Gene Expression Profiling of the Irinotecan Pathway in Colorectal Cancer

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ABSTRACT
The exact mechanism responsible for large variation of response to chemotherapy remains unclear. This study profiled the gene expression for the entire irinotecan pathway to provide insights into individualized cancer therapy. The RNA expressions of 24 irinotecan pathway genes were measured in paired tumor and normal tissues from 52 patients with Dukes’ C colorectal cancer using a real-time quantitative reverse transcription-PCR assay. The relative expression levels across the 24 pathway genes varied considerably, with a 441-fold range from highest to lowest expression levels for the tumor tissues and a 934-fold range for the normal tissues. Interpatient variability was also quite large, with a 33.6 median fold change in the tumor tissue genes and a 30.1 median fold change in the normal tissue genes. Six of the 24 irinotecan pathway genes had dramatically lower expression levels in the tumor samples than did the genes in the normal tissues (median range, 1.28-4.39 folds; \( P = 0.001-0.029 \)). Eight genes had significantly higher levels (median range, 1.35-2.42 folds; \( P = 0.001-0.011 \)). Using hierarchical clustering, three gene clusters and three patient groups were observed with high similarity indices by the RNA expressions in colorectal tumors. The three patient groups had no unique clinical pathologic features but could the RNA expressions in colorectal tumors. The three patient groups were observed with high similarity indices by the RNA expressions in colorectal tumors. The three patient groups had no unique clinical pathologic features but could

INTRODUCTION
Irinotecan, a semisynthetic derivative of the natural alkaloid camptothecin, was approved by the Food and Drug Administration in 1996 for the treatment of colorectal cancer. Irinotecan belongs to the topoisomerase I interactive class of anticancer agents, which targets the DNA-topoisomerase I complex, preventing the reannealing of the nicked DNA strand and thus arresting DNA replication and subsequent cell death (1, 2). Clinical studies with irinotecan have shown a broad spectrum of efficacy against solid tumors (1–4) and tolerable side effects. Of special interest was a demonstration of considerable activity against 5-fluorouracil-refractory colorectal cancer, leading to a comprehensive evaluation program of irinotecan both as a single agent and as part of combination therapies (5, 6). The results of the various studies have showed irinotecan to be one of the most active drugs in the first- and second-line treatment of colorectal cancer (5–8).

Irinotecan acts as a prodrug; it is converted in vivo primarily by CES2 to an active metabolite, SN-38, that is generally 100 to 1,000 times more potent than its parent drug (Fig. 1; refs. 1, 2, 9–11). SN-38 itself is glucuronidated in the human liver by UGT1A1 to an inactive compound, SN-38G. Recently, other quantitatively important inactive metabolites of irinotecan (whose formation are dependent on CYP3A) have been identified (12–14). Of these, 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino] carbonyloxy camptothecin is one of the most important. It is detectable in plasma and is formed by CYP3A-mediated oxidation of the distal piperidine group at C10 of irinotecan. 7-Ethyl-10-(4-amino-1-piperidino) carbonyloxy camptothecin is also formed through this pathway, by cleavage of the distal piperidine group of irinotecan. Recent studies have shown that the subtype CYP3A4 is the main isoenzyme involved in formation of both 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino] carbonyloxy camptothecin and 7-ethyl-10-(4-amino-1-piperidino) carbonyloxy camptothecin, although CYP3A5 has shown weak catalytic activity (13, 14).

Irinotecan and its metabolites are eliminated mainly through biliary and renal excretion (12). Transport occurs via ABCB2, a canalicular multispecific organic anion transporter, located mostly on hepatic cells and the bile canalicular membrane (15). Efflux of irinotecan and SN-38 in tumor cells is recognized as a potential determinant of anticancer activity; several ATP-binding cassette transmembrane proteins (ABC) have been shown able to efflux camptothecins (16–18). On the other hand, overexpression of ABC proteins such as ABCB1 and ABCB1 seems to trigger multidrug resistance, a major obstacle in cancer chemotherapy. Another member of the ABC family of drug transporters, the breast cancer resistance protein ABCC2, also mediates resistance to camptothecins (18).

Many studies have shown that chemotherapy-induced cell death involves a number of cellular pathways, such as apoptosis and DNA damage repair systems (19, 20). Deficiency in the DNA repair systems has been shown to affect both intrinsic and acquired resistance to several drugs, including irinotecan (20–24). For example, functional complementation of MLH1 in an MLH1-defective cell line resulted in resistance to topoisomerase
inhibitors irinotecan and etoposide, proving that MMR is a critical determinant for chemosensitivity (21). In addition, irinotecan enhanced chemotherapy activity on human colon cancer cell lines when combined with oxaliplatin, via either reducing ERCC1 and XPA mRNA expression or poisoning topoisomerase I activity, showing an important role of DNA repair enzymes in cancer chemotherapy (22). Pharmacogenomic analysis also supports that polymorphisms in nucleotide excision repair genes ERCC2 and XRCC1 have been an important determinant in predicting the clinical outcome of irinotecan-containing chemotherapy (23, 24).

A number of genes, ADPRT, CDC45L, DRG1, FDXR, NFkB1, TDP1, TNFSF6, and TP53, have been implicated in the regulation of irinotecan activity through the apoptosis pathway (25–39). For example, ADPRT has been identified as a key enzyme in ADP ribosylation: this process of eukaryotic post-translational modification of proteins is strongly induced by the presence of DNA strand breaks and plays a role in DNA repair and the recovery of cells from DNA damage (25, 26). Other studies have indicated that the activation of NFKB1 is initiated by the formation of single- and double-strand breaks in DNA induced by topoisomerase poisons such as irinotecan. Inhibition of NFKB1 enhances gemcitabine’s antitumor activity (31–33) but diminishes therapeutic response in tumors that retain wild-type p53 (33). In addition, two recent studies have indicated that FDXR is a putative contributor to p53-mediated apoptosis from anticancer drugs through the generation of oxidative stress in the mitochondria (37, 38). Yet another study has suggested that DRG1 may modulate sensitivity to irinotecan in colon cancer cells (39). All these downstream elements associated with chemotherapy-induced apoptosis were included in this study to be further assessed with gene expression profiling.

Although genes regulating irinotecan metabolism and transport and several downstream elements have been defined for yeast, bacteria, or mammalian cell lines, little information exists on the expression of most of these genes in human tumors. In addition, there has been no comprehensive analysis of the entire drug pathway in both neoplastic and normal tissues. In this study, we have done a comprehensive analysis to the irinotecan pathway using gene expression data for 24 irinotecan pathway genes in human colorectal neoplastic and normal tissues. This will allow us to further understand differential tumor and normal gene expression, intraindividual variation, and coregulation/coexpression of the irinotecan pathway genes and provide insights into the use of gene expression profiling for individualized cancer therapy.

**MATERIALS AND METHODS**

**Patients and Samples.** In this study, gene expression was profiled in tumor specimens and paired normal tissues from 52 consecutive patients with Dukes’ C colorectal cancer. The age of the patients ranged from 32 to 96 (median, 69.5); 29 males and 23 females were included. Samples were snap frozen in liquid nitrogen immediately after surgery and stored at −80°C. None of the patients had received preoperative radiation or chemotherapy. Histologic examination was done in all of the cases to

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Fig. 1 Illustration of the irinotecan pathway, along with the quartile of median T/N ratios from the RNA expressions of 24 pathway genes in 52 colorectal cancer patients. The four colors of the pathway genes are matched to each quartile.
evaluate tumor histotype (41 enteric and 11 mucinous) and grade of differentiation (1, 38, 13 in grades 1, 2, 3, respectively) according to WHO criteria. Twenty-seven tumors were localized in the right colon, 19 in the left colon, and the remaining six in the rectal portion. Written informed consent was obtained from all patients to bank tumor tissue and to perform genetic analysis. This study was approved by the Washington University Human Subjects Committee.

**Reverse Transcription for Preparation of cDNA.** Regions of high tumor cellularity were selected for RNA extraction (median, 86.3%; range, 65-95%). Tissue total RNA was isolated from the tumor or adjacent normal mucosa using a TRIzol RNA isolation kit (Invitrogen, Carlsbad, CA). The quality of RNA (A_{260/280} > 1.8; clear RNA bands for 28S, 18S, and 5S) was confirmed in the Siteman Cancer Center Tissue Procurement Core. cDNA was prepared in a 20-μL reaction containing 1 μg total RNA, 0.5 μg oligo(dT)_{20}VN primer, and 100 units of Superscript II reverse transcriptase (Invitrogen). The cDNA samples were then adjusted to a concentration of 10 ng/μL.

**Quantitative Real-time PCR.** Primers and Taqman probes used in this study were designed using Primer Express version 1.5 (ABI, Foster City, CA). The sequence of primer and probe specific for each gene is displayed in Table 1. The specificity of each primer/probe set was determined with gel visualization. The 10-μL reaction mixture was composed of 5 μL of 2× Taqman universal PCR master mix (ABI), 3 μL of primer and probe mix (600 nmol/L each forward and reverse primers, 200 mmol/L specific Taqman probe), and 2 μL of cDNA. All real-time PCR assays were done in triplicate in MicroAmp optical 384-well reaction plates closed with MicroAmp optical adhesive covers (ABI) on an ABI PRISM 7700 Sequence Detector System (ABI) according to the following:

1. Denaturing at 95°C for 2 minutes to activate uracil-N-glycosylase enzyme, 95°C for 10 minutes to denature uracil-N-glycosylase and activate DNA polymerase, 40 cycles at 95°C for 20 seconds and at 60°C for 1 minute.

**Measurement of Relative Expression of mRNA.** The relative RNA expression levels were calculated via a modified comparative C_{T} method (40, 41), which uses actual real-time PCR amplification efficiency instead of assuming all sets of gene primers and probes have approximately equal efficiency. Thus, a standard curve for each gene was established according to the equation $E = 10^{(C_{T}\text{target} - C_{T}\text{calibrator-sample})/(C_{T}\text{reference} - C_{T}\text{calibrator-sample})}$ to obtain PCR amplification efficiency. A mathematical model was applied to determine expression levels of the target gene in individual samples, relative to a reference gene and a calibrator sample, using the following formula:

$$(E_{\text{target}})_{\text{ΔC}_{T}}/E_{\text{reference}} = (E_{\text{target}})_{\text{ΔC}_{T}}/E_{\text{calibrator-sample}}$$

where $E_{\text{target}}$ is PCR efficiency of the target gene transcript and $E_{\text{reference}}$ is PCR efficiency of reference gene transcript. The reference gene used in this study was the amyloid β precursor protein, as it had nearly identical expressions between colon tumor and normal tissues (46:31 copies per cell) in previous SAGE analysis (42) and <3-fold change between the tumor and normal samples in our study. To allow comparison of gene expression in the 52 paired RNA samples, as well as comparison of the 24 target genes, all assay $C_{T}$ values were standardized to a calibrator sample (also called 1× sample). This calibrator sample had the largest $C_{T}$ value of any target gene from the 104 RNA samples, which was the tumor sample from patient 23, detected with ABC2. In addition, a pooled RNA sample from each of the 104 samples was run on every PCR plates as quality control for reproducibility of the real-time PCR assay. The coefficient of variance in $C_{T}$ value was 0.1% to 5.7% (mean, 2.4%) for intra-assay variability (from triplicate reactions each sample) and 0.2% to 7.6% (mean, 4.3%) for interassay variability (from the pooled RNA sample on four runs each gene) in this study.

**Hierarchical Clustering Analysis.** Unsupervised cluster analysis of gene expression was done using the hierarchical clustering software Spotfire DecisionSite (Spotfire, Inc., Somerville, MA). The clustering method used was unweighed pair-group with arithmetic mean, including the similarity measure of correlation and the ordering function of averaged average value. Profiles with identical shape have maximum similarity index of correlation (+1.0); and perfectly mirrored profiles have the minimum similarity index of correlation (−1.0).

**Statistical Analysis.** Descriptive statistical analyses were done using the software STATISTICA from StatSoft, Inc. (Tulsa, OK). The ratio of tumor (T) to matched normal sample (N) RNA expression values (T/N) was considered increased when T/N > 1.2 (i.e., tumor higher than normal), or decreased when T/N < 0.8 (tumor lower than normal). The significance of difference between paired tumor and normal samples was evaluated via the Wilcoxon matched pairs test. ANOVA was used to determine whether or not there was a significant difference in the pathway gene expression between different patient groups. The influence of gender, tumor location, or pathologic variables on RNA expression was evaluated with the Mann-Whitney or the Kruskal-Wallis tests. Spearman rank correlations were used to compare the variables; and a $P < 0.001$ was chosen to highlight the correlations between the pathway genes for hypothesis formation purpose.

**RESULTS**

**Differential Expression of the Pathway Genes.** With the Wilcoxon matched pairs test, six genes (ABCB1, ABCG2, CES1, CES2, MLH1, and UGT1A1), or 25%, had significantly lower RNA expression levels in the 52 colorectal tumor samples than the paired adjacent normal tissues (median range, 1.28-4.39 fold lower; $P = 0.001$-0.029). In contrast, eight genes (ABCC1, CDC45L, DRG1, ERCC1, ERCC2, FDXR, TP51, and TP53) in the tumors, or 33%, had significantly higher expression levels than those in the normal tissues (median range, 1.35-2.42 folds higher; $P = 0.001$-0.011). There were no significant differences between paired tumor and normal samples in 10 of 24 (42%) genes ($P = 0.259$-0.764). Figure 2 shows the RNA expression levels of the 24 pathway genes in the colon tumor and normal samples.

**Variability of RNA Expression of the Pathway Genes.** Variability for each gene was quite large; the coefficient of variance ranged from 57.0% to 110.6% (median, 90.4%) in the
<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Description</th>
<th>Forward primer 5’ to 3’</th>
<th>Reverse primer 5’ to 3’</th>
<th>Taqman probe 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABCB1</strong></td>
<td>ATP-binding cassette, subfamily B (MDR/TAP), member 1 (MDR1)</td>
<td>GCTGGCACAGA</td>
<td>CAGAGTTCACT</td>
<td>TCCAGCCTTGAGAC</td>
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<td><strong>ABC1</strong></td>
<td>ATP-binding cassette, subfamily C (CFTR/MRP), member 1 (MRP1)</td>
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<td>AATAAATATATGCGTGGT</td>
<td>CCAGACTTCAAAAC</td>
</tr>
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<td>ATP-binding cassette, subfamily G (MDR/TAP), member 1 (MRP1)</td>
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<td>TCGGAAATC</td>
<td>CATACGTTGAATTACGA</td>
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<td>TCGGAAATC</td>
<td>CATACGTTGAATTACGA</td>
</tr>
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<td><strong>ABCG2</strong></td>
<td>ATP-binding cassette, subfamily G (MDR/TAP), member 1 (MRP1)</td>
<td>AGGTTCTGCT</td>
<td>TCGGAAATC</td>
<td>CATACGTTGAATTACGA</td>
</tr>
<tr>
<td><strong>ADPR</strong></td>
<td>ADP-ribose transferase (NAD+; poly (ADP-ribose) polymerase)</td>
<td>AGGTTCTGCT</td>
<td>TCGGAAATC</td>
<td>CATACGTTGAATTACGA</td>
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<td><strong>CDC45L</strong></td>
<td>CDC45 cell division cycle 45-like (Saccharomyces cerevisiae)</td>
<td>GCTGGCACAGA</td>
<td>CAGAGTTCACT</td>
<td>TCCAGCCTTGAGAC</td>
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<td><strong>CES1</strong></td>
<td>Carboxylesterase 1 (monocyte/macrophage serine esterase 1)</td>
<td>CCAAGACTCAGACTT</td>
<td>GCTAAGAATTACG</td>
<td>TGTGAATCTGG</td>
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<tr>
<td><strong>CES2</strong></td>
<td>Carboxylesterase 2 (intestine, liver)</td>
<td>AATCCCAGCTAT</td>
<td>TGGGAAGGAA</td>
<td>CAGTCAGGACA</td>
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<tr>
<td><strong>CYP3A4</strong></td>
<td>Cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 1</td>
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<td>TCGGAAATC</td>
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<tr>
<td><strong>CYP3A5</strong></td>
<td>Cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 1</td>
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<td>TCGGAAATC</td>
<td>CATACGTTGAATTACGA</td>
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<td><strong>DRG1</strong></td>
<td>Developmentally regulated GTP binding protein 1</td>
<td>CCAAGACTCAGACTT</td>
<td>GCTAAGAATTACG</td>
<td>TGTGAATCTGG</td>
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<tr>
<td><strong>ERCC1</strong></td>
<td>Excision repair cross-complementing rodent repair deficiency, complementation group 1</td>
<td>AGGTTCTGCT</td>
<td>TCGGAAATC</td>
<td>CATACGTTGAATTACGA</td>
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<td><strong>ERCC2</strong></td>
<td>Excision repair cross-complementing rodent repair deficiency, complementation group 1 (XPD)</td>
<td>AGGTTCTGCT</td>
<td>TCGGAAATC</td>
<td>CATACGTTGAATTACGA</td>
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<tr>
<td><strong>FDXR</strong></td>
<td>Ferredoxin reductase</td>
<td>AGGTTCTGCT</td>
<td>TCGGAAATC</td>
<td>CATACGTTGAATTACGA</td>
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<td><strong>MLH1</strong></td>
<td>Mismatch repair homologue 1, colon cancer, nonpolyposis type 2</td>
<td>GGTGCTTCTGG</td>
<td>TGTGAATCTGG</td>
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<tr>
<td><strong>MSH6</strong></td>
<td>MutS homologue 6 (Escherichia coli)</td>
<td>AGGTTCTGCT</td>
<td>TCGGAAATC</td>
<td>CATACGTTGAATTACGA</td>
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<td><strong>NFKB1</strong></td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B cells 1 (p105)</td>
<td>AGGTTCTGCT</td>
<td>TCGGAAATC</td>
<td>CATACGTTGAATTACGA</td>
</tr>
<tr>
<td><strong>TP53</strong></td>
<td>Tumor protein p53</td>
<td>AGGTTCTGCT</td>
<td>TCGGAAATC</td>
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<td><strong>UGT1A1</strong></td>
<td>UDP glycosyltransferase 1 family, polypeptide A1</td>
<td>AGGTTCTGCT</td>
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<td><strong>XPA</strong></td>
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<td>AGGTTCTGCT</td>
<td>TCGGAAATC</td>
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<td><strong>XRCC1</strong></td>
<td>X-ray repair complementing defective repair in Chinese hamster cells 1</td>
<td>AGGTTCTGCT</td>
<td>TCGGAAATC</td>
<td>CATACGTTGAATTACGA</td>
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<td><strong>APP</strong></td>
<td>Amyloid beta precursor protein (reference gene)</td>
<td>AGGTTCTGCT</td>
<td>TCGGAAATC</td>
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</table>
tumor tissues and from 50.9% to 108.3% (median, 82.3%) in the normal tissues. Five genes (ABCG2, CES1, TDP1, TP53, and UGT1A1) all had >100% coefficient of variance in the tumors, as did three genes (CDC45L, TDP1, and TP53) in the normal colons. Similarly, the fold change of gene expression in the 52 colon cancer patients was wide for most of the 24 pathway genes; it ranged from XRCC1 8.8 to ABCC2 118.7 (median, 33.6) in the tumor and from XRCC1 11.3 to TP53 96.9 (median, 30.1) in the normal tissue (Table 2; Fig. 2).

**Relative Expression/Contribution of the Pathway Genes.** As shown in Fig. 2, the median RNA expression of the 24 pathway genes ranged from the highest DRG1 (6,923.3 units) to the lowest TNFSF6 (15.7 units; 441-fold) for the tumor samples, and from CES2 (15,129.3 units) to TNFSF6 (16.2 units; 934-fold) for the normal samples. The median T/N ratio of RNA expression may reflect the relative contribution of each single gene to drug pathway activity. To assess this relative contribution (as a balance between efficacy and toxicity), we ordered the median T/N ratio of RNA expression for the 24 pathway genes and compared quartiles. The ratios ranged from 0.23 (CES1) to 2.42 (TP53); each quartile had six genes (Fig. 1).

**Coexpression/Coregulation of the Pathway Genes.** Of the 24 irinotecan pathway genes in the colon tumor tissues, three groups were found to have a Spearman rank score of ≥0.45 (all P < 0.001). For instance, the DNA damage repair–related genes (ADPRT, CDC45L, MSH6, NFkB1, and TDP1, and the drug transporter ABCC2) correlated closely with one another. These genes were found on chromosomes 1q41, 22q11, 2p16, 4q24, and 14q32. In addition, ABC11, ERCC2, TP53, and XPA formed a group, and TOP1 had a closely correlation with XRCC1. The Spearman rank score of all 24 pathway genes is shown with a matrix table of correlation (Table 3).

**Clinicopathology and RNA Expression of the Pathway Genes.** For 22 of the 24 genes in this study, no significant correlation was found between tumor RNA level and patient age. The Spearman rank scores ranged from /C0.27 to 0.26. (For the two other genes, CDC45L and TDP1, the scores were 0.36 and 0.47, P < 0.01 and 0.001, respectively.) Also, statistically there was no significant difference in the tumor RNA expression levels with respect to gender, tumor location, pathologic grade, or classification for most of the 24 genes studied (P = 0.07-0.97).

**Hierarchical Clustering of the Pathway Genes.** Gene clustering analysis of RNA expression may provide insights into functional correlation or coregulation within the pathway genes. The higher the similarity index is, the greater the possibility that such correlation or coregulation occurs between the clustered genes. The unweighed pair-group method with
### Table 2
Variation and T/N category of the RNA expression in 52 colon cancer patients

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>CV% of RNA expression in tumor (median)</th>
<th>CV% of RNA expression in normal (median)</th>
<th>Fold change of RNA expression in tumor (median)</th>
<th>Fold change of RNA expression in normal (median)</th>
<th>n of case in category of T/N &lt; 0.8</th>
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<td>ABCB1</td>
<td>95.2</td>
<td>85.2</td>
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<td>31.7</td>
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<tr>
<td>TDP1</td>
<td>110.6</td>
<td>104.8</td>
<td>47.7</td>
<td>31.1</td>
<td>12</td>
<td>8</td>
<td>32*</td>
</tr>
<tr>
<td>TNFSF6</td>
<td>80.6</td>
<td>81.7</td>
<td>15.3</td>
<td>25.4</td>
<td>19</td>
<td>13</td>
<td>20</td>
</tr>
<tr>
<td>TOP1</td>
<td>69.2</td>
<td>82.0</td>
<td>24.3</td>
<td>30.4</td>
<td>20</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>TP53</td>
<td>103.6</td>
<td>108.3</td>
<td>101.7</td>
<td>96.9</td>
<td>12</td>
<td>4</td>
<td>36*</td>
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<tr>
<td>UGT1A1</td>
<td>109.0</td>
<td>84.5</td>
<td>34.1</td>
<td>47.8</td>
<td>40</td>
<td>7</td>
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<tr>
<td>XPA</td>
<td>74.2</td>
<td>69.1</td>
<td>18.0</td>
<td>17.1</td>
<td>16</td>
<td>9</td>
<td>27*</td>
</tr>
<tr>
<td>XRCC1</td>
<td>57.0</td>
<td>54.6</td>
<td>8.8</td>
<td>11.3</td>
<td>25</td>
<td>10</td>
<td>17</td>
</tr>
</tbody>
</table>

Abbreviation: CV, coefficient of variance.

* $x^2$ test: $P < 0.001$ when comparing between categories of T/N < 0.8 and T/N > 1.2.

$\chi^2$ test: $P < 0.05$, when comparing between categories of T/N < 0.8 and T/N > 1.2.

### Table 3
The Spearman rank correlation between 24 pathway genes in 52 colon cancer patients

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>ABCB1</th>
<th>ABCC1</th>
<th>ABCC2</th>
<th>ABCG2</th>
<th>ADPRT</th>
<th>CDC45L</th>
<th>CES1</th>
<th>CES2</th>
<th>CYP3A4</th>
<th>CYP3A5</th>
<th>DRG1</th>
<th>ERCC1</th>
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<tr>
<td>ABCB1</td>
<td>1.000</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCC1</td>
<td>−0.132</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
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<td>ABCC2</td>
<td>0.042</td>
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<td></td>
</tr>
<tr>
<td>ABCG2</td>
<td>0.106</td>
<td>−0.083</td>
<td>−0.094</td>
<td>1.000</td>
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<td></td>
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<td></td>
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<td></td>
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<tr>
<td>ADPRT</td>
<td>−0.154</td>
<td>0.099</td>
<td>0.564</td>
<td>0.054</td>
<td>1.000</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>CDC45L</td>
<td>−0.132</td>
<td>0.071</td>
<td>0.511</td>
<td>0.062</td>
<td>0.724</td>
<td>1.000</td>
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<tr>
<td>CES1</td>
<td>0.256</td>
<td>−0.176</td>
<td>0.124</td>
<td>0.160</td>
<td>0.182</td>
<td>−0.027</td>
<td>1.000</td>
<td></td>
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</tr>
<tr>
<td>CES2</td>
<td>0.179</td>
<td>−0.214</td>
<td>0.108</td>
<td>0.164</td>
<td>0.113</td>
<td>0.082</td>
<td>0.033</td>
<td>1.000</td>
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<tr>
<td>CYP3A4</td>
<td>0.303</td>
<td>−0.053</td>
<td>0.311</td>
<td>0.052</td>
<td>0.258</td>
<td>0.459</td>
<td>0.161</td>
<td>0.215</td>
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<td>CYP3A5</td>
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<td>0.145</td>
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<td>NFKB1</td>
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<tr>
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<td>0.036</td>
<td>0.032</td>
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<td>−0.019</td>
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<td>0.086</td>
<td>0.263</td>
<td>0.016</td>
<td>0.256</td>
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<tr>
<td>TNFSF6</td>
<td>−0.025</td>
<td>0.448</td>
<td>−0.184</td>
<td>0.098</td>
<td>−0.075</td>
<td>−0.109</td>
<td>0.138</td>
<td>0.052</td>
<td>0.045</td>
<td>0.024</td>
<td>−0.236</td>
<td>0.481</td>
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<tr>
<td>TOP1</td>
<td>0.350</td>
<td>−0.047</td>
<td>−0.090</td>
<td>0.228</td>
<td>0.109</td>
<td>0.166</td>
<td>0.156</td>
<td>0.190</td>
<td>0.187</td>
<td>0.081</td>
<td>−0.036</td>
<td>0.041</td>
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<tr>
<td>TP53</td>
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<td>0.606</td>
<td>0.093</td>
<td>0.265</td>
<td>0.034</td>
<td>0.062</td>
<td>0.009</td>
<td>−0.179</td>
<td>0.170</td>
<td>0.112</td>
<td>0.134</td>
<td>0.377</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>0.159</td>
<td>0.037</td>
<td>−0.206</td>
<td>0.265</td>
<td>−0.016</td>
<td>−0.017</td>
<td>−0.198</td>
<td>0.200</td>
<td>0.005</td>
<td>0.262</td>
<td>0.078</td>
<td>0.195</td>
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<td>0.692</td>
<td>0.284</td>
<td>−0.170</td>
<td>0.186</td>
<td>0.210</td>
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<td>0.246</td>
<td>0.463</td>
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<td>0.160</td>
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<tr>
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<td>0.168</td>
<td>0.286</td>
<td>0.277</td>
<td>0.286</td>
<td>0.045</td>
<td>0.260</td>
<td>0.085</td>
<td>0.064</td>
<td>−0.160</td>
<td>−0.190</td>
<td>0.123</td>
</tr>
</tbody>
</table>

NOTE: The score is in bold if the $P < 0.001$. The range of the higher scores is between 0.448 and 0.730.
0.178; it had a four-gene subcluster (ABCC1, ERCC2, TP53, and XPA) with a much higher similarity index (0.574).

**Clustering Analysis of Gene Expression in Cancer Patients.** Consistent with what is generally known regarding response to chemotherapy, there was no significant correlation between the gene expression and the clinicopathology in this study. The gene expression pattern itself, however, may be valuable in tailoring therapy for cancer patients. As shown in Fig. 3, 52 patients were divided into three groups based upon the RNA expression of the pathway genes. The similarity index was 0.744, 0.635, and 0.545, respectively, for groups 1, 2, and 3. The three groups were 17% (9 of 52), 23% (12 of 52), and 60% (31 of 52) of the patient population. The ANOVA revealed significant differences ($P = 0.001-0.036$) between the groups for 7 of the 24 pathway genes (CES1, CES2, CYP3A5, DRG1, FDXR, TP53, and XPA). Analysis also revealed no statistically unique clinicopathology ($P = 0.057-0.909$) for gender, tumor location, grade, and classification but did show particular RNA expression patterns for the seven genes (Fig. 3).

**DISCUSSION**

Tumor response in patients in the same stage of colorectal cancer varies widely even with the use of uniform chemotherapy. The reason for this variation in chemotherapy activity remains unclear. The evaluation of single genes, or a small panel of candidate genes, has promise for predicting therapeutic benefit from chemotherapy (43, 44). However, it is clear that a polygenic disease such as colorectal cancer requires a polygenic approach to predict outcome. In this study, we carried out a comprehensive analysis to profile gene expression of the irinotecan pathway. Our data reveal both large interpatient and intergene variations in RNA expression levels. This large interpatient variability could be a major source for predicting the diverse responses of cancer patients to chemotherapy; it certainly indicates a need for individualized chemotherapy. The large variability in transcription levels of drug pathway genes demands identification of the key determinants in whole pathway genes. Individual difference in gene expression may be based on many factors, including variable physiologic and pathophysiologic states, environmental stimuli (such as smoking, drug intake, or diet), genetic variants, as well as technological variation in the RNA expression measurement.

The difference in RNA expression between tumor and normal tissues may help determine a strategy for individualized treatment of cancer patients, either to obtain maximal benefit, or to avoid toxicity from chemotherapeutic agents. Our results show statistically significant differences between the colon tumor and the normal tissues for 14 of the 24 irinotecan pathway genes (58%), which is comparable to the fundamental difference in biological behavior between tumor and normal tissues. In general, each of the pathway genes can be classified as either a drug response gene or a drug resistance gene, according to their known functions. Higher expression of the drug response genes or lower expression of the drug resistance genes in tumors may benefit patients. At the same time, if the normal tissues have higher expression of the drug resistance genes or lower expression of the drug response genes, the patients may experience little toxicity. For example, a patient with higher tumor RNA expression for CES1 or CES2 may have a better response of tumor cells to the same dose of irinotecan chemotherapy than a patient with the lower tumor expression of those activating enzymes. Furthermore, those with lower CES1/CES2 expression in normal tissues will be able to tolerate higher doses of irinotecan than those with high expression; those with high CES1/CES2 expression in normal tissues will likely have more toxicity to the same dose of irinotecan.

**Table 3** Continued

<table>
<thead>
<tr>
<th>ERCC2</th>
<th>FDXR</th>
<th>MLH1</th>
<th>MSH6</th>
<th>NFkB1</th>
<th>TDP1</th>
<th>TNFSF6</th>
<th>TOP1</th>
<th>TP53</th>
<th>UGT1A1</th>
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<td>0.015</td>
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<td>0.253</td>
<td>1.000</td>
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<td>0.185</td>
<td>–0.032</td>
<td>–0.070</td>
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<td>0.121</td>
<td>–0.127</td>
<td>–0.224</td>
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<td>–0.057</td>
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<td>–0.292</td>
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<td>–0.253</td>
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<td>0.236</td>
<td>1.000</td>
<td>0.007</td>
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<td>0.236</td>
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<td>0.007</td>
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<td>0.169</td>
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<tr>
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<td>0.189</td>
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</tr>
<tr>
<td>0.143</td>
<td>–0.018</td>
<td>0.169</td>
<td>–0.189</td>
<td>–0.064</td>
<td>–0.090</td>
<td>0.162</td>
<td>0.377</td>
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<td>1.000</td>
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</tr>
<tr>
<td>0.509</td>
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<td>0.015</td>
<td>0.002</td>
<td>–0.009</td>
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</table>
Irinotecan. Indeed, gut toxicity from irinotecan can be due in part to direct drug conversion by local CESs present within the small intestine (45). However, it should be pointed out that the expression of hepatic CESs is a major determinant of toxicity of irinotecan.

The gene expression level is a reflection of gene function status. For the 52 patients in this study, individual expression values varied widely, with up to a 119-fold change. It is difficult to tell how gene function affects drug pathway activity, based only on the expression level of a single gene in an individual patient. Thus, we were trying to evaluate the irinotecan pathway activity based on the RNA expressions of whole pathway genes. In addition, because some of the pathway genes may physiologically have constitutive expression, a gene with a higher expression level may not have a greater effect on drug pathway activity than a gene with a lower expression level. Nonetheless, the T/N ratio of RNA expression reflects a kind of normalized relative level in colon tumor tissues, and could thus be more valuable for evaluation of effect of the pathway genes on tumor chemotherapy activity. Consequently, we assessed the relative contribution of each of the 24 irinotecan pathway genes to drug pathway activity based upon the median T/N ratio of RNA expression. The top (high T/N ratio) and bottom quartile (low T/N ratio) genes (Fig. 1) may make more difference in direction of pathway movement than the genes in the midquartiles, with regard to the response or resistance of tumor tissue, or the toxicity of normal tissue to the irinotecan chemotherapy. Because TP53 had the highest median T/N ratio (2.42) in the pathway, which is favorable to the antitumor activity of the drug, it may be a critical variable in the killing of tumor cells. In addition, as CES1 and CES2 had the lowest median T/N ratios (0.23 and 0.35, respectively), the conversion of irinotecan to SN-38 may take place mainly in the normal tissue rather than the colon tumor. In fact, the conversion of irinotecan to its active form SN-38 has been found to occur heavily in liver and intestinal cells (9, 10). Moreover, ABCC1 had a higher median T/N ratio (2.00) than other drug transporters (e.g., ABCB1, ABCC2, and ABCG2). Thus, the relative functional contributions of ABCC1 to irinotecan effect need to be evaluated and ranked to understand the clinical relevance of this finding. It is commonly accepted that FDXR contributes to TP53-mediated apoptosis; in addition, DRG1 has recently been shown a target for modulating sensitivity to CPT-11 in colon cancer cells (37–39). Both FDXR and DRG1 had higher median T/N ratios in pathway gene expression, suggesting they may play an important role in the antitumor activity of the drug.

Gene expression profiling and clustering analyses have become one of the most useful tools to characterize classification and prediction markers for cancer and other
diseases (46–50). In our study, we made an effort to assess
coregulation or coexpression of the irinotecan pathway genes.
Analysis of the RNA expression of the 24 irinotecan pathway
genes revealed three gene clusters. These clusters are not groups
of gene family members, but the genes in the largest clusters
(ADPRT, CDCA4SL, MSH6, NFKB1, and TDP1) are involved in
the cellular DNA repair reaction initiated by irinotecan-induced
DNA strand breaks. TOP1, MLH1, and XRCC1, which
participated in the process of DNA replication and repair, were
also grouped together. Further study is needed, however, to
determine if these findings imply functional associations
between irinotecan pathway genes. Three patient groups were
also observed after unsupervised clustering. These three patient
groups had no unique clinical pathologic features but could be
differentiated using the particular expression patterns of the
seven genes (CES1, CES2, CYP3A5, DRG1, FDXR, TP53, and
XP4). These seven genes had statistically significant differences
in RNA expression among the three patient groups. Based on the
particular RNA expression profiles of the seven genes, we may
be able to predict tumor response to irinotecan (sensitive or
resistant). For example, high expression of CES1/CES2 will
increase the production of active SN-38; low expression of
CYP3A5 will decrease the formation of inactive 7-ethyl-10-[4-
N-(5-aminopentanoic acid)-1-piperidino] carbonyloxy campto-
thecin and 7-ethyl-10-(4-amino-1-piperidino) carbonyloxy
camptothecin. Moreover, low DRG1 expression will increase the
sensitivity of tumor cells to apoptosis, whereas low XPA
expression will decrease the repair of tumor cells with drug-
induced DNA damage. We chose not to analyze the association
between gene expression and outcome, because of the limited
number of patients and the large number of treatment variables.
Therefore, clinical trials are needed to study if these seven genes
can be used as markers for determining individualized treatment
for cancer patients. This and other studies (46, 48) suggest that
large-scale gene expression profiling (DNA microarrays, high
throughput real-time reverse transcription-PCR, etc.) can provide
more information for tailoring therapy to individual cancer
patients than will the patients' clinical features. The data from
this and related studies can now form the basis for constructing a
clinical trial to evaluate the relevance of these drug pathway
groups.

In conclusion, our data show that there is quite large
interpatient and intergene variability in the RNA expression of
irinotecan pathway genes. The data in this study also provides
preliminary evidence for the use of gene expression profiling as
an approach to predicting response to irinotecan chemotherapy
and for tailoring therapy individual cancer patients.

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irinotecan (CPT-11) by human hepatic microsomes: participation of
cytochrome P-450 3A and drug interactions. Cancer Res 1998;58:
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repair system in the cytotoxicity of the topoisomerase inhibitors
camptothecin and etoposide to human colorectal cancer cells. Cancer

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cell lines: activity in vitro and in vivo. Anticancer Drugs 2001;12:
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clinical outcome to platinum-based chemotherapy in patients with

XRCC1 gene predicts for response to platinum based treatment in


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Jinsheng Yu, William D. Shannon, Mark A. Watson, et al.


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