Varicance in the Expression of 5-Fluorouracil Pathway Genes in Colorectal Cancer

Elizabeth A. Kidd, Jinsheung Yu, Xi Li, William D. Shannon, Mark A. Watson, and Howard L. McLeod

Abstract

Although colorectal cancer has the third highest cancer mortality rate, the treatment remains far from optimized with patients showing variable responses to standard treatment. Molecular differences in pharmacologically relevant genes may contribute to the variability in response. This study used Taqman PCR to investigate the expression of 24 5-fluorouracil (5-FU) pathway genes in colorectal cancer using paired nontumor and tumor samples from 52 patients with Dukes’ C colon cancer. In comparing tumor versus nonmalignant tissue, 14 of the 24 genes showed significant variation in gene expression. For 11 of these same genes (FPGS, DHFR, GGH, NME1, NME2, RRM2, UMPH2, UNG, UMP5, TP53, and TK1), a significant proportion of the patients showed an over expression of the particular gene in tumor tissue with a tumor-to-nonmalignant (T/N) ratio >1.2, whereas one gene (DPYD) showed the converse with a large number of patients showing a lower expression in the tumor tissue (T/N < 0.8). Multiple gene correlations for the genes of the 5-FU pathway were found with the Spearman rank correlation of >0.6 (all P < 0.001), suggesting possible coregulation mechanisms. Hierarchical clustering analysis created at least three groups of genes, which were consistent with groupings by the other statistical methods. Additionally, the hierarchical clustering showed two distinct groups of patients based on their gene expression. These variations in gene expression could provide valuable insights for optimizing treatment selection for patients with colorectal cancer.

Despite medical advances in diagnosis and treatment, colorectal cancer is the third leading cause of cancer death for both men and women in the United States (1). Studies from the past decade showed adjuvant chemotherapy with 5-fluorouracil (5-FU) results in a significant reduction in mortality (relative reduction, ~20%) and absolute reduction in mortality, (~10%) for patients with Dukes’ C colorectal cancer (2–8). An improvement in survival is also observed after systemic chemotherapy for metastatic colorectal cancer (9, 10). Unfortunately, patients respond very differently to the treatment. For some patients, the standard dose is too toxic, whereas other patients have minimal side effects. There is also substantial variability in tumor response or survival after chemotherapy. The mechanistic basis for the different reaction and/or unsuccessful treatment is rarely known. An understanding of the molecular differences between tumor gene expression will help explain some of the variation in patient response (11–13). Although many different chemotherapeutic regimens are used to treat cancer, frequently, the biological mechanism of how the drug works is poorly understood. Defining distinct gene expression patterns associated with chemotherapy may provide a basis for treatment optimization (14).

One of the major chemotherapy drugs used against colorectal cancer is 5-FU. Although 5-FU, an analogue of the pyrimidine uracil, represents a rationally designed chemotherapy agent based on the molecular biology of tumors, many aspects of its functioning are not fully characterized (15). With the knowledge that tumors preferentially take up uracil, 5-FU was synthesized as a means of interfering with nucleic acid synthesis and thus slowing tumor growth. To achieve its pharmacologic effects, 5-FU must be activated. As 5-FU mimics uracil, being catabolized and anabolized by the same biological pathway as naturally occurring pyrimidines, the significance of particular genes or target enzymes can be postulated (15). Thus, some specific targets of 5-FU are known, such as inhibition of thymidylate synthase activity, and incorporation into RNA (FUMP may substitute for UTP), or DNA (FdUTP may substitute for dTTP). At the same time, it is unclear how much each of these events contributes to the toxicity of 5-FU and whether additional aspects of 5-FU and its metabolism potentiate other antitumor activity. Many of the 5-FU pathway genes are known but have not previously been investigated in human tissues (Fig. 1). This project aimed to characterize the patterns of gene expression of 5-FU pathway genes in colon cancer and thereby gain a better understanding of potential opportunities for optimizing therapy selection.
To investigate the variance in gene expression, the expression of the 24 genes of the 5-FU pathway was examined in 104 samples (52 tumors matched with adjacent nonmalignant tissue) from patients with Dukes’ C colorectal cancer using real-time PCR. Bioinformatic analysis was then conducted using statistical strategies and hierarchical clustering investigation. The distinct gene expression pattern exhibited in colon tumors can now be used to design clinical trials to evaluate predictive markers of 5-FU sensitivity.

**Materials and Methods**

**Tissue samples.** The tumor tissue and paired nonmalignant colon tissue sample were collected by the Siteman Cancer Center Tissue Procurement Core from 52 consecutive patients with Dukes’ C colorectal cancer, under protocols approved by the Washington University Human Subjects Committee. Following surgery, the tissue samples were snap frozen in liquid nitrogen and stored at −80°C. All frozen tissue specimens were sectioned and reviewed by H&E staining to ensure adequate cellular representation (i.e., absence of tumor cells in nonmalignant tissue and high tumor cellularity (median, 86.3%; 65-95%). Serial 50-μm sections were then used for RNA preparation. See Table 1 for patient and tumor characteristics. Some of the patients (30 of 52) received 5-FU-based adjuvant therapy. However, the paucity of recurrences to date (12 patients) make it inappropriate to conduct clinical outcome analysis.

**Preparation of complementary DNA.** Using a Trizol RNA isolation kit (Invitrogen Co., Carlsbad, CA), the total RNA was isolated from the normal and tumor tissue samples by the Siteman Cancer Center Tissue Procurement Core. The integrity, concentration, and purity of the RNA were verified using an Agilent Bioanalyzer 2100 Nano RNA assay and UV spectroscopy. All samples were adjusted to a final concentration of 10 ng/μL. This RNA was reverse-transcribed into cDNA.

**Real-time quantitative PCR.** The primers and Taqman probes for the 24 genes were all designed using Primer Express Software (Applied Biosystems, Foster City, CA). Primer and probe sequences are shown in Table 2. Additionally, the primers and probes were screened for secondary structure and homology to other genes. Probes were labeled with 5′ FAM reporter and 3′ TAMRA quencher. Initially, probes were tested in standard PCR to ensure they amplified a DNA fragment of appropriate size. For real-time PCR, Taqman Universal PCR Master Mix was used, and two to three replicates for each reaction were plated into 384-well plates. The PCR program was 40 cycles of 95°C for 20 seconds and 60°C for 1 minute in the ABI Prism 7700 (Applied Biosystems).

**Relative expression of mRNA.** Relative expression of the 24 genes was calculated using the comparative CT method (16). To standardize all the samples, amyloid precursor protein (APP) was used as an endogenous reference gene, as APP expression does not vary in tumor versus nontumor samples. To compare expression of the different genes an equal molar mixture of all 104 samples was used as a calibrator sample. The relative expression calculation (2−ΔΔCT) takes into account the difference between the expression of the target gene and reference gene APP and between the target gene and calibrator sample.

**Statistical analysis.** STATISTICA (StatSoft, Tulsa, OK) was used to perform statistical analysis. The Wilcoxon matched pairs test was used to evaluate the significance between the gene expression of tumor and nonmalignant tissue samples. Additionally, patients were grouped by their tumor to nonmalignant ratio for each gene: T/N ratio <0.8 (nonmalignant tissue shows higher expression of gene), 0.8 to 1.2, >1.2 (tumor sample shows higher expression of gene). These cut points were selected to be greater than thrice the coefficient of variation of the assay. To evaluate the significance of the different proportions of patients in the three T/N categories a χ2 test was done (P < 0.05, after Bonferroni correction for multiple comparisons). Gene correlations for the 5-FU pathway genes were evaluated using the Spearman rank correlation, with a value >0.6 deemed to be a biologically significant correlation. Additionally, unsupervised hierarchical clustering was used to examine gene expression relationships and patient groupings.
Table 2. Gene symbol, common name, and forward, reverse, and probe sequences

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<th>Gene symbol</th>
<th>Common name</th>
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| **DHFR**    | Dihydrofolate reductase | Forward: CCTAAATATGGTAACTACTGGGACAA  
Reverse: GGATGAGTGATGAGGAGGATTAGCTCTTT  
Probe: CACCATCAATCTCAGCTTCCATTCACCATGAA |
| **DPYD**    | Dihydropyrimidine dehydrogenase | Forward: ATCTTGAATCGTTGGATCTAGATCGA  
Reverse: GAGTAGTAGCTAGTCGCTACTGAT  
Probe: ACCGTACTCATGAGTCTGACATGAA |
| **DPYS**    | Dihydropyrimidinase | Forward: GCTGCCATTCCCTTCACA  
Reverse: TAAAGGATCCTAGGGAGTAAGGTGAAGCA  
Probe: CACCATCACAATCTTCACTTTTCCTCCCAT |
| **DUT**     | DUTP pyrophosphatase | Forward: CCTCTGCTGTTTAGGGAAGGAA  
Reverse: GGATATGACAGATGAGGATTAGCTCTT  
Probe: CACCATCACAATCTTCACTTTTCCTCCCAT |
| **ECGF1**   | Endothelial cell growth factor 1 (platelet-derived)/thymidine phosphorylase | Forward: GGTTCCTGCGGACGGAAT  
Reverse: GAGTAGTAGCTAGTCGCTACTGAT  
Probe: ACCGTACTCATGAGTCTGACATGAA |
| **FDXR**    | Ferredoxin reductase | Forward: CCTCGTCTTCAGCTGCATTTC  
Reverse: GGGACTAGGTGGCTGGAAGAT  
Probe: CATGCCTTGCAATGGATCAGCCAA |
| **FGP**     | Folypolyglutamate synthase | Forward: CCCTTCTGGGTGTTATGGGAAGA  
Reverse: CCCAGTCTTCAAACCCTGCTTACCA |
| **GGH**     | γ-glutamyl hydrolase | Forward: GCAATGCCGCTGAACTTCA  
Reverse: ATGACAGCAACAAACTCAGTAGGAAA  
Probe: TCGTGACATTCTCAGCTTCCAGT |
| **MTHFR**   | 5,10-methylene tetrahydrofolate reductase | Forward: GTGATCAGCAGAGGTGCAAAGT  
Reverse: GGATATGACAGATGAGGATTAGCTCTT  
Probe: CACCATCACAATCTTCACTTTTCCTCCCAT |
| **NFKB1**   | Nuclear factor of kappa light polypeptide gene enhancer in B cells | Forward: AGCAAATAGACGAGCTCCGAGA  
Reverse: GGCACCACTGGTCAGAAAGTCA |
| **NME1**    | Nucleoside diphosphate kinase 1 | Forward: TTCCCTCTGAGGAAGCTGTTAGATT  
Reverse: GAGTAGTAGCTAGTCGCTACTGAT  
Probe: ACCGTACTCATGAGTCTGACATGAA |
| **NME2**    | Nucleoside diphosphate kinase 2 | Forward: AGCACGGCGTGGTGTGT  
Reverse: GCTCTATAAAAGAATGATCAATCCTGGT  
Probe: CCCTGGACACAGCTCTTCATTCCACTG |
| **RRM1**    | Ribonucleotide reductase M1 polypeptide | Forward: CATCCACACTGGTCAGCTTCCAGTAAC  
Reverse: CCCAGTCTTCAAACCCTGCTTACCA |
| **RRM2**    | Ribonucleotide reductase M2 polypeptide | Forward: TCTGGGTCTTAGTGATCAGTCTACAGGC  
Reverse: CAAGACTGGCATTGTCACTGGCTA  
Probe: TAAAGGATCCTAGGGAGTAAGGTGAAGCA |
| **TK1**     | Thymidine kinase 1 | Forward: GCGGCTTTCACTGAGTGTATT  
Reverse: GTGTGGGCCAAGGCTCAA  
Probe: TCTCCCTGGGAAGCCTGTGCCA |
| **TNFSF6**  | Tumor necrosis factor (ligand) superfamily, member 6 | Forward: AGACTGGGTCTCGTTATTGGGAAGAAGGAA  
Reverse: TGGCAAAACTCAGTAGTGAAGAGCTTACAGGC  
Probe: TAAAGGATCCTAGGGAGTAAGGTGAAGCA |
| **TP53**    | Tumor protein p53 | Forward: AGACTGGGTCTCGTTATTGGGAAGAAGGAA  
Reverse: TGGCAAAACTCAGTAGTGAAGAGCTTACAGGC  
Probe: TAAAGGATCCTAGGGAGTAAGGTGAAGCA |

(Continued)
Results

Interpatient variability of mRNA gene expression. A high degree of interpatient variation in the relative expression of the 24 5-FU pathway genes was observed in the tumor and nonmalignant samples (Fig. 2). For the tumor samples, FPGS showed the largest range of relative expression values (~16-fold), whereas DUT exhibited the smallest overall range of relative expression (~3-fold). For the nonmalignant tissue samples, RRM1 showed the greatest overall range of relative expression among the 52 samples (~26-fold), whereas GGH expression varied the least (~2-fold). The difference among interpatient gene expression suggests the presence of subgroups within the histologically homogeneous patients.

Differences in mRNA expression of paired tumor versus nonmalignant tissues. Fourteen of the 24 genes showed significant variation in gene expression in the tumor versus paired nonmalignant samples (FPGS, DHFR, GGH, NME1, NME2, RRM2, UMPH2, UNG, FDXR, UMPS, TP53, UP, TK1, and DPYD). To further differentiate tumor versus nonmalignant relative expression patterns, patients were grouped by their tumor to normal ratio into one of three groups: T/N <0.8 (nonmalignant tissue shows higher expression of gene), 0.8 to 1.2 (no difference between tissues), or T/N >1.2 (tumor shows higher expression than normal; Fig. 3). To evaluate the differences in proportions of patients in the three T/N categories, a χ² test was done. Twelve of the 24 genes yielded a significant χ² P for distribution of patients T/N ratios (Fig. 3). FPGS, DHFR, GGH, NME1, NME2, RRM2, UMPH2, UNG, UMPS, TP53, UP, TK1, and DPYD) To further differentiate tumor versus nonmalignant relative expression patterns, patients were grouped by their tumor to nonmalignant ratio into one of three groups: T/N <0.8 (nonmalignant tissue shows higher expression of gene), 0.8 to 1.2 (no difference between tissues), or T/N >1.2 (tumor shows higher expression than normal; Fig. 3). To evaluate the differences in proportions of patients in the three T/N categories, a χ² test was done. Twelve of the 24 genes yielded a significant χ² P for distribution of patients T/N ratios (Fig. 3). FPGS, DHFR, GGH, NME1, NME2, RRM2, UMPH2, UNG, UMPS, TP53, and TK1 all had higher tumor expression compared with normal tissue. DPYD showed significantly lower expression in tumor compared with normal.

Spearman rank correlation. Multiple gene correlations for the genes of the 5-FU pathway were found with a Spearman rank correlation of >0.6 (Table 3; all P < 0.001). DHFR, RRM2, NME1, UNG, NME2, FPGS, GGH, MTHFR, and UMPH2 each showed correlations with eight to 10 other genes, mostly each other. UPB1 and DPYS were correlates, as were DPYD and ECGF1.

Hierarchical clustering analysis. Using the unweighed pair grouping method, unsupervised hierarchical clustering of the 24 genes for the tumor tissues of the 52 patients was done using the relative expression data (Fig. 4). At least three groups of genes and two groups of patients were elucidated. There was no significant difference in demographic or pathologic features between the two patient groups. The specific gene expression patterns that are critical for defining the patient groupings could become a useful marker for determining which treatment course is best for a particular patient.

Discussion

Human cancers show great variability in response to chemotherapy, even in the context of the same tumor stage and a uniform treatment approach. Differences at the molecular level might help explain some of the variation in patient response, either due to the influence of genes on the course of the disease or on a patient’s response to treatment. Recent studies have shown a relationship between a patient’s genetic profile and cancer treatment outcome for breast cancer and diffuse large B-cell lymphoma (17–19). There are studies of gene expression in colorectal cancer. However, there are not yet clear molecular predictors of response to therapies for colorectal cancer (20–22). Standard of care treatment for colorectal cancer...
Dukes’ C colorectal cancer relies heavily on 5-FU (9). However, several additional cytotoxic agents are now available. Therefore, looking at how expression of the 5-FU pathway genes vary in tumor and normal tissue is valuable. Unfortunately, the expression pattern of most genes of the 5-FU pathway has not previously been studied in human tumor. Previous studies have shown FDXR to be induced by TP53 after 5-FU treatment and that disruption of FDXR caused decreased sensitivity to 5-FU-induced apoptosis (16, 23). High levels of thymidylate synthase has previously been associated with resistance to 5-FU therapy (24, 25). Similarly, low expression of thymidylate synthase, DPYD, and thymidine phosphorylase were associated with a good response to 5-FU and better survival in patients with colorectal cancer (26). In contrast, another study found that high levels of thymidylate synthase correlated with a benefit from adjuvant chemotherapy (27). Given the conflicting results and limited study of the genes in the 5-FU pathway, more knowledge about the expression patterns of these genes in tumor and normal tissue should be valuable for future directions of colon cancer therapy.

Fig. 2. Variability in expression of pathway genes. Interpatient variability among the 52 patients for the tumor (A) and normal tissue (B) samples for the 24 5-FU genes. Long bars, range; larger rectangle, middle 50% of patient samples; smaller rectangle, median value.
The relative expression level of the 24 genes of the 5-FU pathway in 52 paired tumor and normal tissue samples could provide useful insights into 5-FU therapy and colon cancer. The gene expression pattern established during tumor development may provide an opportunity for better treatment selection or modulators of therapy. The differences in gene expression in tumor versus nonmalignant tissue might provide some basis for the predicting and/or modeling variability in toxicity and tumor response to treatment. Using unsupervised hierarchical clustering, three major groups of genes and two groups of patients were elucidated (Fig. 4). Not surprisingly, the gene groupings showed similarity to the Spearman rank correlation results, with many of the genes which showed correlations being closely linked on the hierarchical clustering. Additionally, our data showed interpatient variability of the expression of the 24 5-FU pathway genes supporting the presence of distinct subpopulations of patients. Therefore, different treatment might be more appropriate for different patients, if these variabilities can be correlated with therapy response and outcome. The hypothesis can now be tested that the two distinct patient groups identified by the 5-FU pathway genes are associated with treatment outcome. For example, Van de Vijer et al. and Rosenwald et al. used gene expression profiles as a predictor of survival for breast cancer and diffuse large B-cell lymphoma, respectively, identify patients who do not achieve adequate tumor control from current standard patient management (17, 18).

Many of the 24 genes in the 5-FU pathway have significant differences in expression between the tumor and normal tissue samples. Fourteen of the 24 genes showed statistically significant variation in gene expression between the tumor and normal samples (FPGS, DHFR, GGH, NME1, NME2, RRM2, UMPH2, UNG, FDXR, UMPS, TP53, UP, TK1, and DPYD), perhaps treatment can be better optimized to take advantage of these differences between tumor and normal tissue (Table 3). Twelve of those 14 genes additionally showed a significant difference between T/N category proportions (FPGS, DHFR, GGH, NME1, NME2, RRM2, UMPH2, UNG, FDXR, UMPS, TP53, UP, TK1, and DPYD). The two other genes, FDXR and UP, were just below the level of significance. Most all of the 12 genes showed a significant number of patients with increased tumor expression (T/N > 1.2), with the exception of DPYD which had a majority of patients with decreased expression of the gene in tumor samples (T/N < 0.8). DPYD, the first and rate-limiting enzyme, catabolizes >80% of given 5-FU. Therefore, patients with T/N < 0.8 for DPYD should experience greater tumor response, as the tumor can less easily catabolize 5-FU as compared with the normal tissue, which is consistent with previous studies (28, 29). The likely outcome for higher or lower expression of most of the genes is less well understood.

![Gene Expression Distribution in Tumor vs. Normal Tissue](image)

**Fig. 3.** Gene expression distribution. Proportion of patients with T/N < 0.8, 0.8 to 1.2, or >1.2 for the 24 5-FU genes and the corresponding \( \chi^2 \) Ps. Genes with a significant proportion of patients with either high (T/N > 1.2) or low (T/N < 0.8) expression (underlined).
Eight of the 12 genes that show significant variation in expression between tumor and normal tissue samples were consistently linked together in correlation analysis (DHFR, FPGS, RRM2, NME1, NME2, GGH, UNG, and UMPH2; Table 3). These genes are all in a similar region of the 5-FU pathway (Fig. 1). The mechanistic basis for this apparent coregulation needs to be evaluated but likely reflects the known role of these genes in folate biochemistry. Initial analysis of the region 10 kb upstream from the transcription start site reveals interesting regulatory domains that may reflect a common control mechanism.

The current study focused on RNA expression for 5-FU pathway genes. However, RNA may not always serve as an adequate biomarker for tumor protein level or functional activity. This work represents an initial evaluation of the distinct patient groups unraveled by analyzing drug pathways. The next step will include correlation of pathway-defined groups with clinical outcome, to refine this model and develop similar models for the other active agents used to treat colorectal cancer. The goal could be either the identification of those patients needing only single agent therapy or assist in the selection of the best therapeutic pairing out of the available agents. Hopefully, this study will provoke and stimulate greater assessment of gene pathways, as we move toward the goal of tools for the individualization of patient therapy. Prospective studies, in the context of modern combination chemotherapy, now need to be conducted to push this field forward.

Table 3. Correlation of 5-FU pathway gene expression

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NOTE: Gene correlations for 5-FU pathway (Spearman rank correlation > 0.6). Only Spearman rank correlation > 0.6 (< 0.001) are shown.

References

Fig. 4. Hierarchical clustering. Using unweighed pair grouping method with arithmetic mean hierarchical clustering using the relative expression data for the 24 genes of the 52 patients was performed, yielding at least three groups of genes (left; specific genes, right) and two groups of patients (across the top).
Variance in the Expression of 5-Fluorouracil Pathway Genes in Colorectal Cancer

Elizabeth A. Kidd, Jinsheng Yu, Xia Li, et al.


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