compared with Wild-Type UGT1A1. The relative expres-
sion level of the novel variant UGT1A1/P229L in the membrane fraction of COS-1 cells was determined by Western blotting using a polyclonal anti-human UGT1A antibody (Fig. 2). Because two bands, one at the same position as the wild-type and the other at a higher molecular weight, were detected for P229L, the total chemiluminescence of the two bands was used to calculate the protein expression level for P229L. The protein expression level of P229L was approximately 60% of the wild type, and this difference was statistically significant by the $t$ test ($p < 0.044$). The $UGT1A1$ mRNA expression level was then measured by real-time reverse transcription-PCR (RT-PCR) using SYBR Green. As shown in Fig. 3, however, no significant difference in the mRNA level was detected among the COS-1 cells transfected with the expression plasmids carrying wild-type and P229L $UGT1A1$ cDNAs.

The glucuronidation activity of SN-38 by P229L expressed in COS-1 cells was compared with that of the wild-type under 11 substrate concentrations ranging from 2.5 to 150 $\mu$M. The representative curves of the Michaelis-Menten kinetics are shown in Fig. 4, and the estimated apparent kinetic parameters ($K_m$, $V_{max}$, and $V_{max}/K_m$) are summarized in Table 4. $V_{max}$ values normalized...
to the expression levels are also shown. Wild-type UGT1A1 catalyzed SN-38 glucuronidation with an average apparent $K_m$ value of 8.67 $\mu$M, whereas P229L catalyzed SN-38 glucuronidation with a $K_m$ value of 37.6 $\mu$M. The average $V_{\text{max}}$ values were 71.2 and 5.27 pmol/min/mg membrane protein for the wild-type and P229L, respectively. The average intrinsic clearances of SN-38 by glucuronidation ($V_{\text{max}}/K_m$) normalized to expressed UGT1A1 protein levels were 8.26 and 0.24 $\mu$l/min/mg protein for the wild-type and P229L, respectively.

Discussion

There have been previous reports of the allele frequencies of variants in the TATA box in various ethnic groups (Beutler et al., 1998; Fertrin et al., 2002; Innocenti et al., 2002; Sugatani et al., 2002; Ki et al., 2003; Premawardhena et al., 2003; Sai et al., 2004). According to these studies, allele frequencies of the wild-type $TA_9$ were highest in Polynesians (0.97–0.99), followed by Asians, including Japanese, Koreans, and Taiwanese (0.81–0.92), and Caucasians (0.61–0.73). Allele frequencies were lowest in Africans (0.44–0.52). The allele frequencies of $TA_9$ and $TA_8$ in Africans were reported as approximately 0.05, but were very rare in Caucasians and were not detected in Asians. The allele frequencies of the four genotypes in the TATA box in African-American, Caucasian, and Japanese populations observed in this study were in the ranges previously reported. Allele frequencies of other $UGT1A1$ variations have not been as extensively studied as those in the TATA box. The allele frequencies for the variant $-3297T>G$ were 0.17 to 0.27 in Japanese and Koreans (Sugatani et al., 2002; Ki et al., 2003; Sai et al., 2004), 0.47 in Caucasians (Innocenti et al., 2002), and 0.85 in African-Americans (Innocenti et al., 2002). Thus, our data (0.257 for Japanese, 0.550 for Caucasians, and 0.847 for African-Americans) are comparable with previously reported frequencies of this allele. The allele frequency of the variant $211G>A$ observed in this study was comparable to previously reported values for Asian people (0.09–0.21) (Huang et al., 2000; Sugatani et al., 2002; Ki et al., 2003; Sai et al., 2004). The variant is considered to be characteristic for Asians. We found only two individuals with this allele out of 150 Caucasians, and no African-Americans had this allele.

Innocenti et al. (2002) reported on the haplotype frequencies of $UGT1A1$ in African-Americans and Caucasians using $-3297T>G$, the TA repeat, and other SNPs in the transcription-regulating region. Although they did not use the marker SNPs $211G>A$ and $686C>A$ (likely due to their rarity in both populations), frequencies of the $*28$, $*36$, $*37$, and $*60$ haplotype groups in African-Americans can be calculated according to their data as 0.35, 0.04, 0.12, and 0.33, and the calculated frequencies in Caucasian as 0.36, 0.01, 0.01, and 0.09. These frequencies were also comparable to our data. We found two individuals carrying $211G>A$ in the Caucasian group; however, we could not infer haplotypes for these individuals with high certainty. The observed haplotype $*28$ group frequency in our study of 0.100 ($*28b$ plus $*28c$) was slightly lower than the reported values of 0.131 in Japanese patients (Sai et al., 2004) and 0.127 in Koreans (Ki et al., 2003). However, the previously reported values are included in the 95% confidence interval of our data. The observed haplotype $*6$ group
frequency (allele plus allele) was also comparable to the previously reported data in Japanese and Koreans. A strong association of TA7 such as phosphorylation or glycosylation, which may occur due to the result of products with different post-translational modifications was also observed in Western blots of variant UGT1A1. The low molecular weight in SDS-polyacrylamide gel electrophoresis is considered to be higher than the wild-type UGT1A1 was detected by the study (less than 3% of that for the wild type). The low glucuronidation pathway using drugs, such as CPT-11, that are metabolized by different ethnic groups may cause different toxicity profiles during therapy using drugs, such as CPT-11, that are metabolized by UGT1A1.

A novel SNP 686C>A was found in an African-American sample in this study, leading to an amino acid change, P229L, which is different from the known SNP 686C>A (P229Q). Studies using a larger sample size may be necessary to elucidate an accurate frequency. The known SNP 686C>A (P229Q) was reported to be related to Gilbert’s syndrome and to have very low bilirubin glucuronidation activity (Koiwai et al., 1995). However, Jinno et al. (2003a,b) reported that protein expression and mRNA levels of P229Q expressed in COS-1 cells were comparable to those of the wild type, and the decrease in its SN-38 glucuronidation was marginal. Since the association of P229Q with TA7 has been suggested previously (Huang et al., 2000; Sai et al., 2004) and confirmed in this study, hyperbilirubinemia observed in Japanese and Taiwanese patients carrying the P229Q variant can be mainly attributed to the TA7 variation. On the other hand, the novel variant P229L expressed in COS-1 cells was found to have extremely low SN-38 glucuronidation efficacy in this study (less than 3% of that for the wild type). The low glucuronidation activity of P229L was not caused by its low transcription levels, because a significant difference in the mRNA level was not observed between the wild type and variant. The low UGT1A1/P229L expression level in COS-1 cells suggests that this enzyme is unstable compared with the wild-type enzyme. Moreover, a band shifted to a higher molecular weight than the wild-type UGT1A1 was detected by a rabbit anti-human UGT1A antibody in a Western blot. Such additional bands were also observed in Western blots of variant UGT1A1 having extremely low enzyme activities, such as F83L and Y486D in our group (Jinno et al., in press). The band shift to a higher molecular weight in SDS-polyacrylamide gel electrophoresis is considered to be the result of products with different post-translational modifications such as phosphorylation or glycosylation, which may occur due to conformational changes caused by amino acid substitution. This may also contribute to the extremely low catalytic activity of the variant enzyme compared with that of the wild type as shown in Table 4. Although the clinical significance of 229L remains to be confirmed, this novel SNP may be a potential cause of diseases such as hyperbilirubinemia or jaundice and may cause severe adverse effects after administration of CPT-11.

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**References**


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