Supplementary Material

Genotyping Methods for Some Polymorphisms in "Pharmacogenetic Pathway Analysis of Irinotecan."

UGT1A7 genotyping method:

The UGT1A7 387G>T and 391C>A variants were genotyped by sequencing. SNPs covered were 98938 G>T, 98942 A>C, 98943 A>G, and 99173 C>T. PCR amplification and sequencing were done using one set of primers [UGT1A7* (forward): 5'-TTGCCTATGCTCGCTGGAC-3', UGT1A7* (reverse): 5'-TTTCAGGGGCTATTTCTAAGA-3'] to generate a 423-bp fragment covering all SNPs for both variants. PCRs were set up in a 25 µl volume containing 2.5 mM MgCl2, 200 µM each dNTP, 500 nM forward and reverse primers, 0.5 units AmpliTaq Gold (Applied Biosystems, Foster City, CA) and 75 ng of DNA. The PCR reaction was cycled for 38 cycles at 94°C for 45 s, 59°C for 30 s and 72°C for 1 min. In preparation for sequencing, PCR products were purified using QIAquick® PCR Purification Kit (QIAGEN, Inc., Valencia, CA). Purified products were eluted in 35 µl elution buffer. DNA cycle sequencing reactions were carried out in 10 µl reactions using 2 µl of purified PCR product, 400 nM forward or reverse primer, and BigDye® Terminator Version 3.1 Cycle Sequencing Kit (Applied Biosystems). Cycle sequencing was performed using standard PCR conditions and reactions were run on a 3100 DNA Sequencer (Applied Biosystems).

SLCO1B1 genotyping method:

The polymorphisms of SLCO1B1 *1b (388 A>G) and *5 (521 T>C) were genotyped by a single base extension (SBE) method. The SLCO1B1 genomic sequence from GenBank AC022335.8 was used for designing the primers. The PCR and SBE primers used for 388 A>G were: 5'- TTC AGT AcA TAA GCA AAA TGT T -3' (forward PCR primer), 5'- CAC AAC AAgTcT TAG AGA TG -3' (reverse PCR primer) and 5' -AGG TAT TCT AAA GcA ACT AAT ATC -3' (upstream extension primer). The modified bases in the 388 A>G primers are indicated in lower case. The PCR and extension primers used for 521 T>C were: 5'- TGA AAC ACT CTC TTA TCT AC -3' (forward PCR primer), 5'- TTA CCT AAA TAC AAA GAA GAA T -3' (reverse PCR primer) and 5'- CGA AGC ATA TTA CCC ATG AAC -3' (downstream extension primer). The PCRs for 388 A>G (239 bp fragment) and 521 T>C (175 bp fragment) were performed separately in a 15 ml volume containing 125 nM of each primer, 2.5 mM of MgCl2, 50 mm of each dNTP, 0.375 U of AmpliTaq Gold polymerase (Applied Biosystems) and 30 ng of genomic DNA. Amplification was performed in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems) with an initial denaturation step of 15 min at 95 0C followed by 42 cycles of 95 0C for 15 s, Ta 0C for 15 s and 72 0C for 30 s, and a final extension step of 72 0C for 10 min. The annealing temperatures (Ta) were 54.5 0C and 54 0C, respectively. PCR products were purified by treatment with shrimp alkaline phosphatase and exonuclease I at 37°C for 45 minutes prior to the SBE reaction. The SBE reactions were carried out separately for 388 A>G and 521 T>C in a 12 ml volume containing 1 mM of SBE primer, 250 mM each of four ddNTPs, 1.5 U of ThermoSequenase (Amersham Pharmacia Biotech) and 7.2 ml of purified PCR products. Reactions were run in a 9600 thermal cycler under the following conditions:

96 0C for 2 min, followed by 60 cycles of 96 0C for 30 s, 55 0C for 30 s and 60 0C for 30 s. The reactions were denatured at 96 0C for 4 min and held at 4 0C prior to separation of the SBE products on a WAVE 3500HT DHPLC system (Transgenomic Inc). Each SBE products for 388 A>G and 521 T>C were pooled and 18 ul was injected onto the WAVE 3500HT system for analysis. An oven temperature of 70 0C was used and samples were run on an HT (high throughput) column (Transgenomic Inc) with a buffer B (Transgenomic Inc) gradient range of 27.1% to 39.6% over 2.5 minutes (buffer B contains 25% acetonitrile). The extended products were eluted in the order of C<G<T<A dependent on the hydrophobicity differences of the four bases. Both SBE products and unextended primers were eluted at different times and could be distinguished. Positive controls with known genotypes at the 388 A>G and 521 T>C loci were included in each run.

BCRP (ABCG2) genotyping method:

The two SNPs 34 G>A and 421 C>A in ABCG2 gene(1, 2) coding for BCRP were genotyped by duplex PCR and duplex Single Base Extension (SBE) analyzed on DHPLC. The PCR and SBE primers used for 34 G>A were: 5'-GCA ATC TCA TTT ATC TGG ACT A-3' (forward PCR primer), 5'-AAT AGC CAA AAC CTG TGA GG-3' (reverse PCR primer) and 5'-CCA TTG GTG aTT CCT aGT GAC A-3' (downstream extension primer). The modified bases are indicated in lower case to reduce 3' end dimmers and hairpins. The PCR and SBE primers used for 421 C>A were: 5'-ACT AAA CAG TCA TGG TCT TAG A-3' (forward PCR primer), 5'-ATC AGA GTC ATT TTA TCC ACA C-3' (reverse PCR primer) and 5'-CCG AAG AGC TGC TGA GAA CT-3' (downstream

extension primer). The amplified PCR fragments are 344 bp and 296 bp in size, respectively. Duplex PCRs were performed in a 15 ml volume containing 125 nM of each primer for the two PCR amplicons, 30 ng of genomic DNA, 2.5 mM of MgCl2, 100 mm of each dNTP and 0.375 U of AmpliTag Gold polymerase (Applied Biosystems) in the buffer provided by the manufacturer. Amplification was performed in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems) with an initial denaturation step of 15 min at 95 0C followed by 40 cycles of 95 0C for 15 s, 56 0C for 15 s and 72 0C for 45 s, and then an extension step of 72 0C for 10 min. PCR products were purified by treatment with shrimp alkaline phosphatase (Roche) and exonuclease I (USB) at 37°C for 45 mins prior to the SBE reaction. Duplex SBE reactions were carried out in 12.6 ml containing 1 mM of each SBE primer, 250 mM each of four ddNTPs, 7.2 ml of purified PCR product and 1.5 U of ThermoSequenase (Amersham Pharmacia Biotech) in 1x Reaction buffer provided by the manufacturer. Reactions were run in a 9600 thermal cycler (Applied Biosystems) under the following conditions: 96 0C for 2 min, followed by 60 cycles of 96 0C for 30 s, 55 0C for 30 s and 60 0C for 30 s. Wave 3500HT DHPLC system (Transgenomic Inc) was used for separating SBE products. Prior to run on DHPLC, the samples were denatured at 96 0C for 4 min and hold at 4 0C. For analyzing SBE on Wave DHPLC, 8 ml of SBE products of each sample was injected. We used “Mutation Detection” application type as a template, selected “Normal” clean as clean type (i.e. 100% buffer B clean off step after each injection) and manually set the following variables for this application: the flow rate was at 1.5 ml/min by using a HT column, oven temperature was set at 70 0C and the gradient used for elution of the SBE products was from 24% to 36.5% buffer B over 2.5 min (buffer B contains 25%

acetonitrile). The extended products were eluted in the order of C<G<T<A dependent on the hydrophobicity differences of the four bases. In duplex SBE, two extension primers were designed to be able separating un-extended primers and each extended products. The known genotype controls were included in each run.

CES2 Genotyping Method: The CES2 three SNPs 5'UTR -363 C/G, intron 1 +1361 A/G and 3'UTR +108 C/G were genotyped by PCR and SBE-DHPLC for the CPT11 trial patients (n=85). The SNPs 5'UTR -363 and 3'UTR +108 were done by a duplex PCR and a duplex SBE reaction, then the SBE products were mixed with intron 1 +1361 SBE products and run together in a triplex format on DHPLC.

The genotyping primers are listed in an attached Table. The duplex PCRs were set up in a 15 ml volume containing 140 nM of each 5'UTR -363 primer and 125 nM of each 3'UTR +108 primer (the ratio of two sets of PCR primers was adjusted to 1.12:1 to achieve even amplification of the two PCR fragments 187 bps and 672 bps), 30 ng of genomic DNA, 1.5 mM of $MgCl_2$, 100 mm of each dNTP and 0.375 U of AmpliTag Gold polymerase (Applied Biosystems) in the buffer provided by the manufacturer. Amplification was performed in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems) with an initial denaturation step of 15 min at 95 ^{0}C followed by 40 cycles of 95 ^{0}C for 15 s, 60 ^{0}C for 15 s and 72 ^{0}C for 45 s, and then an extension step of 72 ^{0}C for 10 min. The PCR fragment for intron 1 +1361 is 789 bps and the PCR was done by a touch down cycling conditions. The PCRs were set up in a 15ml volume containing 125 nM of each primer, 1.5 mM of $MgCl_2$, 100 mm of each dNTP, 0.375 unit of AmpliTaq Gold and 30 ng of

genomic DNA. Amplification was performed in a 9600 thermal cycler with an initial denaturing step at 95 ^{0}C for 15 min followed by 14 cycles of 95 ^{0}C for 30 sec, 71 ^{0}C for 30 sec (touch down - 0.5 ^{0}C per cycle) and 72 ^{0}C for 1 min, then 25 cycles of 95 ^{0}C for 30 sec, 64 ^{0}C for 30 sec and 72 ^{0}C for 1 min, and a final extension step at 72 ^{0}C for 10 min. Prior to SBE reaction, PCR products were purified by treatment with shrimp alkaline phosphatase (Roche) and exonuclease I (USB) at 37°C for 45 mins. SBE reactions were carried out in a 10 ml volume containing 1 mM of SBE primer, 250 mM each of four ddNTPs, 1.25 U of ThermoSequenase (Amersham Pharmacia Biotech) and 6 ml of purified PCR products. For the duplex SBE reaction, 1 mM of each extension primer was added to the total volume. Reactions were run in a 9600 thermal cycler under the following conditions: 96 ^{0}C for 2 min, followed by 60 cycles of 96 ^{0}C for 30 sec, 55 ^{0}C for 30 sec and 60 ^{0}C for 30 sec. Samples were denatured at 96 ^{0}C for 4 min and held at 4 ^{0}C prior to separation of the SBE products on a WAVE 3500HT DHPLC system (Transgenomic Inc). 16 ml mixed SBE products per sample were injected onto the DHPLC for analysis. Samples were run on a HT (high throughput) column (Transgenomic Inc) at 70 ^{0}C oven temperature using a start gradient of 23 %B for 2.5 min (slope at 5%B per min). The extended products were eluted in the order of C<G<T<A dependent on the hydrophobicity differences of the four bases. Three extension primers for a triplex format on DHPLC were designed to be eluted at different times and to be able separating each extended products. The known genotype controls were included in the run of the patient samples.

References for Supplementary Material:

(1) Imai, Y., Nakane, M., Kage, K., Tsukahara, S., Ishikawa, E., Tsuruo, T. *et al.* C421A polymorphism in the human breast cancer resistance protein gene is associated with low expression of Q141K protein and low-level drug resistance. *Mol Cancer Ther* **1**, 611-6 (2002).

(2) Zamber, C.P., Lamba, J.K., Yasuda, K., Farnum, J., Thummel, K., Schuetz, J.D. *et al.* Natural allelic variants of breast cancer resistance protein (BCRP) and their relationship to BCRP expression in human intestine. *Pharmacogenetics* **13**, 19-28 (2003).