CORRELATING IRINOTECAN AND CAPECITABINE TREATMENT FOR COLORECTAL CANCER TO GENE EXPRESSION, POLYMORPHISMS, AND CLINICAL OUTCOMES

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ABSTRACT

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Colorectal cancer is the third most common type of cancer and the third most common cause of cancer-related mortality. There are three types of treatment available to patients, either individually or in combination. Treatments are radiation, chemotherapy, and surgery. In a Phase II clinical trial at IUSM, a multimodality approach was chosen. The patients with locally advanced rectal cancer received preoperative treatment with capecitabine and irinotecan (CPT-11) combination followed by chemoradiation with capecitabine and finally surgery to improve response and decrease local recurrence. Irinotecan and Capecitabine are both prodrugs activated *in vivo* to SN-38 and 5-FU, respectively. Identification of the molecular markers for 5-FU and Irinotecan efficacy and toxicity is important for the development of more efficient and less toxic treatment strategies for patients with colorectal cancer. The goal of this study was to determine the expression levels of the genes involved in activation and metabolism of capecitabine and irinotecan in pre and post treatment specimens from these patients. The genes quantitated by real-time PCR were carboxylesterase 1 and 2 (CES1 and CES2), thymidylate synthase (TS), β-glucoronidase (β-GUS), thymidine phosphorylase (TP), dihydropyrimidine dehydrogenase (DPD) and topoisomerase I (Topo I). The UGT1A1*28 polymorphism in

UDP glucuronosyltransferase 1 is associated with SN-38 toxicity. Therefore, the UGT1A1*28 polymorphism status in patients was determined by PCR-sequencing. Correlative analysis of gene expression and UGT1A1*28 mutation with clinical outcome in this Phase II study was completed.

Maureen A. Harrington, Ph.D., Chair

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INTRODUCTION

I. Colorectal Cancer

Colorectal cancer is the third most common type of cancer and the third most common cause of cancer related mortality. Although incidence has been steadily decreasing, the NIH estimated colorectal cancer to cause 49,960 deaths in 2008 (1). Colorectal cancer is an uncontrolled proliferation of cells in the largest part of the large intestine, the colon or rectum. Most colorectal cancers are adenocarcinomas, meaning they originate from the glandular cells that line the intestine (2).

Staging is the categorization of cancer according to the extent that it spreads. It is used for diagnostic, therapeutic, and prognostic purposes. There are several staging systems, but the most common system is the American Joint Committee on Cancer (AJCC) system, also called the TNM system. This system uses Roman Numerals I-IV to describe the extent of the primary **T**umor (T), the absence or presence of metastasis to nearby lymph **N**odes (N), and the absence or presence of distant **M**etastasis (M) (3).

Tumor
T1: Tumor invades submucosa
T2: Tumor invades muscularis
T3: Tumor invades serosa
T4: Tumor invades other organs/structures
Node
N0: No regional lymph node invasion
N1: Metastasis in 1-3 regional lymph nodes
N2: Metastasis in 4 or more regional nodes
Metastasis
M0: No distant metastasis
M1: Distant metastasis present

Table 1: TNM System: (American Cancer Society. *Detailed Guide: Colon and Rectum Cancer, How is Colorectal Cancer Staged).*

Stage I: T1 N0 M0; T2 N0 M0.
Cancer is contained to inner lining.
Stage II: T3 N0 M0; T4 N0 M0.
Cancer has spread to other nearby organs, but not reached lymph nodes.
Stage III: any T, N1-2, M0.
Cancer has spread to lymph nodes, but has not been carried to distant parts of the body
Stage IV: any T, any N, M1.
Cancer has been carried through the lymph system to distant parts of the body. The most likely organs are the lungs and liver

Table 2: Staging of Colon Cancer (American Cancer Society. *Detailed Guide: Colon and Rectum Cancer, How is Colorectal Cancer Staged).*

II. Treatment for Colorectal Cancer

There are three types of treatment available for patients with colorectal cancer. They are Primary Surgical Therapy, Adjuvant Chemotherapy, and Adjuvant Radiation Therapy (4). The route of therapy chosen is selected according to the stage of the disease.

Primary Surgical Therapy

Surgery is often the main treatment for colorectal cancer. It is often the best choice, when the cancer has not metastasized. Surgery may be used alone, or it may be employed with other options, such as chemotherapy or radiation therapy. Advanced techniques have greatly improved cure rates and reduced the level of damage to normal tissue (5).

Radiation Therapy

Radiation therapy is one of the most common forms of treatment for cancer. It uses high-energy particles to attack the cancer cells. It may be used by itself, or in conjunction with another form of treatment (6). Unlike Chemotherapy, radiation is localized to the area of the tumor.

Chemotherapy

Chemotherapy is a common method of cancer treatment. It employs the use of chemical/biological compounds to destroy cancer cells. These drugs may be used alone, or in combination with other drugs. And unlike surgery and radiation therapy, chemotherapy is a primarily systemic treatment (7). This means the drugs are not localized to a specific region. Rather, they are administered to the patient in such a way that would allow them to travel throughout the body, reaching the cancer cells wherever they may have spread.

Chemotherapy Options for Colorectal Cancer

A variety of drugs are available for patients with colorectal cancer. The most common drug is 5-Fluorouracil (5-FU). 5-FU may be administered intravenously or orally, along with Leucovorin. It is frequently given with another drug, such as Camptosar (irinotecan) or oxaliplatin, or with targeted therapies, that involve the use of monoclonal antibodies for specific proteins. These monoclonal antibodies may include Bevacizumab (Avastin), which targets vascular endothelial growth factor (VEGF), or Cetuximab (Erbitux) and Panitumumab (Vectibix), which target epidermal growth factor receptor

(EGFR) (8). Capecitabine, a prodrug of 5-FU, is increasingly used, as it is an oral drug and has minimal side effects.

III. Clinical Trial

This study was part of a Phase II clinical trial conducted at the Indiana University School of Medicine, Indianapolis. Patients were selected according to colorectal cancer staging, using endoscopic ultrasound (EUS). Those with T3/T4 or ≥ N1 rectal cancer were treated with capecitabine 1000 mg/m^2 twice daily for days 1-14 and irinotecan 200 mg/m^2 IV on the first of every 21 days for 2 cycles. This was followed by capecitabine 825 mg/m^2 twice daily days 1-5 weekly with concurrent radiotherapy 50.4 Gy in 28 1.8-Gy fractions. Baseline tumor biopsies were tested for correlative studies of genes expression with clinical endpoints 4-6 weeks after completion of preoperative therapy. The objectives of this trial were to assess pathological success rate, toxicity, rate of recurrence, clinical response to induction chemotherapy, and perform biological correlative studies of the enzymes involved in capecitabine and irinotecan metabolism.

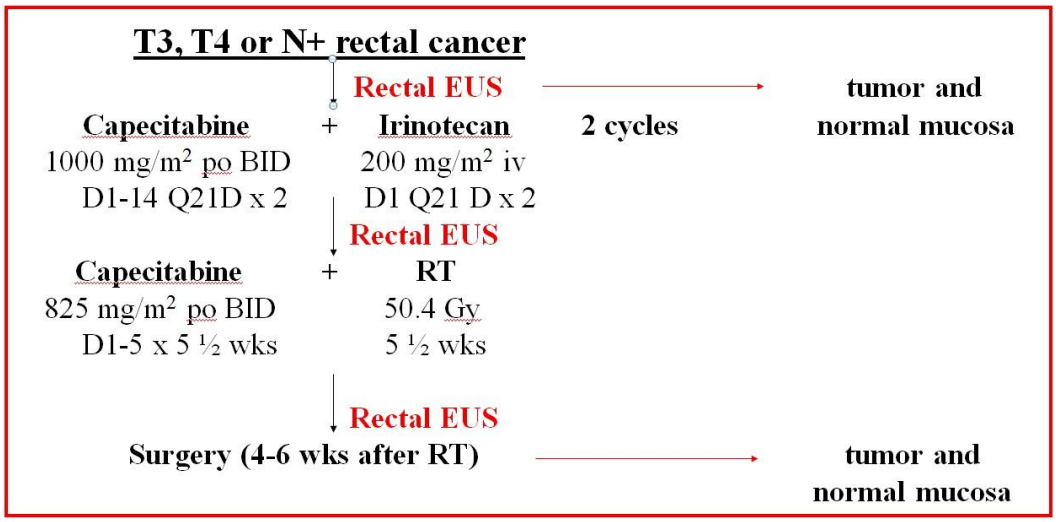

Figure 1. Clinical Trial Schema

IV. Capecitabine and Irinotecan

The first-line treatment of metastatic colorectal cancer is typically Irinotecan, used in conjunction with Capecitabine. As carbamate prodrugs, both require *in vivo* activation by carboxylesterases (9).

Irinotecan: Structure and Function

Irinotecan (Figure 2) is a semisynthetic, water-soluble derivative of the natural alkaloid, camptothecin, and is a carbamate ester prodrug of SN-38 (7-12). It works by inhibiting DNA Topoisomerase I, which is encoded by the gene *TOPO I* (9). Topo I is a nuclear enzyme involved in DNA replication, transcription, and DNA repair and recombination. During DNA replication, topoisomerase works by breaking one DNA strand and covalently binding to the 3'-phosphoryl end. Irinotecan prevents ligation of the nicked strand by stabilizing the DNA-topoisomerase I complex. This eventually leads to a double-strand break and results in apoptosis (10).

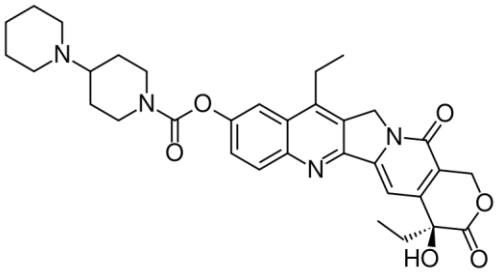

Figure 2. Molecular Structure of Irinotecan

Metabolism of Irinotecan

Irinotecan exists in two forms, an active lactone form and an inactive carboxylate form, that exist in a pH-dependent equilibrium (9). Irinotecan is converted to several different

metabolites by various enzymes. It is converted by carboxylesterases to its active form SN-38, and by the cytochrome CYP3A4 to form the compounds NPC and APC. APC is not further metabolized, but NPC is also converted by carboxylesterases to yield SN-38. (10). SN-38 then undergoes glucuronidation by UGT1A/7 to yield the inactive SN-38G. Various bacteria produce the enzyme β-glucuronidase (β-GUS), which then converts SN-38G back to the active metabolite, SN-38 (Figure 3) (9-14).

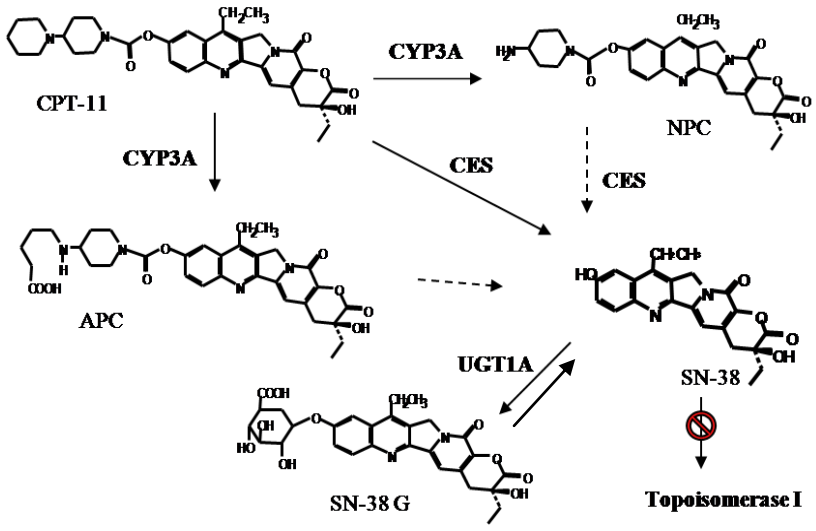

Figure 3. Irinotecan (CPT-11) Metabolism
Irinotecan exists in two forms, an active lactone form and an inactive carboxylate form, that exist in a pH-dependent equilibrium. Irinotecan is converted to several different metabolites by various enzymes. It is converted by esterases to form SN-38, and by CYP3A4 to form the compounds NPC and APC. APC is not further metabolized, but NPC is also converted by carboxylases to yield SN-38. SN-38 then undergoes glucuronidation by UGT1A/7 to yield the inactive SN-38G. Various bacteria produce the enzyme β-glucuronidase, which then converts SN-38G back to the active metabolite, SN-38.

Capecitabine: Structure and Function

Capecitabine (Figure 4) is an orally administered prodrug of the pyrimidine analog 5-fluorouracil (5-FU). 5-FU inhibits the production of the nucleotide thymidine by inhibiting the enzyme thymidylate synthase (TS) (9-14).

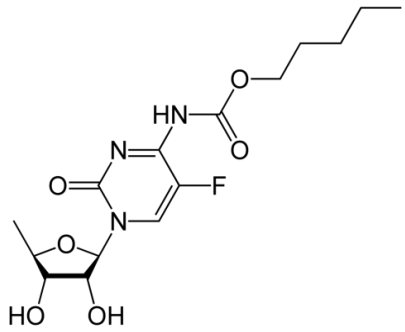

Figure 4. Molecular Structure of Capecitabine

Metabolism of Capecitabine

Capecitabine is first converted to 5'-deoxy-5-fluorocytidine (5'-DFCR) by carboxylesterases. 5'-DFCR is then converted to 5'-deoxy-5-fluorouridine (5'-DFUR) by cytidine deaminase. Next, thymidine phosphorylase (TP) converts 5'-DFUR to 5-FU. 5-FU inhibits the production of the nucleotide thymidine by inhibiting the enzyme thymidylate synthase (TS). 5-FU, however, is inactivated by the enzyme dihydropyrimidine dehydrogenase (DPD) (Figure 5) (15).

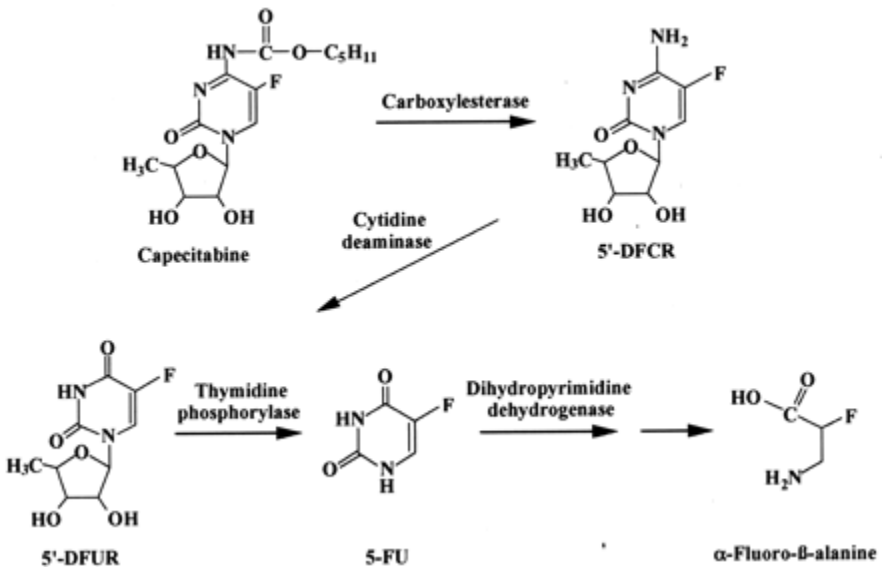

Figure 5. Metabolism of Capecitabine

V. Carboxylesterases

Classification and Function

Carboxylesterases (CES) are members of the α/β hydrolase fold family and are a group of enzymes that function in the metabolism of ester and amide prodrugs (16). They are ubiquitously expressed, but levels are highest in the small intestine, liver, and lungs. There are five genes of carboxylesterases reported in humans, named CES1-CES5. The vast majority are members of the CES1 or CES2 families. CES1 substrates generally contain a large acyl and a small alcohol group, while substrates for CES2 contain a small acyl and a large alcohol moiety (17).

Relationship to Irinotecan and Capecitabine

Irinotecan and Capecitabine are carbamate prodrugs, which require *in vivo* activation. Irinotecan activation is accomplished by CES2, and to a lesser extent, CES1. However, Capecitabine is activated by both CES1 and CES2. Irinotecan is converted to SN-38, while Capecitabine is converted to 5'-deoxy-5-fluorocytidine (5'-DFCR).

VI. Significance of UGT1A1

UDP glucuronosyltransferase 1 family, polypeptide A1 (UGT1A) encodes a specific UDP-glucuronosyltransferase (UGT) in the glucuronidation pathway. UGT1A1 also glucuronidates SN-38, converting it to SN-38G, and renders it inactive. However, glucuronidase activity is significantly decreased by the presence of additional TA repeats in the TATA sequence of the promoter region. Patients with a UGT1A1*28 polymorphism are more likely to experience severe adverse reactions to Irinotecan, such

as severe neutropenia and even potentially fatal diarrhea, due to their lower capacity to inactivate SN-38 to its glucuronide (10). There are several possible UGT1A1 $(TA)_n$ polymorphisms, the most common being variations of $(TA)_6$ and $(TA)_7$ (18).

VII. Biotechniques Utilized

There were several biotechniques employed in this study. DNA and RNA isolation, Real-Time PCR, DNA sequencing, and the use of enzyme activity assays were all employed. Nucleic Acid (NA) isolation was performed on tissues obtained during biopsy. Samples were immediately frozen, upon procurement, to prevent RNA degradation. Samples were then placed in a buffer, homogenized, and purified NAs were obtained with Qiagen kits. RNA integrity was determined using the 2100 bioanalyzer (Agilent) and the RNA integrity number was determined using pico chip. This technology uses picogram quantities of RNAs to determine the RNA integrity and quantity. To quantify gene expression, RNA samples were reverse-transcribed and cDNA was used for real-time PCR. Real-time PCR differs from traditional PCR in that it allows for the detection of amplicon during the initial, exponential phase of amplification, whereas traditional PCR measures amplicon produced in the final, plateau phase. This allows for earlier and more dependable quantitation of gene expression. DNA sequencing was performed on an automated instrument, using a chain-termination method. This method utilizes dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators. Samples are separated into four reactions, each using all four standard deoxynucleotides (dATP, dGTP, dCTP and dTTP). To each reaction is added only one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP) (19). When these

labeled nucleotides are incorporated, elongation ceases. This produces DNA fragments of varying lengths. DNA fragments are denatured and resolved by size. The differences in length are used to determine the sequence of the DNA (20).

VIII. Hypothesis

It was our hypothesis that higher CES2 expression in tumor tissue would result in better patient response, due to localized activation of irinotecan to SN-38. Also, higher CES2 expression in normal tissue may be the cause of severe drug related gastrointestinal toxicity.

AIMS of Thesis

1. The Primary aim of this study was to evaluate the expression levels of the genes responsible for the metabolism of capecitabine and irinotecan in baseline and post-treatment normal and tumor paired samples obtained from colorectal cancer patients enrolled in the Phase II trial.
2. The second aim was to conduct correlative analyses of gene expression in normal and tumor paired samples, and gene expression and clinical outcome.

METHODS

I. Materials

QiaShreddars, Allprep DNA/RNA kits, RNeasy Plus Mini Kits, and QIAquick PCR Purification Kit were purchased from Qiagen (Valencia, CA). Disposable mortars and pestles were purchased from Kontes. SYBR Green kits and GeneAmp Gold RNA PCR kits were obtained from Applied Biosystems (Foster City, CA). All primers were ordered from Integrated DNA Technologies (Coralville, IA).

II. Sequencing of UGT1A1 Region

DNA extraction

Normal tissue samples were used for UGT1A1 sequencing. Tissues were excised, immediately placed in liquid nitrogen, and stored at -70°C for further use. Less than 20 mg of tissue was placed in 350 µL Buffer RLT (Qiagen) and disrupted with disposable mortars and pestles (Kontes). Lysates were homogenized with Qiashredder spin columns (Qiagen) and purified with AllPrep DNA spin columns (Qiagen). DNA was eluted in 50 µL Buffer EB (Qiagen).

DNA sequencing

Approximately 400 ng of DNA from normal biopsy tissue was used as a template for amplifying a 255 bp region flanking the promoter region of the UGT1A1 gene. The forward primer was 5'-AAGTGAACTCCCTGCTACCTT-3'and the reverse primer was 5'-CCACTGGGATCAACAGTATCT -3. Reactions were performed in volumes of 50

μL with 1x buffer, 1.75mM $MgCl_2$, 0.8mM of each dNTP, 0.25 μM of each primer, and 0.05U/ μL AmpliTaq Gold (Applied Biosystems). PCR conditions were based on those of Monaghan et al. (21). Reactions began at 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 s, 58°C for 40 s, and 72°C for 40s. PCR products were electrophoresed on 1 percent agarose gels (Sigma) and bands were excised under UV light. Excised DNA bands were purified with the QIAquick Gel Extraction Kit and sequenced using the forward primer. Chromatograms were used to identify the number of TA repeats in the TATA box region of the promoter in comparison to the normal promoter sequence TATATATATATATAA (18).

III. Gene Expression in Samples

RNA extraction and quantitation

Both normal and tumor samples were collected and processed in a manner similar to the one described above. Tissues were excised, immediately placed in liquid nitrogen, and stored at -70°C for further use. Less than 20 mg of tissue was placed in 350 μL Buffer RLT (Qiagen) and disrupted with disposable mortars and pestles (Kontes). Lysates were homogenized with Qiashredder spin columns (Qiagen) and purified with AllPrep RNA/DNA spin columns (Qiagen). Flowthrough was collected, combined with an equal volume of 70% ethanol, and purified with RNeasy spin columns (Qiagen). RNA was eluted in 30 μL RNase-free water and quantitated using the ND-1000 (Nanodrop).

Reverse transcription and real-time PCR

Gene expression was evaluated using a two-step RT-PCR. Reverse transcription was performed with the GeneAmp Gold RNA PCR kit (Applied Biosystems) "Protocol for Two-Step RNA PCR". Reverse Transcriptions were set up in 50 µL reactions containing 1µg of total RNA, 0.75U/µL MultiScribe reverse transcriptase, 0.5U/ µL RNase inhibitor, 1.25 µM oligodeoxythymidylic acid primer, 250 µM of each dNTP, and 2.5mM $MgCl_2$. Reactions were performed on the GeneAmp PCR System 2700 (Applied Biosystems).

RT conditions were 25°C for 10 minutes, 42°C for 60 minutes, 68°C for 10 minutes, 95°C for 5 minutes, and 40°C hold. Success of all reverse transcription reactions was verified by amplifying a portion of the β-Actin gene, using the GeneAmp Gold RNA PCR kit (Applied Biosystems) and primers for *β-Actin*. The forward primer was 5'-GAAGATCAAGATCATTGCTCCTCC-3'and the reverse primer was 5'-TTTTCTGCGCAAGTTAGGTTTTGTG -3'. PCR parameters were 95°C for 10 minutes; followed by 35 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute; 72°C for 5 minutes, and holding at 40°C. PCR products were electrophoresed on 1% agarose gels (Sigma).

For real-time PCR, cDNA equivalent to 20 ng of RNA was added to each 25 µl PCR reaction. RT was performed using the same conditions described above. This created the cDNA that was subsequently used for the quantitative real-time PCR assays. PCR was performed on the Eppendorf Realplex instrument (Eppendorf) and standard curves were evaluated using the program's software. Standards for each gene were created from

recombinant vectors constructed in our laboratory and were diluted to concentrations of 3 copies/ml, 30 copies/mL, 300 copies/mL, 3000 copies/mL, 3E4 copies/mL, 3E5 copies/mL, and 3E6 copies/mL. Standards and samples were tested in triplicate. The concentration of primers and the reaction conditions were established to ascertain minimal primer dimer and/or non specific product formation and hence increase the specificity of the real-time PCR.

Gene	Primers	Conc. primer (μM)	Melting time at 95°C (sec)	Annealing time at 65°C (sec)	Extention time at 72°C (sec)
CES2	F 5'-CCATGGTGATGAGCTTCCTTTTGT-3' R 5'-AGGTATTGCTCCTCCTGGTCGAA-3'	0.5	30	30	60
CES1	F 5'- AGAGGAGCTCTTGGAGACGACAT-3' R 5'- ACTCCTGCTTGTTAATTCCGACC-3'	0.2	30	30	60
TOPO I	F 5'-CGTTCTACCAGGCAAATTCACTGT-3' R 5'-TGAAATGGGAGAGAGGGAAGGGA-3'	0.3	20	15	40
β-GUS	F 5'-TCAACAAGCATGAGGATGCGGAC-3' R 5'-TACGCACCACTTCTTCCATCACC-3'	0.3	30	30	60
TP	F 5'-AATGTCATCCAGAGCCCAGAGCA-3' R 5-GAACTTAACGTCCACCACCAGAG-3'	0.5	30	30	60
TS	F 5'-TTTACCTGAATCACATCGAGCCAC-3' R 5'-GACTGACAATATCCTTCAAGCTCC-3'	0.5	30	30	20
DPD	F 5'-GGTCTTCAGTTTCTCCATAGTGGT-3' R 5'-GACTCTGTCCATCCCAGTCTTGT-3'	0.5	20	20	45

Table 3. Forward (F) and Reverse (R) Primers for Real-Time PCR: Reaction parameters were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles PCR with the temperature and times listed in the table.

IV. Patient Data

There were 22 patients enrolled in the study. Ages ranged from 36-67, with the median age being 54 years. Samples were obtained for 21 patients. Of these patients, high-quality RNA was obtained from 18 samples.

V. Correlative Analysis

Correlative analysis was done using graphpad prizm. Data was analyzed using Wilcoxin matched paired t-test for comparing normal and tumor paired samples. The entire group of patients was analyzed as a single group. In addition, post-treatment surgical samples were obtained from some patients and the gene expression levels were compared before and after treatment. Unpaired t-test analysis of samples was also done, based on patient response.

RESULTS

I. Analysis of Isolated DNA

We found that most of the DNA samples had an A260/A280 ratio of around 1.8. Table 4 lists DNA data for normal and tumor specimens. Figure 6 provides an example of a typical DNA analysis by spectrophotometer.

	NORMAL DNA		TUMOR DNA	
Sample	Total Amount (µg)	A260/A280	Total Amount (µg)	A260/A280
GI53-001	181.10	1.87	641.11	1.84
GI53-002	164.86	1.89	154.89	1.92
GI53-003	517.10	1.92	475.20	1.88
GI53-007	97.07	1.86	518.55	1.78
LO53-502	396.7	1.82	100.83	1.93
LO53-501	382.78	1.85	298.31	1.83
LO53-504	353.14	1.86	217.47	1.90
LO53-503	470.96	1.84	247.14	1.83
LO53-500	446.49	1.81	446.49	1.85
LO53-505	284.5	1.86	285.18	1.84
LO53-506	67.33	1.83	386.11	1.81
LO53-511	96.85	1.82	665.55	1.90
LO53-510	554.90	1.82	1075.73	1.86
LO53-523	309.62	1.86	263.30	1.86
LO53-515	615.11	1.90	98.40	1.86
LO53-525	278.80	1.87	156.76	1.85
LO53-529	391.62	1.92	391.62	1.92
LO53-528	622.38	1.92	620.98	2.11
LO53-531	268.58	1.89	125.28	1.90

Table 4. Summary of Isolated DNA Data from Normal and Tumor Samples

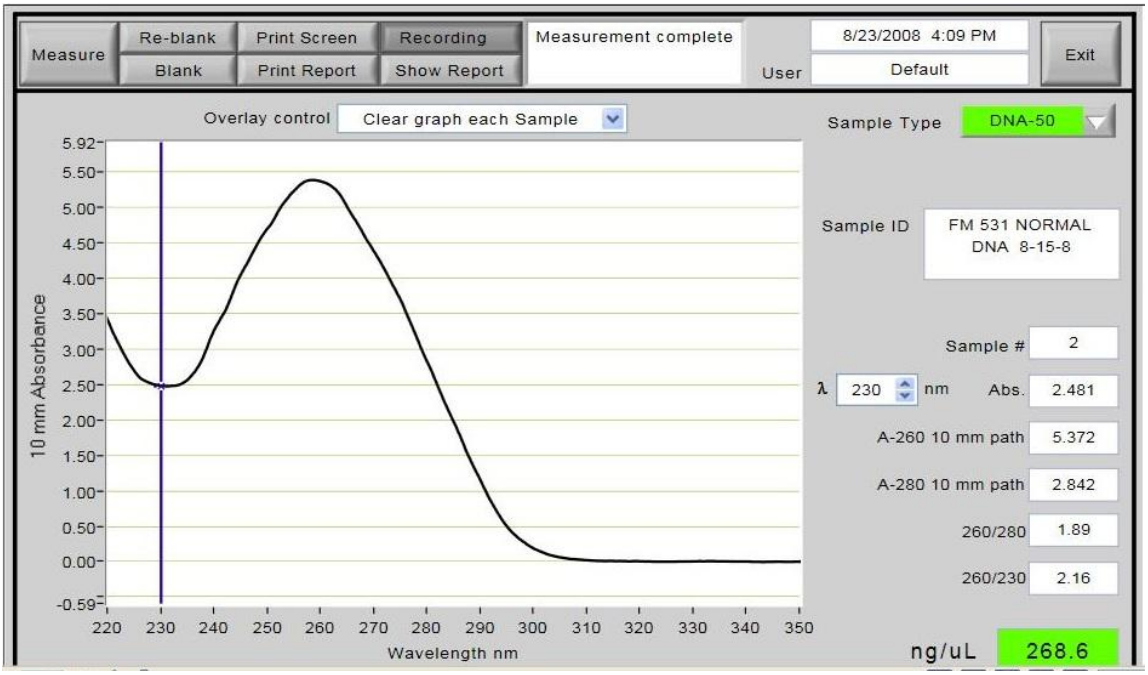

Figure 6. Example of Typical DNA Spectrophotometric Analysis

II. Analysis of Isolated RNA

We found that most of the RNA samples had an A260/A280 ratio of around 2.0. Table 6 lists RNA data for normal and tumor specimens. Figure 7 provides an example of a typical RNA analysis by spectrophotometer, while Figure 8 depicts typical data obtained from degraded RNA.

	NORMAL RNA		TUMOR RNA	
Sample	Total Amount (µg)	A260/A280	Total Amount (µg)	A260/A280
GI53-001	73.01	2.02	1133.79	2.00
GI53-002	174.40	2.08	197.82	2.07
GI53-003	219.42	2.01	335.01	1.99
GI53-007	235.42	1.86	627.03	2.02
LO53-502	484.88	2.18	595.94	2.13
LO53-501	111.88	2.03	294.25	2.10
LO53-504	201.45	2.04	563.86	2.16
LO53-503	256.84	2.06	351.59	1.96
LO53-500	117.01	2.02	131.05	2.06
LO53-505	284.51	2.01	267.66	2.04
LO53-506	178.66	2.06	42.56	2.02
LO53-511	521.15	2.09	1010.61	2.08
LO53- 510	674.49	2.09	875.31	2.08
LO53- 523	362.28	2.07	224.42	2.09
LO53- 515	173.90	2.06	266.43	2.07
LO53- 525	156.00	2.09	115.64	2.07
LO53-529	380.71	2.05	712.5	2.11
LO53- 528	477.89	2.11	854.25	2.09
LO53- 531	174.44	2.08	287.07	1.89

Table 5. Summary of Isolated RNA Data from Normal and Tumor Samples

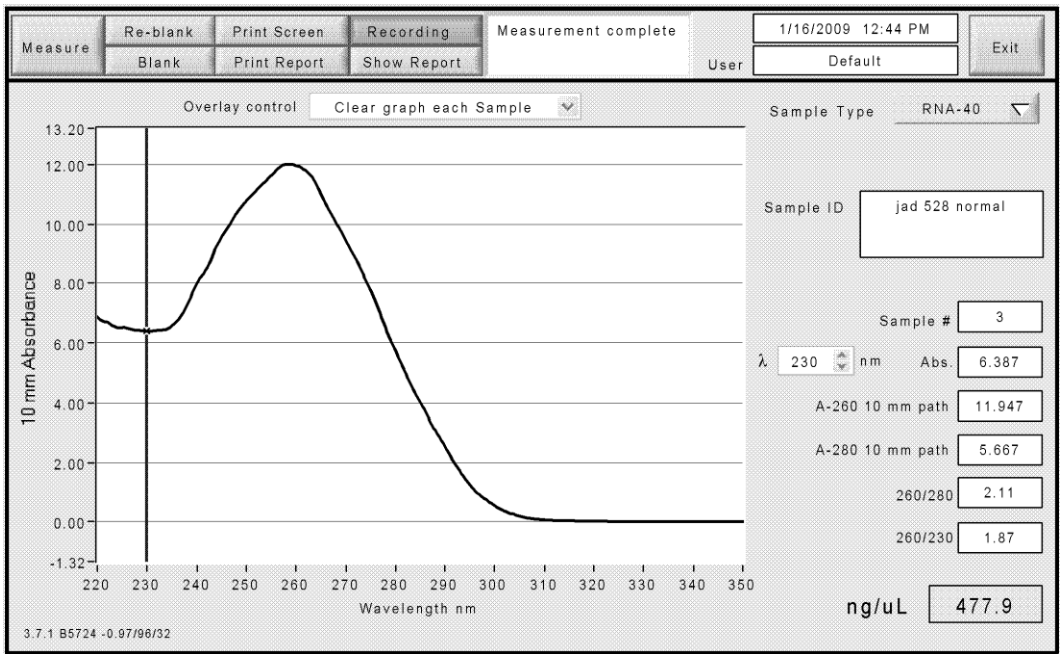

Figure 7. Example of Typical RNA Spectrophotometric Analysis

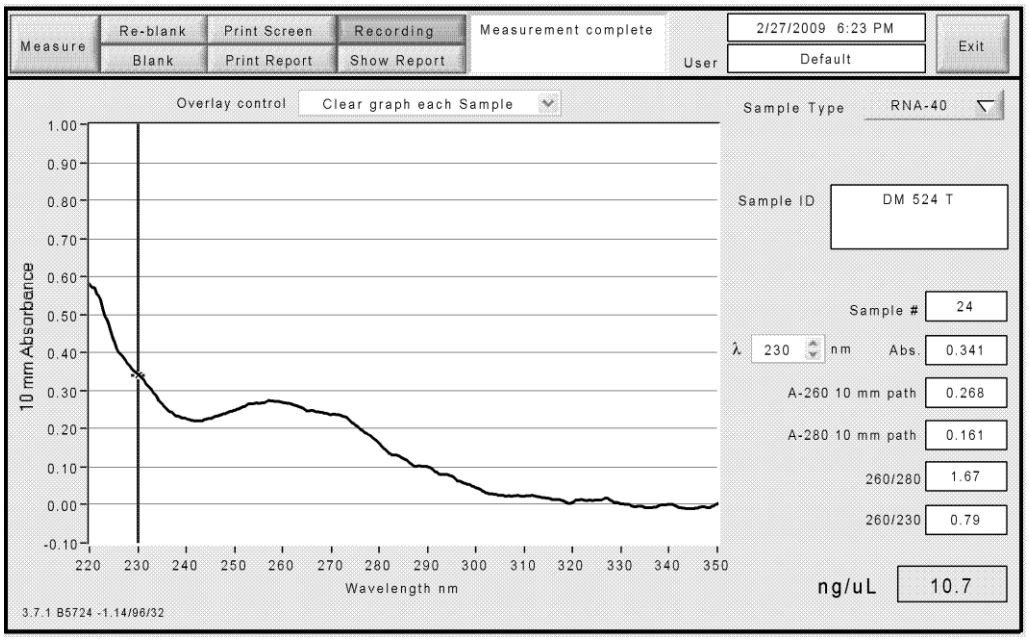

Figure 8. Example of Degraded RNA Spectrophotometric Analysis

III. Real-Time PCR

Overall gene expression was compared between the 18 paired tumor and normal samples. All Real-time PCR reactions employed the use of laboratory-generated standard curves. (Figure 9) Data is summarized in tables 6 and 7. Data for baseline standard curves are provided in table 8.

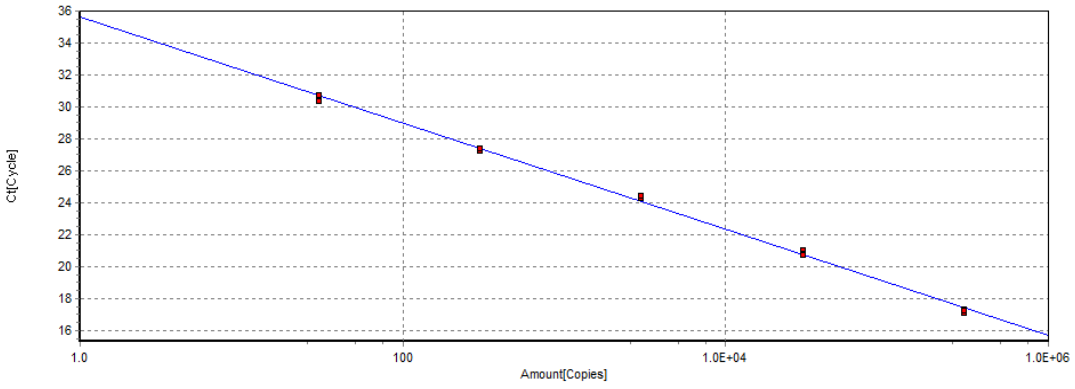

Figure 9. Real-Time PCR Standard Curve: The example shown here is for CES2. The r^2 value is 0.998. The x-axis represents cycle number and the Y-axis represents copy number.

GENE COPY NUMBER							
Sample Name	CES2 Normal	CES1 Normal	TP Normal	TS normal	β-GUS Normal	DPD Normal	TOPO I Normal
GI53-001	15287	144	1319	3207	1763	23.6	1983
GI53-002	32059	38.9	1315	2380	1633	4.15	2728
GI53-003	27488	226	7606	3255	1500	26.4	1848
GI53-007	28636	94.4	2518	795	1848	25.6	1453
LO53-502	12599	19.3	382	441	549	8.95	950
LO53-501	16871	89.7	386	1238	563	5.47	912
LO53-504	21292	56.2	1447	867	1498	11.4	1139
LO53-503	7795	11.6	478	689	353	2.59	774
LO53-500	10695	61.3	348	400	530	6.09	673
LO53-505	11017	43.8	88.6	476	603	13.8	583
LO53-506	5729	9.94	86.2	55.3	231	3.39	196
LO53-511	26506	11	1028	117	771	14.9	2694
LO53-510	20676	43.2	2323	302	1263	55.5	5158
LO53-523	14702	87.5	254	283	3485	16.4	2443
LO53-515	43934	30.4	698	1654	4346	57.5	4879
LO53-525	34759	185	1227	195	1119	51.6	4002
LO53-529	32044	34.7	429	901	939	38.2	2887
LO53-531	21888	28.5	1581	154	793	26.7	2486

Table 6: Gene Expression in Baseline Normal Samples

GENE COPY NUMBER							
Sample Name	CES2 Tumor	CES1 Tumor	TP Tumor	TS Tumor	β-GUS Tumor	DPD Tumor	TOPO I Tumor
GI53-001	3996	29.3	1807	15991	3611	16.3	6029
GI53-002	8403	15.3	550	733	525	42.9	634
GI53-003	2175	202	1256	3513	506	13.6	1013
GI53-007	14872	33	1205	829	930	94.6	1381
LO53-502	16531	69.7	2309	860	1615	138	1463
LO53-501	1853	142	6659	1676	516	118	1026
LO53-504	8151	631	5445	3364	2912	205	4624
LO53-503	15804	584	2009	1204	1058	133	3773
LO53-500	13778	116	3242	1171	970	133	4938
LO53-505	5329	3359	1848	3280	719	42	1416
LO53-506	4927	55.1	11838	2775	1498	635	1696
LO53-511	14049	204	2857	319	1050	0	7538
LO53-510	17171	345	2651	1657	1099	0	5723
LO53-523	2118	7.89	579	240	318	3.02	1114
LO53-515	8625	93.7	2684	1919	4467	193	5268
LO53-525	39743	352	427	215	2081	30	2019
LO53-529	16783	53.7	3156	23448	2068	53.6	11726
LO53-531	26410	19	427	80	4356	51.6	1421

Table 7: Gene Expression in Baseline Tumor Samples

GENE	SLOPE	INTERCEPT	R^2	
CES1	-3.406	36.74	0.997	Baseline
TP	-3.295	35.42	0.999	
TS	-3.337	37.69	0.996	
CES2	-3.563	37.56	0.995	
TOPO I	-3.626	40.78	0.995	

Table 8. Standard Curve Equations for Real-Time PCR Assays

IV. Clinical Trial Outcome Data

Of the 18 patients who completed all therapy, 10 had complete response (CR), 0 had partial response (PR), 7 had stable disease (SD), and 1 had progressive disease (PD).

Best Response	n	%
CR	10	45.50%
PR	0	0.00%
SD	7	31.80%
PD	1	4.50%

Table 9. Summary of Clinical Trial Data

V. Sequencing of UGT1A1 Region

Normal tissue samples were used for UGT1A1 sequencing. Chromatograms demonstrating two sequences in this region were deemed heterozygous. Eight patients were wild-type homozygous for $(TA)_6TAA/(TA)_6TAA$ (6/6). Nine patients were heterozygous for $(TA)_6TAA/(TA)_7TAA$ (6/7). Three patients were homozygous for $(TA)_7TAA/(TA)_7TAA$ (7/7). And one patient was heterozygous for $(TA)_5TAA/(TA)_6TAA$ (5/6). (See Table 4)

Polymorphism	Number of Patients
6/6	8
6/7	9
7/7	3
5/6	1

Table 10. UGT1A1*28 Polymorphism Status in Patients

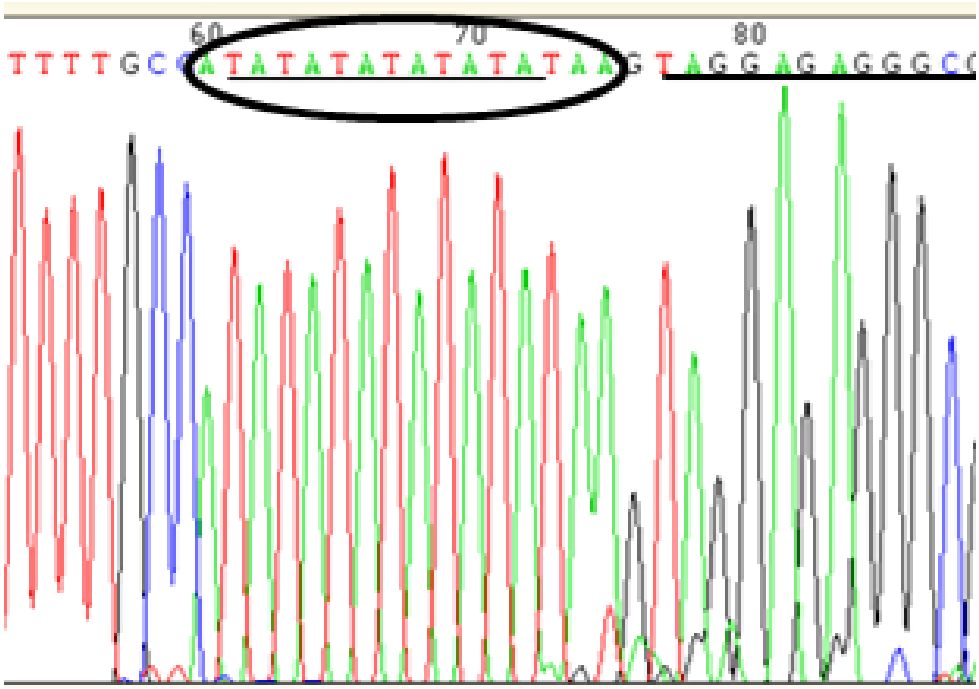

Figure 10. Wild-Type UGT1A1 $(TA)_6TAA/(TA)_6TAA$ (6/6) Chromatogram

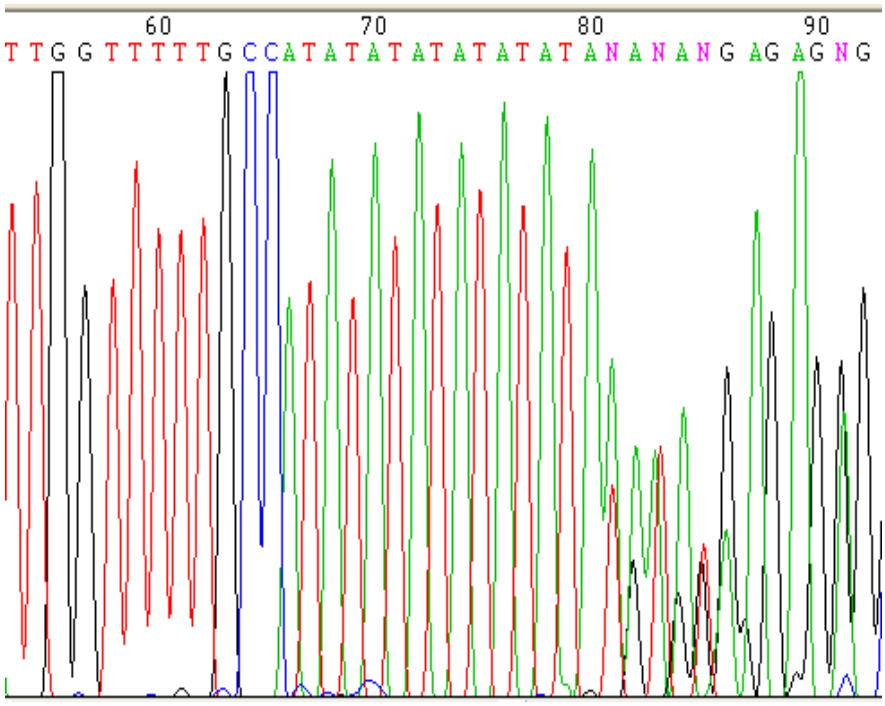

Figure 11. Heterozygous $(TA)_6TAA/(TA)_7TAA$ (6/7) Chromatogram

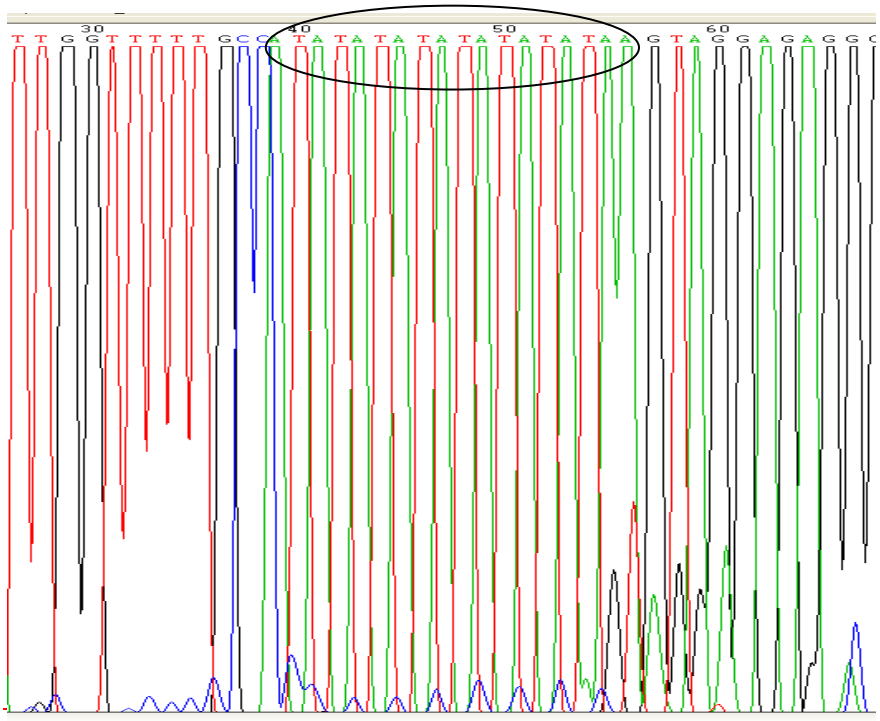

Figure 12. Homozygous $(TA)_7TAA/(TA)_7TAA$ (7/7) Chromatogram

VI. Gene Expression Analysis

Expression levels in paired CR Tumor and Normal, SD Normal and Tumor, Tumor CR and SD, and Normal CR and SD samples were analyzed, using two-tailed t-tests. As seen in Figure 13a, a paired t-test analysis of paired samples showed that the expression of CES2 was significantly higher in normal samples in comparison to tumor samples (p=0.0051). Further analysis of patient groups based on response showed the same trend in both the complete response group and patients with stable disease, but the p-values were higher. (Figures 13b and 13c) Unpaired t-test analysis of CES2 expression in tumor and normal samples based on patient response is shown in Figures 13d and 13e. CES2 expression was found to be higher in normal sample in comparison to tumor samples, but there was no difference in expression of CES2 based on patient response. There was significant increase in TP expression in tumor samples, as compared to normal samples. Similar analysis was done for all the genes. Table 7 summarizes the t-test results.

	p Value				
	CR Normal Vs Tumor, n=6	SD Normal Vs Tumor, n=9	Tumor CR Vs SD	Normal CR Vs SD	Tumor Vs Normal, n=18
CES1	0.257	0.3503	0.208	0.262	0.143
CES2	0.0068	0.2444	0.0755	0.2937	0.0049
TP	0.0528	0.6723	0.0299	0.1906	0.096
TS	0.0568	0.3408	0.7187	0.1866	0.082
TOPO I	0.1481	0.2323	0.7488	0.3412	0.0542
β-GUS	0.9742	0.2571	0.3954	0.8799	0.297
DPD	0.1811	0.0361	0.2697	0.2241	0.0338

Table 11. Summary of p Values in Paired Samples

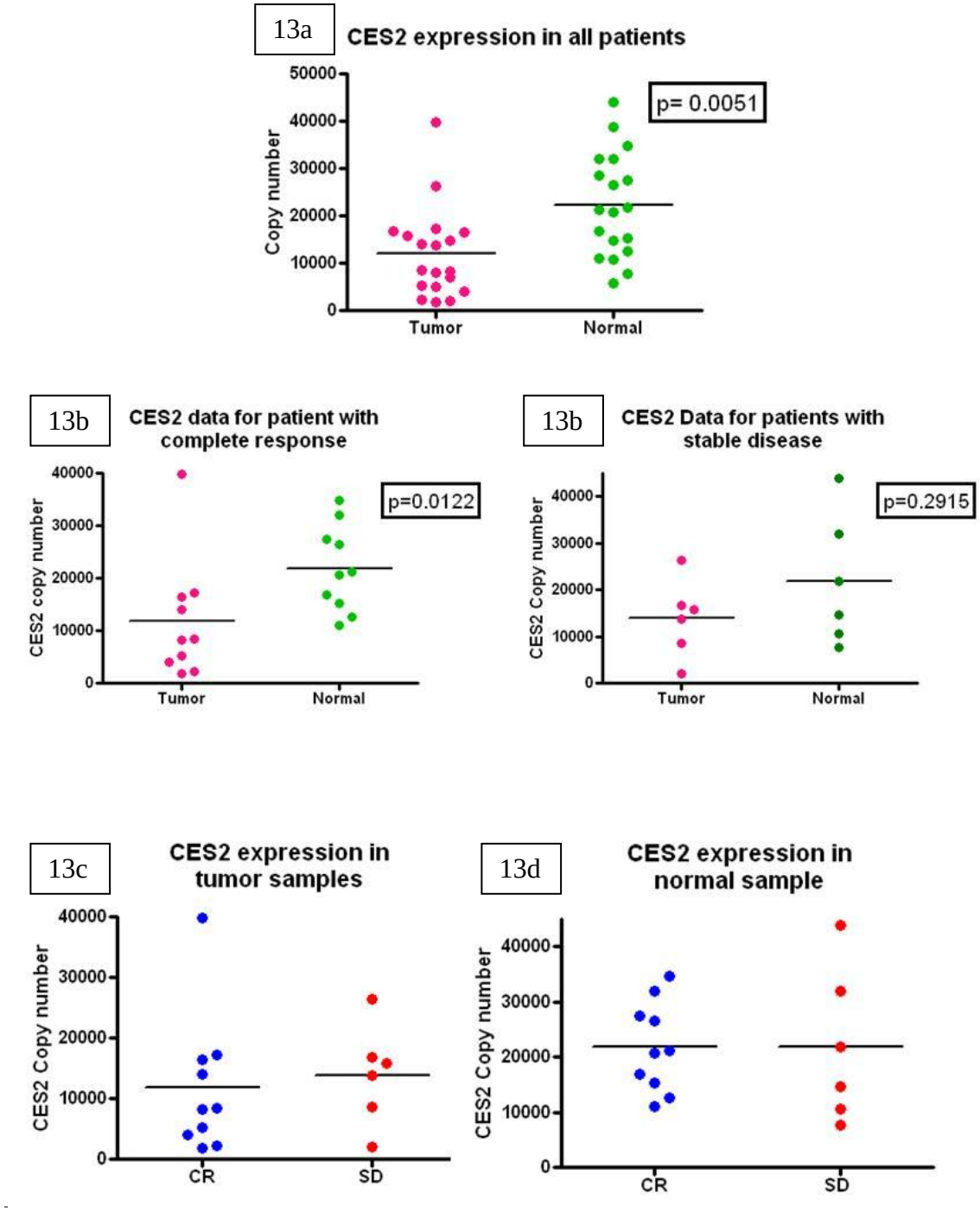

Figure 13. Comparisons of CES2 Expression

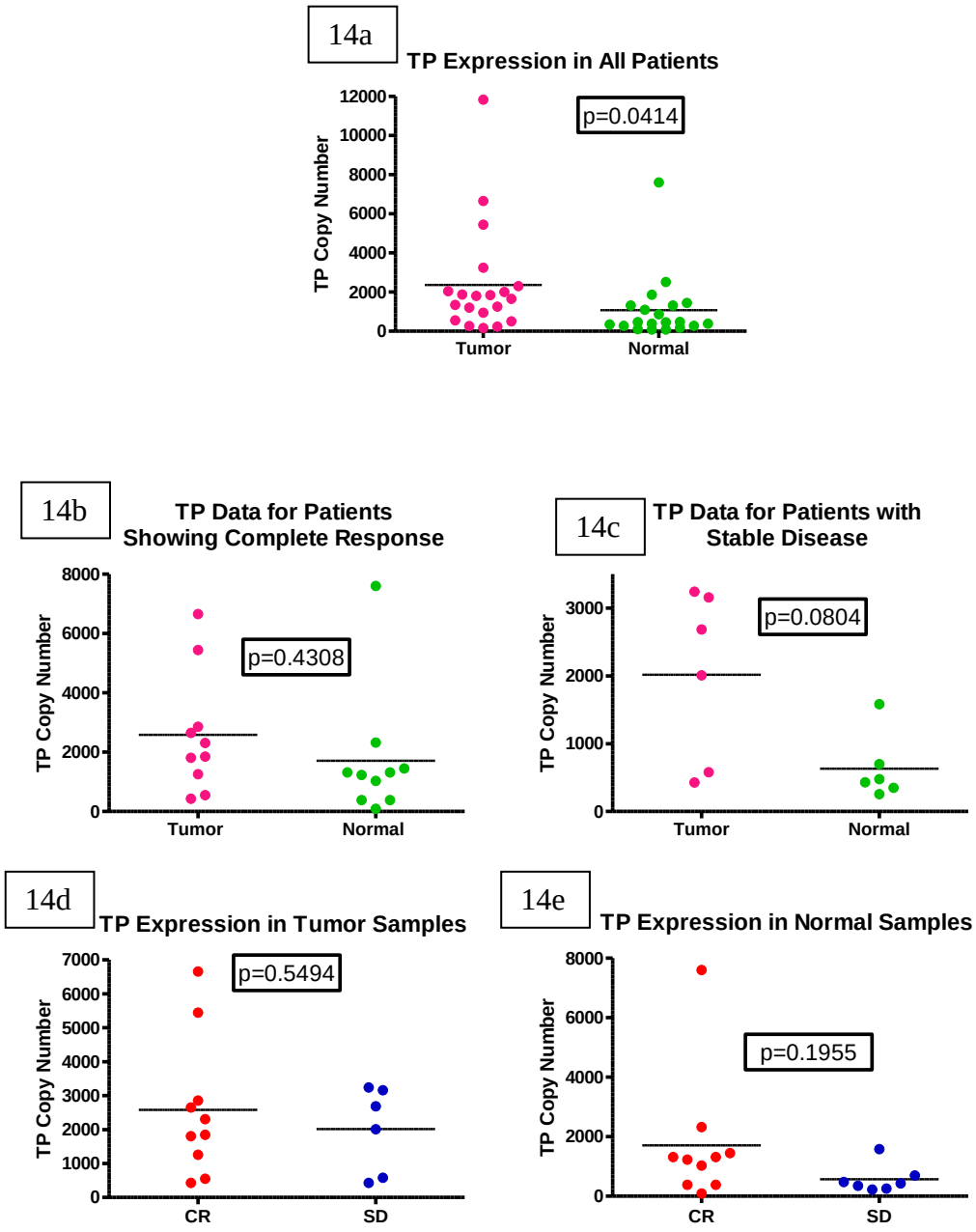

Figure 14. Comparisons of TP Expression

DISCUSSION

I. Gene Expression in Paired Normal and Tumor Samples

About 60% of intravenously administered irinotecan is excreted via feces, of which 32% is in the unchanged form (22). Therefore, there is a great potential for activation of irinotecan in the GI tract by local carboxylesterases and this may be responsible for the life threatening slow onset diarrhea in some patients. Conversely, presence of high levels of carboxylesterases in the tumor tissue could result in localized activation in the tumor and may be associated with better clinical response. The two most abundant carboxylesterases in humans are CES1 and CES2. CES2 the key carboxylesterase enzyme expressed in the GI tract. In this study, gene expression was compared for 19 paired normal and tumor samples. Contrary to our hypothesis, it was found that CES2 expression was higher in normal samples than in tumor samples. We essentially found CES2 expression to be higher in normal samples than in tumor samples, regardless of clinical response. (Figures 13b and 13d). Earlier in the clinical trial it was determined that the administration of loperamide prior to and during chemotherapy significantly reduced the GI toxicity. Therefore all patients received loperamide. In a previous study, our laboratory determined that loperamide was a very good inhibitor of CES2 (IC50 =0.38 μM) (23). Since CES2 activity is inhibited by the presence of loperamide, we were unable to evaluate the correlation between CES2 and GI toxicity.

CES1 expression is reported to be low in GI tissues (24) in comparison to CES2. In accordance with this, we find very low expression of CES1 in normal and most of tumor tissue samples with one exception (Tables 6 and 7).

For TP, we found that expression was higher in tumor samples than normal samples, for all patients. High basal level expression of thymidine phosphorylase gene was associated with nonresponse to 5-fluorouracil treatment in colorectal tumors (25). However, capecitabine which is the prodrug of 5-FU requires TP for activation and uses this fact to achieve higher 5-FU levels specifically in tumors (27). Miwa et al. studied clinical activity and toxicity of capecitabine plus irinotecan as first-line therapy for patients with metastatic colorectal cancer (mCRC). They reported a significantly higher time to disease progression and overall survival in patients with higher expression of TP as assessed by immunohistochemistry. In the same study, association of the real-time PCR data for TP did not show as strong an association with clinical outcome (27). Here, we found TP expression to be significantly higher in tumor samples than in normal samples. This is consistent with several other studies (26-30).

TOPO I is necessary for controlling the replication of DNA and the synthesis of proteins. It is inhibited by irinotecan, topotecan and camptothecin. It has been reported in the literature that higher levels of TOPO I expression leads to a better clinical response to irinotecan (31). It also has been reported that expression is higher in tumor tissue than in normal tissue. This coincides with our findings, which demonstrated higher TOPO I expression levels in tumor samples than in normal samples, irrespective of the clinical outcome (31, 32).

TS is responsible for the synthesis of thymidine monophosphate (dTMP), which eventually is metabolized into thymidine triphosphate (dTTP) (35). dTTP is essential for DNA synthesis and repair. TS is the target enzyme of 5-FU, as TS inhibition leads to the accumulation of deoxy-uridine-monophosphate (dUMP) and depletion of deoxy-thymidine-monophosphate (dTMP) (36). This results in an arrest of DNA synthesis, as well as increased toxicity. Higher TS expression has been reported to be associated with poor response to 5-FU-leucovorin treatment. Consistent with other studies, we found TS expression to be higher in tumor samples than in normal samples (35, 36).

The relative contributions of carboxylesterases and beta-glucuronidase in the formation of SN-38 in human colorectal tumors were studied in vitro and it was found that both enzymes contributed equally to the formation of SN-38. β-GUS is expressed in the GI tract and is an enteric bacterial enzyme which converts SN-38G back to the active metabolite, SN-38 (37). Therefore, increased β-GUS activity can result in higher SN-38G levels in the gut and, hence, the GI toxicities associated with irinotecan. In our study, there was no correlation between normal and tumor samples.

DPD is responsible for the degradation of the cytotoxic 5-FU. It has been reported in the literature that high DPD expression is an indicator of poor clinical response (38). We evaluated normal and tumor samples to determine if there was a difference in their expression levels and found there to be greater expression in tumor samples than in normal samples.

II. Gene Expression in Baseline and Surgical Samples

We evaluated the genes CES1, CES2, TP, TS, and TOPO I in 10 tumor and 11 normal samples. Topo I expression was downregulated in tumor samples, but upregulated in surgical normal samples, when compared to baseline samples. CES2 and TP were both upregulated in surgical normal samples, but not in tumor samples. This is consistent with another study, in which it was reported that TP expression increased for up to four weeks post radiation in rectal cancer patients (39). CES1 was upregulated in both tumor and normal surgical samples and expression in surgical samples was significantly upregulated, overall.

III. Correlation of Gene Expression and Therapeutic Response

Seven genes were analyzed for correlation between expression and clinical outcome. The most significant correlations involved CES2 and DPD expression. When comparing tumor samples to normal samples in patients with CR and SD, we discovered higher CES2 expression in the normal samples. We also found CES2 expression in tumor samples to be higher for patients with SD than those with CR. We found CES2 expression to be higher in normal tissue samples than in paired tumor samples, and also CES2 expression in tumor samples to be higher for patients with SD than those with CR. DPD expression was found to be higher in tumor samples than in normal samples. This correlation was particularly significant among patients with SD and in overall expression. Additionally, there were correlations for TS, TP, and CES1. All demonstrated higher levels of expression in tumor samples than in normal samples. When evaluating for correlations between expression and clinical outcome, we found patients demonstrating

CR to have significantly higher levels of TS expression in tumor tissue than in normal tissue. We also found there to be significant correlation between TP expression and clinical outcome. For patients with CR, TP expression was higher in tumor samples than in normal samples. Additionally, TP expression in tumor samples was found to be higher for patients exhibiting CR than those with SD. This is consistent with several other studies (26-30).

IV. Hypothesis

Our hypothesis was that CES2 expression would be higher in paired tumor samples than in the corresponding normal samples, that higher CES2 expression in tumor tissue would result in better patient response, and that higher CES2 expression in normal tissue would result in toxicity, such as diarrhea. This was not observed. CES2 expression was higher in paired normal samples than in tumor samples. No conclusion could be drawn regarding toxicity, due to pretreatment administration of Loperamide. Loperamide is a strong inhibitor of carboxylesterases and makes irinotecan unavailable for metabolism in the gut, preventing toxicity (23).

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Education

B.S. Cytotechnology. Indiana University, 1999

Graduate Certificate Health Systems Management. Indiana University, 2005

Graduate Certificate Molecular Laboratory Diagnostics. Michigan State University, 2005

M.S. Biotechnology. Indiana University, 2010

Professional Experience

Molecular Technologist/Cytotechnologist, MACL	2006-2010
Molecular Technologist, Diagnostic Cytology Laboratory	2005-2006
Cytotechnologist, Diagnostic Cytology Laboratory	2001-2005
Cytotechnologist, Borgess Medical Center	1999-2001
Lab Assistant, Parkview Hospital	1997-1998
Tutor, Purdue University (Fort Wayne)	1997-1998
Phlebotomist, Parkview Hospital	1995-1997