Prevalence of topoisomerase I genetic mutations and UGT1A1 polymorphisms associated with irinotecan in individuals of Asian descent

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Abstract. Topoisomerase I (TOP-I) mutations have been shown to be correlated to irinotecan resistance in vitro. However, the prevalence of TOP-I germline mutations has yet to be systematically elucidated. On the other hand, polymorphisms of UGT1A1 have been shown to be associated with CPT-11 toxicity in clinical situations. The primary aim of this study was to investigate the prevalence of mutations in the TOP-I exons associated with CPT-11 resistance, including untreated cancer tissue. A secondary aim was to confirm the less frequent UGT1A1*28 and more frequent UGT1A1*6 in individuals of Asian descent compared to Caucasians and individuals of African descent. The prevalence of 5 reported TOP-I mutations in exons was investigated in volunteers (n=236) using DNA sequencing of the PCR products. The prevalence of TOP-I mutations in untreated lung cancer tissues (n=16) was also investigated. Additionally, 3 UGT1A1 polymorphisms, UGT1A1*6, *27 and *28, were investigated in volunteers (n=126). There were no mutations of TOP-I in any of the 236 subjects or in the untreated lung tissues. Among 128 subjects, the distribution of homozygous polymorphisms of UGT1A1 was: UGT1A1*28 in 3 (2.4%) and UGT1A1*6 in 4 (3.2%) subjects, and co-occurrence of heterozygous polymorphisms for both UGT1A1*6 and UGT1A1*28 in 4 (3.2%) subjects, and for UGT1A1*27 and UGT1A1*28 in 1 subject (0.8%). The Hardy-Weinberg deviation test showed there was no significant deviation from the equilibrium, and the association analysis indicated no significant linkage between UGT1A1*6 and UGT1A1*28. In conclusion, TOP-I genetic mutations correlated to CPT-11 resistance were not detected in any of the subjects and untreated lung cancer tissues. Less frequent UGT1A1*28 and more frequent UGT1A1*6 were confirmed in East Asian individuals compared to Caucasians and individuals of African descent. Linkage disequilibrium was not detected between UGT1A1*6 and UGT1A1*28.

Introduction

Irinotecan hydrochloride (CPT-11), a water-soluble semi-synthetic derivative of camptothecin, has been shown to exert marked antitumor activity (1). It is an inactive prodrug, and its major metabolite SN-38 is a potent topoisomerase I (TOP-I) inhibitor. SN-38 stabilizes covalent TOP-I-DNA complexes, causing DNA strand breaks. Several point mutations of TOP-I were identified as being associated with resistance to CPT-11 (2-4), and structural models were introduced to explain how the mutations of TOP-I hinder the docking of camptothecin derivatives in the ternary complex of TOP-I-DNA (5-7).

The cultured cells with the TOP-I mutation showed no obvious or only minor defects in cell function and proliferation (2-4). Consequently, such mutations may be innocent or not fatal to the cells. Therefore, it is reasonable to consider that the mutations may even occur in germlines. CPT-11 exhibits inter-individual variations in terms of both pharmacokinetic and pharmacodynamic behavior (8). CPT-11 is hydrolyzed to yield active SN-38 (9) and detoxified via glucuronidation of SN-38 by uridine diphosphate glucuronosyltransferase (UGTs) to yield its β-glucuronides, SN-38G (10). UGT1A1 is the main isoform of UGTS involved in the formation of SN-38G. Genetic polymorphisms of the UGT1A1 gene were revealed to explain the variability of CPT-11-related toxicity among patients, particularly UGT1A1*28 [(TA)10], the existence of which is known to be predictive of CPT-11-induced neutropenia (11). In addition, the UGT1A1*6 and *27 alleles, two variants in exon 1 of the UGT1A1 gene, are found mainly among individuals of Asian descent, and have also been indicated to affect enzyme function (12,13). The associated phenotype of UGT1A1*28, UGT1A1*6, or UGT1A1*27 is the Gilbert syndrome (14), and a strong association between

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Protein sequence

MSGDLHLDNSQIEADFRLNDSIKHKDKHDREHRIKEHKKEDREKSKISHEKLKDSEKKHK
FEKEKKDKDGSEKDHDKJKHDREHRIKEHKKEDREKSKISHEKLKDSEKKHK

DNA extraction and polymerase chain reaction. DNA was extracted from 200 µl whole blood using the QIAamp® DNA blood mini kit (Qiagen, Valencia, CA, USA). Paraffin-embedded lung cancer tissues obtained from lung cancer patients were stained with hematoxylin and eosin, and the DNA was extracted from LASER-captured microdissected tumor tissue (AS LMD, Leica, Tokyo, Japan) using the QIAamp® DNA Micro kit (Qiagen).

Polymerase chain reaction (PCR) was performed to amplify the targeted regions of TOP-I (or UGT1A1) with a thermal cycler (Takara Thermal Cycler SP, Takara, Shiga, Japan; or PTC-200 DNA Engine, MJ Research, Watertown, MA, USA) using each primer (Table I), HotStarTaq DNA polymerase and the Q-Solution Kit (Qiagen) in accordance with the manufacturer's protocol, with the exception of the case of UGT1A1*28 (see below). The PCR products were identified by gel electrophoresis. After excising the DNA fragment band from the agarose gel, high final concentrations of DNA were extracted using a MinElute® gel extraction kit (Qiagen).

Genetic analyses of TOP-I. Five TOP-I mutations correlated to CPT-11 resistance were previously reported (2-7), as follows [see Redinbo et al (6) for the numbering of each region]: region 1, exon 20 (541-558); region 2-1 and 2-1, exon 12 (501-530); region 2-2, exon 15 (186-194); region 3, exon 20 (577-579); and region 4, exon 15 (99-101) (Fig. 1A and B). The 5 mutation-hotspot regions of TOP-I were amplified using the relevant primer pairs (Table I). Direct sequencing of the PCR products was performed in both the forward and reverse directions.

Genetic analyses of UGT1A1. The following variant sequences were investigated: a 2-extraneous nucleotide insertion (TA) within the TATA box in the promoter, resulting in the sequence
(TA)\_TAA (-39 to -53, UGT1A1\*28: rs8175347); transition (+295 from the initial site of transcription, G to A) at codon 71 that is associated with a substitution of glycine to arginine (G71R, UGT1A1\*2: rs4148323); and a transversion (+770, C to A) at codon 229 in exon 1 that alters proline to glutamine (P229Q, UGT1A1\*27: rs35350960). The variant longer sequence of UGT1A1\*28 was distinguished from the wild-type sequence using poly-acrylamide gel electrophoresis (PAGE) with a DNA-sequencer (Long-Read Tower™, Visible Genetics, Suwanee, GA, USA) following amplification of the targeted sequence using PCR with Pfu DNA Polymerase (native) (Fermentas Life Sciences, Crt Burlington, Ontario, Canada). The primers (Table II) were designed to amplify a 216-bp segment of UGT1A1\*28 variant-type sequence as compared to the wild-type sequence using poly-acrylamide gel electrophoresis (PAGE) following amplification of the targeted sequence using PCR with Pfu DNA Polymerase (native) (Fermentas Life Sciences, Crt Burlington, Ontario, Canada).
with the 214-bp segment of the wild-type sequence. The separation ability was previously verified using guaranteed wild-type and variant-type genome DNA obtained from Daiichi Pure Chemicals (now Sekisui Medical Co. Ltd., Japan). For analysis of \textit{UGT1A1}*, direct sequencing of the PCR amplification product obtained using specific primers (Table I) was performed using the ABI PRISM 3100 genetic analyzer.

**Results**

\textbf{Mutations of TOP-I related to CPT-11 resistance.} Since no TOP-I mutations were observed in any of the 126 subjects, an additional 110 healthy volunteers, as well as untreated lung cancer tissue specimens in patients (n=16), were investigated. The results revealed that no genetic mutations correlated to CPT11-resistance in exons 12, 15, 16 or 20 of TOP-I.

\textbf{UGT1A1 genetic polymorphism associated with CPT-11 toxicity.} Homozygous polymorphisms of \textit{UGT1A1} were detected in 126 subjects, and were distributed as follows: \textit{UGT1A1}*28 in 3 (2.4%) subjects and \textit{UGT1A1}*6 in 4 (3.2%) subjects (Table II), and co-occurrence of heterozygous polymorphisms for both \textit{UGT1A1}*6 and \textit{UGT1A1}*28 in 4 subjects (3.2%), and for both \textit{UGT1A1}*27 and \textit{UGT1A1}*28 in 1 (0.8%) subject (Table III). The frequency of the \textit{UGT1A1}*28 variant allele was found to be 19.8%. In general, either homozygous or heterozygous polymorphisms of \textit{UGT1A1}*6 were detected in 30 (23.8%) subjects, and either homozygous or heterozygous polymorphisms of \textit{UGT1A1}*6, *27 or *28 were detected in 55 (43.7%) subjects. The Hardy-Weinberg deviation test showed that \textit{UGT1A1}*6 (p=0.43), *27 (p=0.96) and *28 (p=0.19) was not significantly deviated from the equilibrium. The linkage analysis revealed that normalized linkage disequilibrium coefficient D’ for \textit{UGT1A1}*6 and \textit{UGT1A1}*28 was 0.05. However, the linkage disequilibrium was not detected (p= 0.64).

\textbf{Discussion}

This is the first study to examine the frequency of TOP-I mutation associated with CPT-11 resistance in healthy subjects and in untreated lung cancer tissue specimens. Although \textit{in vitro} reports have demonstrated that CPT-11-resistant cancer cell lines exposed to CPT-11 possessed TOP-I mutations with no obvious or only minor defects in cell function, these changes were undetectable in healthy subjects and in untreated lung cancer tissue as mutation. Therefore, in the initial chemo-

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### Table I. Primers for topoisomerase I and \textit{UGT1A1}.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward primer 5’-3’</th>
<th>Reverse primer 5’-3’</th>
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</thead>
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<tr>
<td>20</td>
<td>TGGGTAGTAGGAGTCAAGAAGAA</td>
<td>GCCGAAGTTCCTCCCCAGAGG</td>
</tr>
<tr>
<td>12</td>
<td>GACTTTCCTCCTACCTTGACTTA</td>
<td>GACGCCCTCCACCCCTTTTT</td>
</tr>
<tr>
<td>15</td>
<td>TTCCAT ATGCTCATCCTTTTCTT</td>
<td>TGTGCCCTGTTGCTGTCTCA</td>
</tr>
</tbody>
</table>

\textit{TOP-I}, topoisomerase I; \textit{UGT1A1}, UDP-glucuronyltransferase 1A1; \textit{UGT1A1}*6: G→A on exon 1 (protein, G71R); \textit{UGT1A1}*27, 770C→A on exon 1 (protein, P229Q); \textit{UGT1A1}*28, (TA)7→TAA on the promoter (protein, reduced expression of \textit{UGT1A1}).

### Table II. Co-occurrence of \textit{UGT1A1}*28, \textit{UGT1A1}*6 and \textit{UGT1A1}*27 polymorphisms in healthy volunteers (n=126).

<table>
<thead>
<tr>
<th>Number of cases among the 126 subjects</th>
<th>\textit{UGT1A1}*28</th>
<th>\textit{UGT1A1}*6 or *27</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild</td>
<td>Hetero</td>
</tr>
<tr>
<td>\textit{UGT1A1}*6</td>
<td>71</td>
<td>18</td>
</tr>
<tr>
<td>Hetero</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>Homo</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>\textit{UGT1A1}*27</td>
<td>101</td>
<td>21</td>
</tr>
<tr>
<td>Hetero</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Homo</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total in \textit{UGT1A1}*28</td>
<td>101</td>
<td>22</td>
</tr>
</tbody>
</table>

\textit{UGT1A1}, UDP-glucuronyltransferase 1A1 gene; wild, wild-type; hetero, heterozygous; homo, homozygous.
therapy with CPT-11, the resistance-related TOP-I mutation is unlikely to occur.

Tsurutani et al (22) examined 16 samples obtained from 8 CPT-11-treated patients with lung cancer, and detected 2 types of TOP-I mutations in exon 21 in 1 tumor specimen. In a human colon cancer cell line (HCT-15) (23), one exonic mutation was detected in a heterozygous state in exon 19. Since the mutations cased on exon 19 and 21 have not been reported in previous in vitro CPT-11-resistant cancer cell lines, these regions were not examined in our study. However, the results indicated that the development of some acquired CPT-11 mutations was possible in patients in the course of treatment with CPT-11.

According to the SNP database (http://www.ncbi.nlm.nih.gov/SNP/), missense mutations in exons 4 (1 locus), 9 (1 locus), 15 (3 loci), and 21 (1 locus) are currently registered. These SNPs are not correlated to CPT-11 resistance, although a haplotype-tagging SNP in the intervening sequence region has been found to be associated with toxicity (grade 3/4 neutropenia) in patients treated with CPT-11 (17).

Determination of the UGT1A1 genotypes is clinically significant for the prediction of CPT-11-related severe toxicity (11). Individuals who have at least 1 variant (heterozygous) allele for the UGT1A1*28 may exhibit reduced elimination of SN-38 and increased probability of development of dose-limiting neutropenia (11).

The frequency of the UGT1A1*28 variant allele has been reported to be 30-45% in Caucasian, African and Indian populations, which is approximately twice that of the 10-20% reported in East Asian populations (17-22), as confirmed by the frequency of this study (19.8%). On the other hand, UGT1A1*6 has been detected in 16-40% of Asian individuals, as confirmed by the frequency of 23.8% found in this study; UGT1A1*, however, was extremely rare in the Caucasian and African populations (17). The UGT1A1*27 allele detected only in Asian individuals was reported to be harboured exclusively by a UGT1A1*28 haplotype, as findings of this study show. Either homozygous or heterozygous polymorphisms of UGT1A1*6, *27 or *28 were detected in 55 (43.7%) subjects in this study; the risk of UGT1A1 polymorphism-related toxicity of CPT-11 is considered to be high in East Asian individuals.

The Food and Drug Administration in the United States has approved an amendment of the label for Camptosar (irinotecan hydrochloride), to which a warning to reduce the starting dose of irinotecan for UGT1A1*28 homozygous patients has been added. In East Asian individuals, both UGT1A1*6 and UGT1A1*28 require examination, since there is no linkage disequilibrium between the two polymorphisms (24), as indicated in the present study.

As a clinical relevance, the risk of TOP-I mutation-related resistance to CPT-11 is unlikely and it is not necessary to test for TOP-I mutation prior to chemotherapy with CPT-11. However, the risk of UGT1A1 polymorphism-related toxicity of CPT-11 is markedly higher in East Asian individuals, and UGT1A1 polymorphisms, not only of UGT1A1*28, but also of UGT1A1*6, should be tested prior to treatment with CPT-11 to avoid severe adverse effects.

In conclusion, the main findings in this study were twofold. First, TOP-I genetic mutations related to CPT-11 resistance were not detected in any of the subjects or in untreated lung cancer tissues. Second, compared to Caucasians and individuals of African descent, it was observed that UGT1A1*28 was less frequent and UGT1A1*6 was more frequent in Japanese subjects. Moreover, linkage disequilibrium was not noted between UGT1A1*6 and UGT1A1*28.

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