Carboxylesterases Expressed in Human Colon Tumor Tissue and Their Role in CPT-11 Hydrolysis

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ABSTRACT

Purpose: **The purpose is to develop new analytical methods to study the expression profile of CPT-11 carboxylesterases and topoisomerase I in colon tumor samples and understand the impact of their expression on CPT-11 metabolism in chemotherapy.**

Experimental Design: **We investigated 24 colon tumors for expression of carboxylesterases *CES1A1, CES2, CES3, hBr-3,* and *topoisomerase I* genes by real-time PCR and correlated the gene expression with activity assays. The relative abundance of the carboxylesterase isoenzymes and topoisomerase I genes was determined by real-time PCR. Activity assays performed on colon tumor extracts included CPT-11 hydrolase, 4-methylumbelliferyl acetate hydrolase, and topoisomerase I activity assays. Additionally, nondenaturing activity gel electrophoresis with activity staining showed the distribution of carboxylesterases.**

Results: **We detect the expression of *CES1A1, CES2,* and *CES3* carboxylesterase genes in human colon tumors. We were unable to detect the *hBr-3* (also called *hCE-3*) in human liver, colon, or brain. We find large interindividual variation, ≥150-fold, for both *CES1A1* and *CES3* genes, 23-fold for *CES2,* and 66-fold for topoisomerase I. Only *CES2* gene expression correlated with the carboxylesterase activity assays ($P < 0.01$) with CPT-11 and 4-methylumbelliferyl acetate as substrates. Nondenaturing activity gel electrophoresis showed that CES2 was the most predominant activity. Topoisomerase I gene expression significantly correlated with topoisomerase I activity ($P < 0.01$) in the colon tumors, but interindividual variation was very high.**

Conclusions: **We conclude that CES2 is the most abundant carboxylesterase in colon tumors that is responsible for CPT-11 hydrolysis. This pilot study reinforces the hypothesis that there is a large interindividual variation in expression of carboxylesterases that may contribute to variation in therapeutic outcome and/or toxicity of CPT-11 therapy for colon cancer.**

INTRODUCTION

CPT-11[2] is a water-soluble camptothecin derivative that is widely used in combination with 5-fluorouracil as a chemotherapeutic agent in the treatment of metastatic colon cancer (1). CPT-11 is a carbamate prodrug that is activated *in vivo* to SN-38, a potent topoisomerase I inhibitor. Studies with cancer cell lines showed that SN-38 is 300–20,000 times more cytotoxic than CPT-11 (2, 3).

Several pharmacokinetic studies indicate that there is large interindividual variation in CPT-11 bioavailability and toxicity (4, 5). Sixty-two percent of the i.v. dose of CPT-11 is excreted through feces and 64% of that is as the parent drug, CPT-11 (6). Hence, the expression of CPT-11 hydrolases in the target colon tumor tissue may play an important role in the therapeutic efficacy and/or toxicity associated with CPT-11 therapy because of the local conversion of CPT-11. The major dose-limiting toxicities of CPT-11 are diarrhea and leukopenia. The incidence of early-stage diarrhea is as frequent as 80% and is dose dependent (7). Late-stage diarrhea, occurring >24 h after CPT-11 administration, occurs in 60–87% of patients. Grade 3 or 4 diarrhea occurs in 20–40% of patients (7). Although early-stage diarrhea is treatable with atropine, late-stage diarrhea is more difficult to manage. The expression of CPT-11 hydrolases in gastrointestinal mucosa may be a determinant of the diarrhea side effect of CPT-11 therapy.

Carboxylesterases (E.C.3.1.1.1), CYP3A, and UGT are the major enzymes responsible for hepatic metabolism of CPT-11.

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[2] The abbreviations used are: CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin or irinotecan; CES, carboxylesterase and the isoenzymes are labeled CES1A1 (gi: 16905523), CES2 (gi: 21536284), and CES3 (gi:7019977), using the method described by Satoh and Hosokawa (14) and Satoh *et al.* (16), and hBr-3 or hCE-3 (gi: 6009627) was cloned from human brain cDNA library and is >98% identical to mouse liver triacylglycerol hydrolase (gi: 14269426); SN-38, 7-ethyl-10-hydroxycamptothecin; CYP3A, cytochrome P450 isoform 3A; UGT, UDP-glucuronosyl transferase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; APC, 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino] carbonyloxy camptothecin; NPC, 7-ethyl-10-[4-(1-piperidino)-1-amino] carbonyloxy-camptothecin; 4-MUA, 4-methylumbelliferyl acetate.

Carboxylesterases hydrolyze CPT-11 to SN-38 (8, 9). CYP3A4 converts CPT-11 to APC (10) and NPC (11), and UGT1A1 is responsible for the inactivation of SN-38 to 10-O-glucoronyl-SN-38. It was suggested that carboxylesterases may also be responsible for hydrolysis of the CYP-oxidized metabolites NPC and APC to SN-38 (11, 12).

The carboxylesterases responsible for CPT-11 hydrolysis belong to a large family of serine-active hydrolases with 60 kDa subunit mass and have a characteristic β-α-β esterase fold and catalytic triad (13). They are classified into four major groups by amino acid sequence alignment (14). They exhibit broad substrate specificity for ester, thioester, and amide hydrolysis to the corresponding water-soluble acids, alcohols, thiols, and amines (15). The most abundant and well-studied human enzymes are CES1A1 and CES2 (16). We have shown that CES2 has a 64-fold higher catalytic efficiency (k_{cat}/K_M) for CPT-11 than CES1A1 (9). Two additional human carboxylesterase genes have been cloned recently, *hBr3* from brain (17) and *CES3* from the colon. Although CPT-11 hydrolase activity is known to be in colon tumor tissue (18), the specific level of expression of individual carboxylesterase genes and isoenzymes in colon tumor tissue are not known.

A recent study using antibodies for CES1A1 and CES2 indicate that both isoenzymes are expressed in colon adenocarcinomas (19). Xie *et al.*, also showed that hBr-3, previously thought to be brain specific, was expressed in colon tumor (19). The expression of CES2 in colon adenocarcinoma was reported to be highly variable among colon tumors by Xu *et al.* (20).

In this study, we report the relative expression of four human carboxylesterase genes, *CES1A1*, *hBr-3*, *CES2*, *CES3*, and the *topoisomerase I* gene, in primary and metastatic colon tumor samples by real-time PCR. The relative expression of the carboxylesterase genes was correlated with the specific activities of the tumor samples for CPT-11 and 4-methylumbelliferyl acetate. We find that CES2 is the most abundantly expressed isoenzyme in colon and that CES2 expression significantly correlates with CPT-11 hydrolase activity, 4-methylumbelliferyl acetate hydrolase activity, and the CES2 enzyme activity as determined by CES2 band intensity on a native-gel stained for esterase activity with 4-methylumbelliferyl acetate. CPT-11 hydrolase activity significantly correlated with 4-methylumbelliferyl acetate activity, CES2 gene expression determined by real-time PCR and CES2 band density ($P < 0.01$). We also find significant correlation between topoisomerase I gene expression as determined by real-time PCR and topoisomerase I activity.

MATERIALS AND METHODS

Colon Tumor Tissue. The collection of colon carcinoma and the study of carboxylesterases therein were approved by an Institutional Review Board. Tumors from various parts of the colon, of all grades and stages, were collected. The specimens were obtained fresh from the operating room as soon as they were available. The tumor samples were dissected by a pathologist, who identified and removed mucosal fragments of tumor, normal colon mucosa, and secondary metastatic sites. These tissues were immediately frozen in liquid nitrogen and stored in a liquid nitrogen freezer until needed.

Northern Analysis. A human multitissue blot (Origene Technologies, Inc.) was sequentially probed with *CES1A1*, *CES2*, and *CES3* gene-specific probes. A 425-bp *Sap*I (New England Biolabs, Inc., Beverly, MA) fragment for *CES1A1* and a 340-bp *Eco*RI and *Nco*I fragment for *CES2* and the PCR product for *CES3* were radiolabeled with ^{32}P-dCTP using the random primed labeling kit (Roche Diagnostics), and the blot was sequentially probed for each carboxylesterase. The blot was prehybridized for 30 min in Quikhyb solution (Stratagene) at 65°C, hybridized for 2 h at 65°C, and washed twice with 2× SSC containing 0.1% SDS for 15 min at room temperature followed by one 30-min wash at 58°C with 0.1× SSC containing 0.1% SDS. The autoradiographs were generated by exposing the X-OMAT AR film (Kodak) to the radioactive blot for a period of 2 h to 8 days at −70°C.

Carboxylesterase Assay. The frozen tumor specimens were weighed and pulverized with a mortar and pestle cooled in liquid nitrogen. Two μl of 1 mM KH_2PO_4 (pH 6.4) containing 0.35 M NaCl, 5 mM $MgCl_2$, 1 mM EDTA, and 0.2 mM DTT were added per mg of tissue and incubated on ice for 30 min. Samples were centrifuged at 100,000 × *g* for 15 min, and the supernatant was collected. Total carboxylesterase activity in tumor extracts was determined by a spectrophotometric method described by Brzezinski *et al.* (21). Briefly, the tissue extract was incubated at 37°C with 0.5 mM 4-methylumbelliferyl acetate in 90 mM KH_2PO_4 and 40 mM KCl (pH 7.3). The formation of product, 4-methylumbelliferone, was monitored spectrophotometrically at 350 nm. Protein was quantitated by Coomassie blue dye-binding method with BSA as standard (Bio-Rad). The specific activity is expressed as μmol mg^{-1} min^{-1}. CES1A1 purified from human liver exhibited a specific activity of 6.8 units/mg (21) and CES2 exhibited a specific activity of 140 units/mg (22) with 4-methylumbelliferyl acetate as substrate.

CPT-11 Hydrolase Assay. The tumor extract was incubated with 50 μM CPT-11 in 50 mM HEPES buffer (pH 7.4) with 10% ethylene glycol, in a volume of 250 μl for 24 h at 37°C after a modification of Humerickhouse *et al.* (9). The reaction was stopped by addition of 250 μl of acetonitrile followed by 10 μl of camptothecin (internal standard) and centrifuged at 1400 × *g* for 8 min. One hundred μl of 2.5% perchloric acid were added to 400 μl of supernatant and subjected to three extractions with 3 ml of $CHCl_3$. The $CHCl_3$ phase from each extraction was pooled, dried under nitrogen, and reconstituted in 100 μl of 0.005% perchloric acid containing 23% acetonitrile. SN-38 standards were prepared similarly, and 45 μl were injected onto Agilent 1100 high-performance liquid chromatography system equipped with a 5μ C18, 150 × 4.6-mm Luna column (Phenomenex). The mobile phase was 28.5% acetonitrile in 0.1 M KH_2PO_4 (pH 4.0) with 3 mM heptane sulfonic acid. The compounds were eluted at a flow rate of 1 ml/min and monitored by fluorescence (excitation = 375 nm, emission = 560 nm). The specific activity for hydrolysis of CPT-11 is defined as pmol of SN-38 formed mg^{-1} h^{-1}.

Analytical Nondenaturing PAGE. Tumor extract proteins were separated by analytical discontinuous nondenaturing PAGE as described by Dean *et al.* (23). Carboxylesterase activity was detected by incubation of the gel with 1 mM 4-methylumbelliferyl acetate in 100 mM potassium phosphate buffer (pH 6.0) for 15 min. Gel images were acquired on a fluorescence

imaging system (Gel Doc 1000; Bio-Rad). The intensity of CES1A1 and CES2 bands were quantitated by densitometry analysis (Quantity One version 1.0; Bio-Rad).

Topoisomerase I Assay. Tumor extracts were made as described earlier, and after protein determination, serial dilution of tumor extracts was made in 0.15 M NaCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EDTA, 0.2 mM DTT (pH 6.4), with 1 μl pHOT1 supercoiled DNA (0.10 mg/ml; TopoGEN, Inc.), 15 μl of H_2O and 2 μl of 10× topoisomerase I assay buffer (TopoGEN, Inc.). Reactions were incubated at 37°C for 60 min and stopped by addition of 5 μl of 5× topoisomerase I stop buffer containing 5% sarkosyl, 0.125% bromphenol blue, and 25% glycerol. Samples were resolved on a 1% agarose gel and stained with ethidium bromide. One unit of topoisomerase I activity is defined as the lowest concentration of protein capable of the complete relaxation of 0.10 μg of supercoiled pHOT1 DNA.

RNA Isolation, Reverse Transcription, and Analytical PCR. Frozen tissue, ~60–150 mg, was pulverized in a mortar and pestle precooled in liquid nitrogen. Total RNA from human tumor samples was isolated using TRIzol reagent (Invitrogen) and purified using RNeasy Kit (Qiagen). Human liver RNA was obtained from Origene Technologies, Inc. A 75-μl reverse transcription reaction containing 3.75 μg of total RNA was set up using the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA). The reaction contained 2.5 μM oligodeoxythymidylic acid primer, 5 mM magnesium, 1 mM of each deoxynucleotide triphosphate, 75 units of RNase inhibitor, and final concentration of 2.5 units/μl of murine leukemia virus reverse transcriptase. The reverse transcription conditions were 10 min at room temperature, 60 min at 42°C, 10 min at 68°C, and 5 min at 95°C. The PCR conditions were optimized using conventional PCR with Sybr green kit (Applied Biosystems) and gene-specific primers. The gene-specific primers for each gene are as follows: *CES1A1* forward 5′-AGAGGAGCTCTTGGAGACGACAT-3′ and reverse 5′-ACTCCTGCTTGTTAATTCCGACC-3′; *hBr-3* forward 5′-ATTGCTGGTCTGGTTGCTACTCTT-3′ and reverse 5′-CTGGTGCCTTTGGCAGAACTACT-3′; *CES2* forward 5′-AACCTGTCTGCCTGTGACCAAGT-3′ and reverse 5′-ACATCAGCAGCGTTAACATTTTCTG-3′; *CES3* forward 5′-CTGGTCCTTAGCAAGAAGCTGAAA-3′ and reverse 5′-CATTTGGCTTGTGCGTCCGAGTT-3′; *topoisomerase I* forward 5′-TCAGCGTTCTACCAGGCAAATTCA-3′ and reverse 5′-AGAGTGACGACTCTAACAGGTGC-3′; and *GAPDH* forward 5′-GACCACAGTCCATGCCATCACT-3′ and reverse 5′-TCCACCACCCTGTTGCTGTAG-3′. The expected PCR product sizes in kb are 0.19 for *CES1A1*, 0.2 for *hBr-3*, 0.31 for *CES2*, 0.31 for *CES3*, 0.21 for *topoisomerase I*, and 0.45 for *GAPDH*.

Real-Time PCR. One μl of the reverse transcription reaction was used as template in a 25-μl PCR reaction except for *GAPDH*, where 0.4 μl of sample was used per reaction, and each sample was analyzed in triplicate for all of the genes. The PCR reaction conditions were 2 mM Mg^{2+}, 0.25 μM of each primer, 0.2 mM of deoxynucleotide triphosphates using the Sybr green kit (Perkin-Elmer). PCR cycling conditions were 95°C for 10 min and 40 cycles of 95°C for 30 s, 64°C for 30 s, and 72°C for 1 min on an ABI 7700 instrument. A standard curve for each gene, except topoisomerase I, was generated using human liver cDNA as template. A topoisomerase I clone generated in our laboratory was used to construct the standard curve. The transcript amount for *CES1A1*, *CES2*, *CES3*, topoisomerase I, and *GAPDH* genes were estimated from the respective standard curves and normalized to GAPDH transcript amount determined in corresponding samples. The amplified products were analyzed by 2% agarose gel electrophoresis to ascertain the size and purity of the products. Cloning and sequencing in a control experiment confirmed the authenticity of amplified products.

Statistical Analysis. The normalized transcript level for each gene was independently subjected to linear regression analysis (JMP version 4.0, SAS Institute Inc., Cary, NC) with each activity assay. The data were considered significant if $P < 0.05$.

RESULTS

The expression of three carboxylesterase genes and topoisomerase I at the mRNA transcript level and the expression of carboxylesterase activity and protein gel electrophoresis pattern were examined in the 24 colon tumor samples.

Colon Tumor Samples. We collected 18 primary tumors from 10 females and 8 males and 6 metastatic tumors from 4 females and 2 males. The tumors were graded based on Tumor-Node-Metastasis staging system as recommended by the American Joint Commission on Cancer (24) and ranged from $T_1N_1M_x$ to $T_4N_1M_x$ with most of them in $T_3N_{0/1}M_x$ category.

Northern Blot Analysis of Carboxylesterases in Colon Tissue. The expression of carboxylesterase genes in normal colon sample was studied by Northern blot analysis. Three carboxylesterases were detected in samples of normal colon tissue upon sequential probing of the human multitissue Northern blot. As shown in Fig. 1, the relative abundance of carboxylesterases was determined after taking into account the half-life of ^{32}P and was as follows: *CES2* = 6x*CES1A1* = 13x*CES3*. Therefore, in normal colon tissue, CES2 is the most abundantly expressed isoenzyme. We detected multiple transcripts for *CES2* and *CES3* on multiple-tissue Northern blot. This multiplicity could arise from differential processing of the message, but the exact reason is not known.

Real-Time PCR. PCR conditions were developed to amplify carboxylesterases *CES1A1*, *hBr3*, *CES2*, *CES3*, as well as topoisomerase I and *GAPDH* genes using human liver cDNA prepared from human liver RNA (Origene Technologies, Inc.). PCR products of expected size for each gene were obtained as shown in Fig. 2. An isoenzyme from brain called *hCE-3* or *hBr3* was originally cloned from human brain cDNA library (17). The nucleotide sequence for *hBr-3* (gi: 6009627) is 99% identical to mouse liver triacylglycerol hydrolase gene (gi: 14269426). We simultaneously attempted to amplify *hBr-3* gene from human brain cDNA (BD Bioscience-Clontech) and mouse liver cDNA using four different primer pairs. The expected sized PCR products were obtained from mouse liver cDNA with all four primer pairs, but we failed to detect any PCR products using human brain cDNA. Thus far, we have no evidence for expression of *hBr-3* transcript in human liver (Fig. 2, *Lane 3*), colon, primary colon tumor, metastatic colon tumor (data not shown), or brain. Hence, *hBr-3* expression was not examined further.

The dynamic range for linear amplification of *CES2* gene

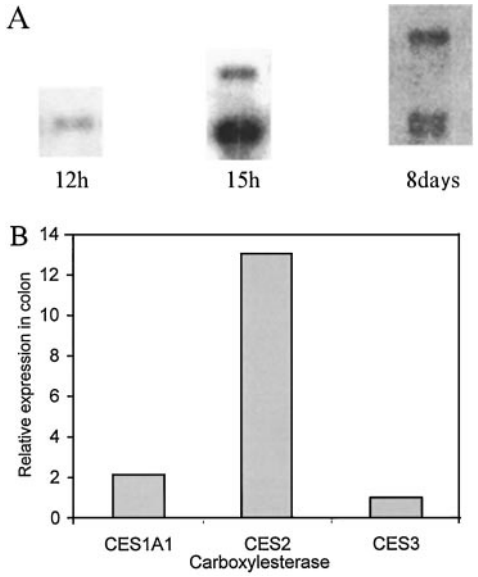

Fig. 1 Northern blot analysis of *CES1A1*, *CES2*, and *CES3* genes in normal colon tissue: *A* shows the results for expression of *CES1A1*, *CES2*, and *CES3* genes in colon tissue after sequentially probing a multitissue mRNA Northern blot (Origene Technologies, Inc.) with gene-specific probes. *CES1A1* blot was developed after 12 h, *CES2* after 15 h, and *CES3* after 8 days. *B* shows the relative abundance of the ~2-kb band in normal colon tissue obtained by densitometric analysis of each film using QuantityOne software (Bio-Rad) and normalized to exposure time.

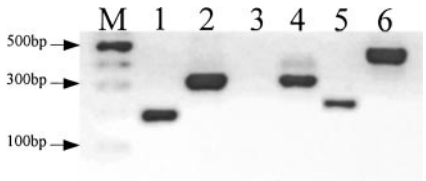

Fig. 2 PCR products separated by 2.0% agarose gel electrophoresis. Four human carboxylesterases, topoisomerase I, and *GAPDH* genes were amplified by PCR from human liver cDNA by gene-specific primers described in "Materials and Methods." Ten μl of PCR reaction were analyzed on 2.0% agarose gel. Samples shown are *Lane M*, 100-bp DNA markers (New England Biolabs, Inc.); *Lane 1*, *CES1A1*; *Lane 2*, *CES2*; *Lane 3*, *hBr-3*; *Lane 4*, *CES3*; *Lane 5*, topoisomerase I; and *Lane 6*, *GAPDH*.

was 300-fold with human liver cDNA as template (Fig. 3). Similar standard curves were obtained for all of the genes. Each sample was analyzed in triplicate, and the SD was typically ≤15% for *CES2* and *CES1A1*. The relative abundance of the transcript for each gene was determined from its standard curve and was averaged and normalized to *GAPDH* signal of the same sample. There was a large variation in the abundance of transcripts for *CES1A1*, *CES2*, *CES3*, and topoisomerase I genes in colon tumor samples. *CES1A1* transcript levels in colon tumors varied by 150-fold, and the signal of 12 samples was <5% of maximum value (Fig. 4*A*). The transcript abundance of the *CES2* gene varied by 23-fold (Fig. 4*B*) and that of *CES3* varied by 275-fold in colon tumor samples (Fig. 4*C*). The relative

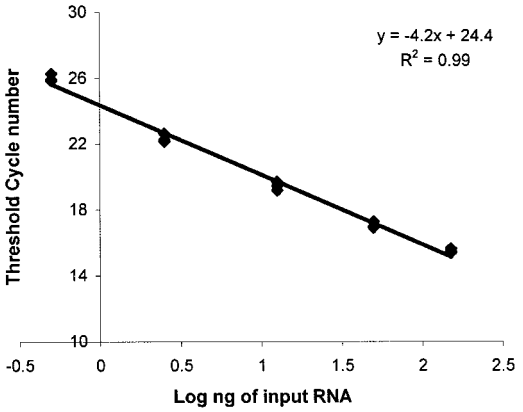

Fig. 3 Real-time PCR standard curve for *CES2*. Total RNA from human liver was reverse transcribed, and cDNA samples equivalent to 0.5–150 ng of input RNA were subjected to real-time PCR in triplicates with *CES2* gene-specific primers. The threshold cycle number (Ct) for each sample was subjected to linear regression analysis as a function of logarithm of input RNA.

abundance of the three individual carboxylesterases in a single sample (Fig. 4) cannot be directly compared. However, based on Northern blot (Fig. 1) and nondenaturing PAGE gel studies (Fig. 5), we find that *CES2* is the most abundant carboxylesterase in colon tumor tissue.

Carboxylesterase Activity Assays. The esterase activity in colon tumor protein extracts was evaluated by three assays: CPT-11 hydrolysis, 4-methylumbelliferyl acetate hydrolysis, and density of activity bands on nondenaturing PAGE gel generated upon activity staining with 4-methylumbelliferyl acetate. All colon tumor extracts displayed CPT-11 hydrolase activity and the specific activity ranged from 0.8 to 45 pmol mg^{-1} h^{-1}, a variation of 56-fold (Table 1). The nonspecific carboxylesterase activity with 4-methylumbelliferyl acetate as substrate ranged from 0.36 to 10.1 μmol mg^{-1} h^{-1} in 24 tumor tissues, a variation of 28-fold (Table 1). The identity of carboxylesterase isoenzymes in human colon tumor tissue extract was examined by nondenaturing PAGE followed by activity staining with 4-methylumbelliferyl acetate using CES1A1 and CES2 proteins from human liver as standards. The nondenaturing gel analysis of 4 colon tumor tissues is shown in Fig. 5. The CES1A1 activity band was very weak in all samples. CES2 was the most abundant activity in all of the samples examined. There is an additional activity band marked with a question mark in Fig. 5 that had mobility between that of CES1A1 and CES2. The expression of the middle band was highly variable among samples. The identity of the protein in this middle band is not known. In agreement with the real-time PCR data, CES2 activity ≫CES1A1 activity. CES2 band intensity quantitated by densitometric analysis (Table 1) varied by 37-fold.

Topoisomerase I Assay. Eighteen primary colon tumors were studied for topoisomerase I activity. The real-time PCR studies demonstrated that the transcript levels for topoisomerase I varied by 67-fold, and this was consistent with the pHOT1 DNA relaxation assays, where the specific activity of nuclear extracts varied from 100 to 3200 units/mg of colon tumor proteins.

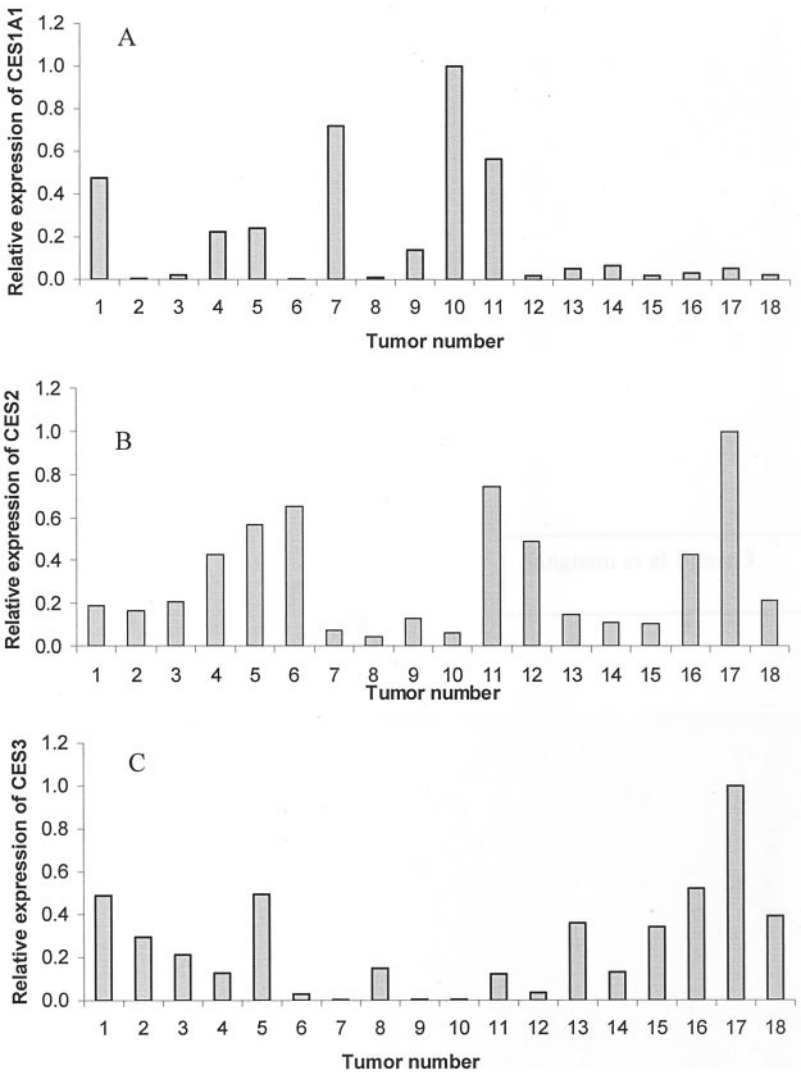

Fig. 4 Relative expression of carboxylesterase genes in primary colon tumor samples as determined by real-time PCR. Each tumor sample was run in triplicate, and the amount of transcript in each sample was determined from its standard curve. The SD for the triplicates was <10% for most samples for *CES1A1* and *CES2*. The transcript amount for each sample for each gene was estimated from its standard curve, averaged, and normalized to its *GAPDH* signal. The sample with maximum signal was set to one and other samples are expressed relative to the most abundant sample. *A* shows relative expression of *CES1A1* gene; *B*, *CES2* gene; and *C*, *CES3* gene.

Correlation Analysis. Transcript levels for *CES1A1*, *CES2*, and *CES3* genes were normalized to *GAPDH* in tissue samples. Relative gene expression of *CES1A1*, *CES2*, and *CES3* was subjected to linear regression analysis individually against specific activities of colon tumor extracts for CPT-11 and 4-methylumbelliferyl acetate and CES2 band density on nondenaturing activity gel electrophoresis (Table 2) using JMP version 4.0 software. Our analysis showed that relative abundance of *CES1A1* and *CES3* transcript determined by real-time PCR did not significantly correlate with any of the esterase activity assays. Only the relative abundance of *CES2* transcript showed significant correlation ($P < 0.01$) for CPT-11 hydrolase, 4-methylumbelliferyl acetate hydrolase, and CES2 band density. The band intensity of CES1A1 and middle activity band on nondenaturing gel did not correlate with expression of *CES1A1*, *CES2*, or *CES3* genes by PCR (Table 2). CPT-11 hydrolase activity positively and significantly ($P < 0.007$) correlated with relative abundance of *CES2* gene by real-time PCR (Fig. 6*A*), CES2 band density on nondenaturing gel (Fig. 6*B*) and 4-methylumbelliferyl acetate hydrolase activity (Fig. 6*C*). Analysis of gender-dependent variation in *CES2* expression using Wilcoxon's rank-sum method (JMP version 4.0) showed greater expression in males in comparison with females ($P < 0.05$). Linear regression analysis showed that topoisomerase I activity significantly correlated with topoisomerase I PCR, $P = 0.002$ and $r^2 = 0.428$ (Fig. 6*D*).

DISCUSSION

Two human carboxylesterase genes in the 60-kDa family, *CES1A1* and *CES2*, have been characterized as CPT-11 hydrolases, and the catalytic efficiency of CES2 is 64-fold greater than that of CES1A1 (9). Both genes are expressed in normal colon as determined by Northern blot (Ref. 1; Fig. 1) and real-time PCR (Fig. 4, *A* and *B*). The expression of *CES2* is much greater than *CES1A1* in colon (Fig. 1). There are other members of this 60-kDa carboxylesterase family that could function as CPT-11 hydrolases. The *hBr-3* cDNA was cloned

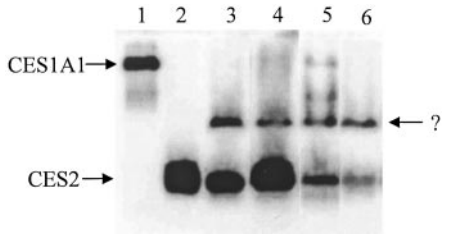

Fig. 5 Nondenaturing activity gel electrophoresis of colon tumor extracts. One hundred fifty μg of protein from four tumor extracts were separated on 10% nondenaturing gel and stained for carboxylesterase activity with 4-methylumbelliferyl acetate. Human liver CES1A1 and CES2 were loaded as controls in *Lanes 1* and *2*, respectively. The samples were loaded as follows: *Lane 3*, tumor number 6; *Lane 4*, tumor number 7; *Lane 5*, tumor number 8; and *Lane 6*, tumor number 4.

from a human brain library (17). Thus far, we have been unable to detect *hBr-3* gene expression in human brain, liver, colon, primary colon tumor, or metastatic colon tumors. Hence, this isoenzyme was not examined further. *CES3* was cloned from colon (gi: 7019977) and is expressed in colon tissue as determined by PCR (Fig. 4), Northern blot (Fig. 1), and cloning (data not shown). However, the level of expression of *CES3* is much less than *CES2* in normal colon such that the relative expression of the three genes is *CES2* > *CES1A1* > *CES3* by Northern blot analysis (Fig. 1). Preliminary studies indicate that CES3 catalyzes the hydrolysis of CPT-11 but much less efficiently than CES2. Hence, CES2 is both the most abundant and efficient CPT-11 hydrolase in colon tumors.

The three carboxylesterase genes are expressed at different levels in human liver as shown by Northern analysis (16). The abundance of carboxylesterases is *CES1A1* = 6x*CES2* and *CES1A1* = 64x*CES3* (unpublished results). The relative abundance of carboxylesterases in colon tumors is *CES2*>*CES1A1*>*CES3* from the Northern analysis data for normal colon (Fig. 1). Real-time PCR cannot be used to determine the relative expression of the 3 carboxylesterases in one tissue sample. The nondenaturing PAGE gel stained for activity using 4-methylumbelliferyl acetate (Fig. 5) confirms that expression of CES2≫CES1A1 in colon tissue.

Xie *et al.* (19) examined the expression of CES1A1, CES2, and hCE-3 or hBr-3 by Western blot analysis using antipeptide antibody in colon adenocarcinoma and adjacent normal colon tissue. This reported expression of hCE-3 does not agree with our studies. The expression of CES1A1 and CES2 protein in colon tissue and message (Fig. 4) are in agreement with Xie *et al.* (19). They additionally suggested that both proteins were more highly expressed in normal colon than tumor tissue. However, we find that the relative expression of *CES2* and *CES1A1* message is not significantly different between paired tumor and normal colon tissues (data not shown). Xu *et al.* (20) reported that CES2 protein was observed in 66% of a variety of tumors ($n = 154$) and 92% of associated normal tissues ($n = 60$). Wu *et al.* (25) demonstrated that overexpression of *CES2* gene in the HT29 colon adenocarcinoma cell line increased the hydrolysis rate and sensitivity of the cells to CPT-11. Our earlier *in vitro* observation demonstrated that the catalytic activity of CES2 is 64 times better than CES1A1 for CPT-11 hydrolysis (9). Hence,

Table 1 Hydrolase activities and CES2 band densities of 24 colon tumors

Colon tumors 1–18 are primary and 19–24 are metastatic tumors. The 4-methylumbelliferyl acetate hydrolase activity was determined in 50 mM sodium phosphate buffer (pH 7.4) at 37°C. Concentration of 4-methylumbelliferyl acetate was set at 0.5 mM, and specific activity is described as μmol mg^{-1} h^{-1}. CPT-11 hydrolase activity was determined in 50 mM HEPES buffer (pH 7.4), with 10% ethylene glycol in a volume of 250 μl for 24 h at 37°C, and CPT-11 concentration was set at 50 μM, and the specific activity is reported in pmol mg^{-1} h^{-1}. The CES2 band densities for carboxylesterase activities on nondenaturing gel were in fluorescence units.

Tumor no.	CPT-11 hydrolase activity pmol mg^{-1} h^{-1}	4-MUA activity, μmol mg^{-1} h^{-1}	CES2 band density
1	5.0	2.88	913
2	2.5	3.77	1789
3	1.7	2.81	1158
4	1.1	2.28	740
5	4.3	5.28	3817
6	12.5	10.08	3614
7	2.6	3.66	888
8	1.2	2.39	721
9	8.1	2.44	785
10	3.4	2.53	1234
11	38.2	8.21	8721
12	45.1	8.75	6347
13	4.4	3	1871
14	3.0	1.09	244
15	0.8	1.56	232
16	2.8	3.24	1268
17	4.6	6.06	2478
18	2.0	3.3	926
19	2.6	0.36	340
20	4.2	1.69	1827
21	9.0	1.8	1586
22	23.1	5.55	5377
23	6.8	2.22	764
24	8.1	2.38	803

CES2 is the most important colon carboxylesterase isoenzyme for CPT-11 metabolism.

In the 18 colon tumor samples and 6 metastatic tumor samples examined in this study, the expression of *CES2* was measured by real-time PCR, and it significantly correlated with CPT-11 and 4-methylumbelliferyl acetate hydrolase activities (Table 2). The expression of *CES1A1* or *CES3* did not significantly correlate with any activity measurements in the study (Table 2). Examination of carboxylesterase isoenzymes on a nondenaturing gel with 4-methylumbelliferyl acetate activity staining revealed two major activity bands in the tumor samples, CES2 and an unknown activity band (Fig. 5). A purified protein marker identified the CES2 band. CES1A1 protein was not highly expressed in any of the tumor tissues in Fig. 5. This is in agreement with real-time PCR data for *CES1A1* where 12 of 18 samples had very low levels of *CES1A1* (Fig. 4*A*). This low expression of CES1A1 does not seem to agree with the Western blot data of Xie *et al.* (19), where CES1A1 protein was abundant in colon tumor tissue. The identity of the middle band (marked with ? in Fig. 5) is not known. The only electrophoretic band that showed a significant intensity and correlation with *CES2* gene expression by real-time PCR is the CES2 band in Fig. 5 (Table 2). CPT-11 hydrolase activity had a significant and

Table 2 Statistical analysis of real-time PCR for colon primary and metastatic tumors

Twenty-five colon tumors, 18 primary and 6 metastatic, were studied. Correlation analysis of the real-time PCR data with activity assays was performed (JMP 4.0) The correlation coefficient and *P* values are tabulated, and the statistically significant values with $P \leq 0.05$ are shown in bold.

	4-MUA activity	CPT-11 hydrolase activity	CES1A1 band density	CES2 band density	Middle band density
CES1A1 PCR	$r^2 = 0.003$	$r^2 = 0.008$	$r^2 = 0.001$	$r^2 = 0.010$	$r^2 = 0.035$
	$P = 0.80$	$P = 0.68$	$P = 0.89$	$P = 0.64$	$P = 0.38$
CES2 PCR	$r^2 =$ **0.462**	$r^2 =$ **0.264**	$r^2 = 0.146$	$r^2 =$ **0.455**	$r^2 = 0.113$
	$P =$ **0.0003**	$P =$ **0.0103**	$P = 0.066$	$P =$ **0.0003**	$P = 0.11$
CES3 PCR	$r^2 = 0.003$	$r^2 = 0.062$	$r^2 = 0$	$r^2 = 0.004$	$r^2 = 0.030$
	$P = 0.79$	$P = 0.24$	$P = 0.97$	$P = 0.76$	$P = 0.42$

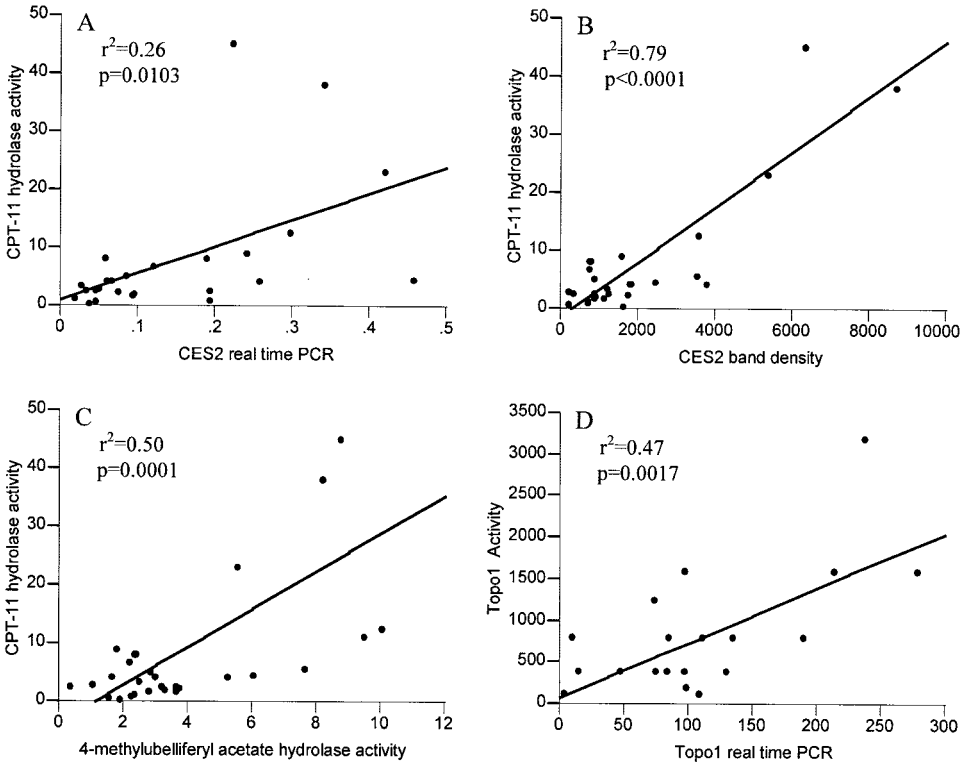

Fig. 6 Correlation analysis: CPT-11 hydrolase activity of colon tumor extracts was subjected to linear regression analysis with other assays (JMP version 4.0). Significant and positive correlation was observed for CPT-11 hydrolase activity with *CES2* real-time PCR (*A*) and CES2 band density (*B*) and 4-methylumbelliferyl acetate activity (*C*). Topoisomerase I real-time PCR significantly correlated with topoisomerase I activity (*D*).

positive correlation with *CES2* PCR, 4-methylumbelliferyl acetate activity, and CES2 band density (Fig. 6, *A–C*). We conclude that CES2 gene and protein expression are significantly correlated with CPT-11 hydrolase activity in colon tumor tissues. CPT-11 hydrolase activity is the best measure for clinical evaluation of CPT-11 metabolism in colon tumors, but the CPT-11 hydrolase assay is technically challenging. We suggest that CES2 real-time PCR is the best choice for analysis of CPT-11 hydrolase activity. Alternatively, CES2 immunohistochemistry can be used when appropriate antibodies become available. Immunohistochemistry is the best choice for analysis of archived tissue blocks from CPT-11-treated patients.

It is important to recognize that there is wide variability in the *CES2* expression among the individual colon tumors (Table 2). The relative expression of CES2 by real-time PCR varied 23-fold, the CPT-11 hydrolase activity 56-fold, and the CES2 band on nondenaturing gel electrophoresis varied 37-fold in the 24 samples. Xu *et al.* (20) reported very high variation in CES2 in tumor tissue and liver microsomes. Hennebelle *et al.* (18) reported very high variation in carboxylesterase activity in colon tumor tissue. One study looking at correlation of CPT-11 cytotoxicity with endogenous carboxylesterase activity in cell lines reported high correlation (3), whereas another reported no correlation (2) between carboxylesterase activity and CPT-11 cytotoxicity. We now conclude that the variability in expression of CES2 in tumor tissue may be a significant determinant of the cytotoxicity and clinical efficacy of CPT-11 treatment for colon cancer. We recommend that studies be conducted in patients treated with CPT-11 to examine the correlation between CES2 gene expression and therapeutic outcome.

There are other factors that could similarly influence CPT-11 treatment outcome such as topoisomerase I expression. As shown in Fig. 6*D*, topoisomerase I activity significantly correlated with topoisomerase I expression in the 18 tumor

samples. There was a 24-fold variation in topoisomerase activity and 66-fold variation in gene expression. Guichard *et al.* (26) has also found a high variation in topoisomerase I activity in colon tumor and normal samples. Decreased topoisomerase I expression and/or protein is associated with camptothecin-resistant cell lines (27, 28). Consistent with this finding, there is a report showing a positive correlation between CPT-11 sensitivity and topoisomerase I activity (2). The expression of other CPT-11 and SN-38-metabolizing enzymes such as CYP3A4 (29) and UGT1A1 (30), as well as enzymes important to combination therapy with 5-fluorouracil such as dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase (31) may be important determinants in the clinical outcome of CPT-11 therapy for colon cancer. The interindividual clinical outcome and toxicity of CPT-11, 5-fluorouracil, and leucovorin therapy for colorectal cancer is highly variable (4, 5). We recommend that studies be initiated to examine the expression of CPT-11 and 5-fluorouracil-metabolizing enzymes to determine whether they can be used to predict therapeutic outcome or toxicity in specific patients.

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