COX-2 INHIBITION IN COLORECTAL CARCINOMA: CHANGES IN GENE EXPRESSION AND IMPACT ON PROSTAGLANDIN METABOLITES

By

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LIST OF ABBREVIATIONS

cDNA - Complimentary Deoxyribonucleic Acid

COX-2 - Cyclooxygenase-2

CRC - Colorectal Carcinoma

FAP - Familial Adenomatous Polyposis

FOBT - Fecal Occult Blood Test

IBD - Inflammatory Bowel Disease

IHC - Immunohistochemistry

KEGG - Kyoto Encyclopedia of Genes and Genomes

NSAID - Non-steroidal Anti-inflammatory Drug

PGE_2 - Prostaglandin E_2

PGE-M - Prostaglandin E_2 Metabolite

RIN - RNA Integrity Number

RNA - Ribonucleic Acid

CHAPTER I

INTRODUCTION

Colorectal Carcinoma

Colorectal carcinoma is the third most common cancer among men and women in the United States. When both sexes are taken together, CRC accounts for 10% of all cancer related deaths. In the year 2005, it is estimated that there will be more than 145,000 new cases and CRC will claim just over 56,000 American lives [1].

Cyclooxygenase-2

Prostaglandin metabolism is important in many cellular processes involved in growth, apoptosis and angiogenesis. Exactly how these processes are influenced by COX-2 is incompletely understood; however, COX-2 up-regulation has been shown to stimulate angiogenesis and tumor growth[2, 3]. A growing body of experimental data implies that COX-2 inhibitors may be therapeutically efficacious in the treatment of gastrointestinal malignancy[4-7]. Unfortunately, clinical evidence regarding COX-2 regulation in human gastrointestinal pathology is limited.

Approximately 85% of colorectal carcinomas exhibit increased expression of cyclooxygenase-2 (COX-2)[8] and evidence suggests this expression may promote tumor survival, invasiveness and angiogenesis[3]. There is evidence to suggest that COX-2 inhibition may be beneficial in preventing progression of existing colorectal polyposis[5-7, 9, 10]; however, the effects of these drugs on colorectal cancer *in vivo*, either individually

or as a component of a multi-modal treatment regimen, are unknown. For this reason, along with their relatively low toxicity profiles, selective inhibitors of COX-2 present potentially promising novel therapeutic agents for the treatment of colorectal cancer.

Tumor promoters and mitogens stimulate increases in COX-2 protein and prostaglandin levels. In colorectal cancer, dysregulation of COX-2 is present nearly 85% of the time[8]. COX-2 disruption in $Apc^{\Delta 716}$ mice results in a five-fold reduction in the number and size of intestinal polyps[5]. Furthermore, COX-2 inhibitors have demonstrated an ability to suppress human colon cancer xenografts in nude mice[4]. This appears to occur in a dose dependent fashion.

Clinical evaluation of COX-2 inhibitors in familial adenomatous polyposis (FAP) has shown therapeutic efficacy in polyp prevention and regression[6, 7, 9-11]. This is noteworthy because this population has essentially a 100% cumulative lifetime risk for developing colorectal cancer unless therapeutic intervention is undertaken. Although this population represents a small percentage (<1%) of colorectal carcinoma cases, the therapeutic efficacy of selective COX-2 inhibition in this group may be applicable to non-FAP cases of colorectal cancer. FAP patients are believed to undergo progression from adenoma to carcinoma at an accelerated rate compared to other non-FAP individuals afflicted with sporadic and hereditary non-polyposis colorectal carcinoma.

Microarray Studies

Recent advances in molecular biology have enabled researchers to evaluate the profile of transcripts present in a given sample of RNA. This has been done in a more or less comprehensive manner using both cDNA chips and oligonucleotide based chips.

While such studies possess the potential to elucidate a great deal of information about certain biological systems under various conditions, they hold clinical potential as well. To harness this technology for clinical application is an ongoing goal of translational researchers around the world.

To date, many studies have been performed on a variety of tumor types in order to better understand the diverse range of cancers. The purposes of these individual studies have been many, but generally they are interested in one of two things: biology or profiling. Those investigators who are interested in identifying the specific genes involved in carcinogenesis and tumor behavior will go to great lengths to obtain a relatively pure population of tumor cells. Others who are interested in tumor transcriptional signatures are more likely to harvest and homogenize gross tumor.

Each of these approaches has advantages as well as disadvantages. The use of microdissection techniques (i.e. laser capture) to obtain “pure” samples of tumor cells does minimize contamination from other non-epithelial cells in the specimen. Thus, it is presumed that the RNA obtained from such preparations reflects the transcriptional pattern of the actual tumor. However, these techniques are labor intensive and time consuming, yielding small amounts of RNA in general; consequently, they are not practical for clinical implementation in their current forms.

On the other hand, preparation and homogenization of a specimen taken from a gross tumor is feasible to implement in a clinical setting and could produce useful transcriptional profiling information that includes other compartments of the tumor microenvironment. Nonetheless, this practice does not provide a high level of homogeneity with regard to the specimen and generalizations about the biology of the

epithelial component of the tumor are not as confidently reached. Alternatively, some investigators will screen grossly dissected specimens by microscopy to select tumor enriched tissue samples and try to achieve the best of both methods.

Since its introduction just before the millennium, gene expression technology has been applied to many tumor cell lines [12-22]. In the clinical arena, it has been applied to tumors from many organs including breast [12, 23-28], head and neck [29-33], stomach [34-37], pancreas [38-42], and prostate [43, 44]. While several studies have been done in colorectal cancer [45-51], few have been comprehensive with regard to the spectrum of stages represented or the platform used. Furthermore, even fewer and similarly limited studies have been done in this field to evaluate the differences between primary tumors and their daughter liver metastases [52].

In 2002, two landmark studies demonstrated the potential for predicting prognosis that this technology might hold [27, 28]. Gene expression patterns were developed from a training set of breast tumors that corresponded to a “poor” prognosis and a “good” prognosis. This profile was subsequently applied to a large group of breast tumors with remarkable performance. This signature was able to identify patients who would do relatively well from those who would do poorly based on the profile into which they most closely fell. Furthermore, this signature was able to differentiate patients who would do well from those who did not even among traditional “poor” prognosis groups (i.e. those with lymph node involvement).

Clearly, it has been recognized that the application of gene profiling technology could hold great potential in understanding the biology of CRC and selection of treatment. Still, controversy over how valuable this technology may be in the clinical

arena exists [53, 54]. As methods of tissue procurement and processing are refined and profiles are developed using larger samples, confidence is likely to grow in this area of translational research. This present proposal has been well designed and potential to generate new understanding about colorectal cancer that is metastatic to the liver. The goal of this project is to continue laying the foundation of tumor profiling by gene expression to develop a system capable of providing useful information about the diagnosis, treatment, prognosis, and biology of stage IV colorectal cancer.

CHAPTER II

URINE PGE-M: A METABOLITE OF PGE_2 AS A POTENTIAL BIOMARKER OF ADVANCED COLORECTAL NEOPLASIA

Introduction

Colorectal carcinoma (CRC) is the second leading cause of cancer-related deaths in the United States. In 2005, it is estimated that there will be more than 145,000 new cases of CRC and over 56,000 people will die of this disease.[1]

Cyclooxygenase-2 (COX-2) is an inducible enzyme associated with inflammation and malignancy.[55] COX-2 is expressed in most CRCs and is associated with increased tumor size, invasion, and poor prognosis.[56-59] A considerable body of evidence supports the notion that inhibition of cyclooxygenase activity, either with non-steroidal anti-inflammatory drugs (NSAIDs) or selective COX-2 inhibitors, provides therapeutic benefit in patients with colorectal neoplasia.[6, 11, 55] Likewise, epidemiologic studies have shown a benefit to NSAID or aspirin use in preventing or decreasing the mortality from CRC.[60-65] Selective COX-2 inhibitors such as celecoxib also inhibit in-vitro and in-vivo tumor cell growth [66-69], angiogenesis[70-72], invasion and metastasis.[73] Furthermore, celecoxib has been shown to decrease the number and size of colorectal polyps in patients with the familial adenomatous polyposis syndrome (FAP).[6, 7, 55]

Experimental evidence in mice links COX-2 expression to increased prostaglandin (PG) activity and subsequent promotion of growth and metastasis in CRC.[74] Specifically, COX-2 induction and subsequent production of PGE_2, are

important for tumor development and angiogenesis.[75] PGE_2 is produced in high quantities among several COX-2 expressing colon carcinoma cell lines and has been shown to enhance malignant characteristics of colon carcinoma cells in vitro.[76] It has been reported that PGE_2 increases the proliferation, migration, and invasiveness of colon cancer cells[76]; conversely, inhibition of COX-2 decreases PGE_2 levels and reduces growth factor mediated cell proliferation.[77]

Although eicosanoids such as PGE_2 can be readily quantified in cell culture by various methods, quantification of endogenous PG production in humans is significantly more challenging.[78, 79] The majority of measured urine PGE_2 is a product of renal metabolism.[80] Therefore, PG metabolites that are excreted in the urine are considered to more accurately reflect the systemic synthesis of their parent compounds.[78-80] We have recently developed methods that utilize mass spectrometry to accurately quantify the major urinary metabolite of PGE_2, PGE-M.[81]

The present study examines the role of urinary PGE-M as an indicator of COX-2 activity in colorectal cancer, colorectal adenomatous polyps, and IBD. Our results suggest that PGE-M may have utility as a biomarker in colorectal cancer and may be useful as a marker of disease activity in Crohn's disease.

Methods

Patient Selection

Urine specimens were collected from 58 patients with colon or rectal cancer, 69 patients who were endoscopically proven to have varying degrees of polyp disease

("large/multiple" - ≥2 polyps or polyp >1.0 cm in diameter; or "simple/small" - <2 polyps and <1.0 cm in diameter), and 72 patients who had no evidence of colorectal disease by colonoscopy (normal controls). Polyp size of >1cm was estimated by comparison to biopsy forceps (7 mm open, 2 mm closed) or obtained from the operative report if the polyps were resected. Patients were selected from participants enrolled from three ongoing prospective clinical studies investigating the role of COX-2 in CRC and colorectal polyp disease. These trials were conducted at Vanderbilt University Medical Center and The University of Alabama at Birmingham (UAB). Patients with benign disease provided detailed medication lists at the time of enrollment; those who had used NSAIDs within 48 hours were excluded from analysis. Patients diagnosed with CRC were asked to abstain from NSAID, aspirin, or selective COX-2 inhibitor use for at least 48 hours prior to submission of their urine specimen. Patients' urine samples were collected prior to surgery or endoscopy. All patients subsequently underwent endoscopy or surgical resection and were diagnosed with one of the aforementioned categories of colorectal disease.

As part of a clinical trial evaluating the role of COX-2 inhibition in rectal cancer, a subgroup of 13 patients underwent 5 days of oral celecoxib therapy. These patients abstained from use of non-selective NSAIDs, aspirin, and selective inhibitors of COX-2 for 7 to 10 days, before providing a pre-treatment urine specimen. Patients then received celecoxib, 400 mg orally, twice daily for 5 days. At the conclusion of the celecoxib treatment, a post-treatment urine specimen was obtained.

A cohort of patients with Crohn's disease and ulcerative colitis was included as a comparison group to represent a benign inflammatory condition of the gastrointestinal

tract. Since only four patients with ulcerative colitis were enrolled, only patients with Crohn's disease were included in this study to prevent any discrepancy between these two different inflammatory conditions. Twenty-eight patients with Crohn's disease were prospectively enrolled and their urine specimens were collected. Patients had previously been diagnosed by colonoscopy and biopsy with histological confirmation by a gastrointestinal pathologist. No treatment intervention was attempted and medication regimens varied at the time of submission of the urine specimen.

Written consent for participation and to provide clinical samples was obtained from every patient included in the aforementioned studies, which were all approved by the Institutional Review Boards of Vanderbilt University and the University of Alabama at Birmingham.

Quantification of Urinary PGE_2 Metabolite

Urine specimens were processed by aliquoting into two 10 mL samples and the samples were immediately stored frozen at -80^{o} C until final analysis. Samples were submitted for analysis in a randomized order and the personnel performing the analyses were blinded to the clinical and disease status associated with each specimen.

Urine PGE-M was quantified using liquid chromatography and mass spectrometry (LC/MS) by the methods previously reported.[81] Briefly, one ml of urine per patient was titrated to a pH of 3 by addition of 1M HCL and then 0.5 ml of methyloxime solution (1600 mg in 10 ml 1.5 M sodium acetate, pH 5) was added. After one hour, the methyloxime was diluted with 10 ml H_20 at pH 3 and each sample was applied to a C-18 Sep Pack (after prepping the Sep Pack with 5 ml MeOH and 5 ml H_20 at pH 3).

Samples were washed with 10 ml H_20 at pH 3 followed by 10 ml Heptane and then eluted from the Sep Pack with 5 mL ethyl acetate. The PGE-M internal standard (6.2 ng [$^2H_6$] O-methyloxime PGE-M in 10 µl) was added to the sample and the solution was dried under N_2 at 37^o C and reconstituted in 50 µl of LC mobile phase A, consisting of 95% 5 mM NH4oAC, 5% ACN, and 0.1% HoAc. Each sample was placed through a Spin X filter bullet tube by centrifugation and transferred to a mass spectroscopy vial. A TSQ Quantum Triple Quadrupole Mass Spectrometer was used to quantify the amount of PGE-M per sample. Urine creatinine was also measured and values of PGE-M were reported as ng PGE-M/mg creatinine.

Data from a time course study indicates that repeated testing of a single sample results in a coefficient of variance of about 15% (for 45 replicate samples).[81]

COX-2 Immunohistochemistry

Immunohistochemistry for COX-2 was carried out for tissues that were available from the cancer and adenoma groups using the DAKO EnVision™ visualization kit. Antigen retrieval was carried out using EDTA and high temperature pressure treatment for 15 minutes. The reaction was quenched by incubating with 0.03% hydrogen peroxide and sodium azide solutions for 5 minutes. Incubation with the primary antibody (Oxford Biomedical Research #PG 27 B rabbit polyclonal, dilution 1:200) was performed for 30 minutes. After washing, incubation with the secondary HRP labeled anti-rabbit antibody with a labeled polymer, provided by EnVision™. After development, tissue expression was scored against a seminal vesicle positive control. A gastrointestinal pathologist (MKW) noted the presence or absence of COX-2 expression

as well as the pattern (epithelial or stromal) of expression; this was subsequently correlated with urine PGE-M levels for these patients.

Statistical Analysis

We characterized the right skewed urine PGE-M distribution using the median and inter-quartile range (25^{th} and 75^{th} percentiles). Urine PGE-M values (reported in ng/mg Cr) were analyzed using non-parametric tests and transformed to their natural log for graphical display for each comparison. The logarithmic transformation reduced skewness of the PGE-M distributions and t-tests and ANOVA were used to assess group differences for log PGE-M values. Alternatively, Wilcoxon rank-sum and Kruskal-Wallis tests were used to determine significant inter-group differences for non-log transformed data; for multiple group comparisons, individual rank-sum tests were carried out, adjusting the p-values accordingly (multiplying by the number of comparisons). The signed-rank test was used for analysis of paired/repeated measures data. Adjusted p-values of <0.05 were considered statistically significant. Multiple logistic regression was used to assess the discriminatory ability of urine PGE-M, while adjusting for the effect of other covariates. PGE-M values were parameterized using restricted cubic splines[82] to allow a flexible structural relationship with classification probabilities. The STATA® (StataCorp, College Station, TX) and SAS® (Cary, NC) statistical software packages were utilized for analysis and graphics preparation.

Results

Urine specimens from a total of 227 patients were analyzed in this study. The set included 58 patients with colon or rectal cancer, 69 patients with varying degrees of colorectal adenomatous polyps (33 with polyps >1 cm, 37 with polyps <1 cm, 11 with ≥2 polyps), 72 patients that had no colorectal pathology on endoscopy, and 28 patients with Crohn's Disease. The clinical characteristics of each group are summarized in Table 1.

Table 1. Demographic characteristics for groups involved in the study.

	CRC Cases (n=58)	Large/Multiple Polyp Cases (n=44)	Small Polyp Cases (n=26)	Polyp-free Controls (n=72)	Crohn's Cases (n=28)
Age, mean (SD)	60.9 (10.0)	59.6 (11.5)	60.5 (7.2)	60.9 (7.5)	38.8 (11.7)
Female (%)	45	25	30.8	26.4	53.6
NSAIDs within 48 hrs	0	8.8	17.1	18.6	n/a
Characteristics of Neoplasia					
Location (%)					
Left colon	61.7	38.2	45.7	--	--
Right colon	38.3	52.9	40	--	--
Both	0	2.9	11.4	--	--
Unknown	0	5.8	2.9	--	--
> 1 Polyp (%)	--	14.7	25.7	--	--
Polyp ≥ 1 cm (%)	--	94	--	--	--
Tubulovillous/villous (%)	--	11.8	--	--	--
Median PGE-M	15.0	15.6	9.69	7.17	21.9
Interquartile Range	9.11 - 26.9	7.79 - 22.9	6.54 - 20.4	4.69 - 15.9	17.4 - 49.1

SD – standard deviation

PGE-M Among Polyp-Free Men and Women Controls

Among our control group of patients with no endoscopic evidence of colorectal pathology, a difference in urine PGE-M was seen between male and female subjects (NSAID users excluded). Otherwise healthy polyp-free men (controls) had a higher level of PGE-M (median 8.59 [IQR 5.67 – 22.3]) than polyp-free women (controls) (median PGE-M 4.25, IQR 2.35 – 6.03). This difference was significant (Wilcoxon rank-sum, p = 0.0027) and is consistent with our previous findings of men having higher baseline PGE-M levels than women.[81] No significant difference was noted with respect to age (Spearman's rank correlation p=0.19), or race (whites vs. non-whites, p=0.98) among control individuals. Smoking as a potential confounder could not be evaluated due to the low number of smokers in the control group (n=4).

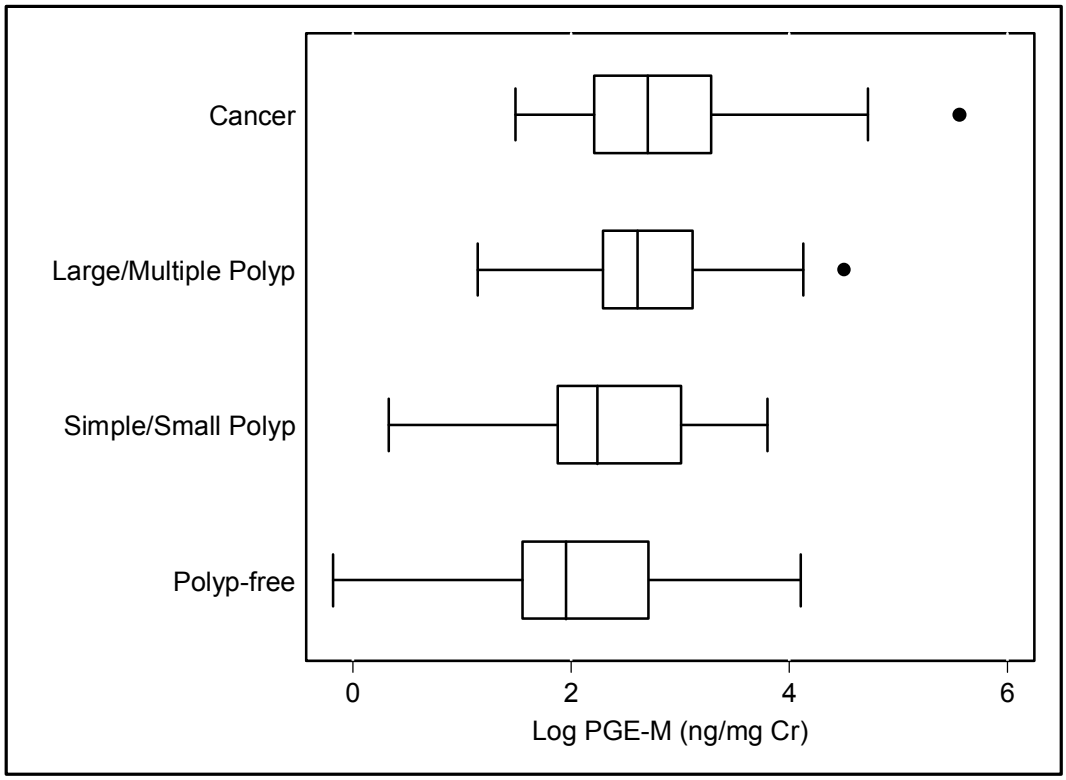
COX-2 Expression by Immunohistochemistry

To evaluate COX-2 expression in neoplastic tissue, immunohistochemistry using a COX-2 specific antibody was performed on tissue sections taken from paraffin embedded blocks of 32 pathological specimens (CRC n=26, large adenoma n=6) for which corresponding urine PGE-M data was available. Specimens were scored as either expressing COX-2 (to any degree) or as not expressing COX-2. In total, 77% (20 out of 26) of the CRC and 50% (3 out of 6) large adenoma specimens were found to express COX-2. However, no significant correlation was identified regarding the intensity or pattern of COX-2 expression and urinary PGE-M levels among this group (epithelial or stromal; Wilcoxon rank-sum p=0.55 and p=0.27, respectively).

Patients with CRC

Among the aforementioned classification groups, the CRC group (median PGE-M 14.65, IQR 5.94 - 92.1) and the multiple/large polyp disease group (median PGE-M 15.6, IQR 4.54 - 30.6) had elevated urinary PGE-M levels compared to the simple/small polyp group (median PGE-M 6.92, IQR 3.56 - 22.2), and the polyp-free group (median PGE-M 7.20, IQR 1.55 - 31.5) (figure 1). Urinary PGE-M in the CRC group was significantly elevated compared to the simple/small polyp and polyp-free groups (Wilcoxon rank-sum adjusted for multiple comparisons $p = 0.006$ and $p = 0.0004$, respectively). There was no difference in urine PGE-M with regard to tumor location (right sided tumors n=21, median 11.4 [IQR 8.54 – 37.9]; left sided tumors n=37, median 15.1 [IQR 9.52 – 21.7]; Wilcoxon rank-sum p=0.62). Smoking as a potential confounder counfounder could not be evaluated due to incomplete smoking data on the rectal cancer patients (smoking status at the time of resection available for only 28 out of 58 patients; however, only 7 were actively smoking).

Figure 1.



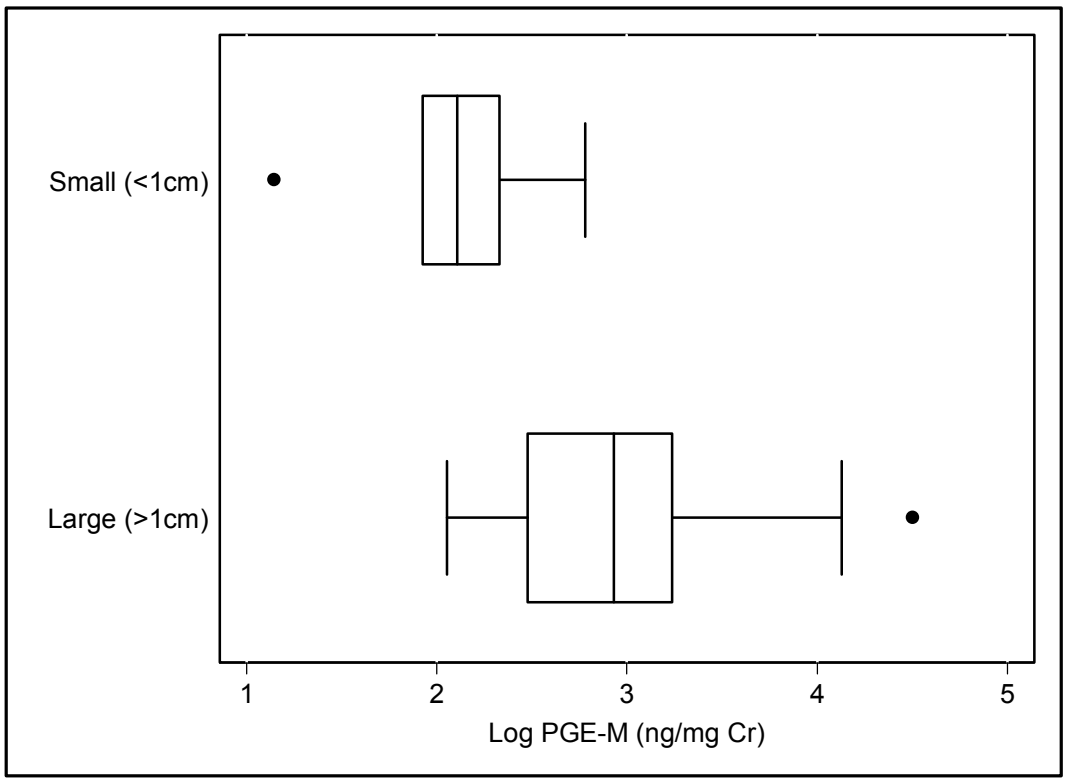
Urine PGE-M by Pathology Group. The groups consisted of patients with CRC, patients with large (>1cm) or multiple (<1cm but ≥2 in number), small polyps or simple polyps (<1cm and <2 in number), and no polyp disease. A significant difference was detected between the groups (p=0.0003); adjusted pair wise comparisons are listed. Log transformed median urine PGE-M values and interquartile range (IQR) are indicated by box plot for each group. The inter quartile range of values is represented by the box, with the median logPGE-M value indicated by a line within the box; the bars represent the most extreme values that are not outliers or 1.5 times the size of the IQR, whichever is smaller. Outliers are represented by small circles

Patients with Polyp Disease

Patients with large/multiple polyps comprised of a heterogeneous group with variable sizes of polyps - either polyps greater than 1.0 cm in size or greater than 2 polyps, regardless of size. Therefore, we sought to determine if polyp size, regardless of number, had any impact on PGE-M levels. Patients with adenomas greater than 1.0 cm in size, regardless of number of polyps, were found to have PGE-M levels that were elevated relative to those with adenomas less than 1.0 cm in size, even if multiple (median PGE-M 16.1 [IQR 6.43 – 30.6] versus 13.2 [IQR 10.3 – 16.1]; Wilcoxon rank-sum p = 0.056; figure 2). The urine PGE-M values of patients with small (<1 cm) polyps, even though they were multiple in number, was similar to those patients who had no detectable colorectal pathology by endoscopy (median PGE-M 10.9 [IQR 6.4 – 20.9] vs. 7.2 [IQR 4.7 – 16.4]; p = 0.56).

Due to the small sample size of tubulovillous and villous adenomas (n=4) in the adenoma group, we were unable to determine whether histological subtype correlated with PGE-M levels. It should also be noted that out of the 33 patients with polyps >1 cm, 20 were sufficiently large that they were referred for resection; the remaining 13 patients had polyps >1cm found on endoscopy. However, among non-NSAID users, there was no significant difference between the resected polyps and endoscopically discovered polyps that were >1cm in size (median PGE-M 18.3 [IQR 7.73 – 24.7] vs. 17.1 [IQR 12.3 – 36.7]; Wilcoxon rank-sum p=0.44).

Figure 2.



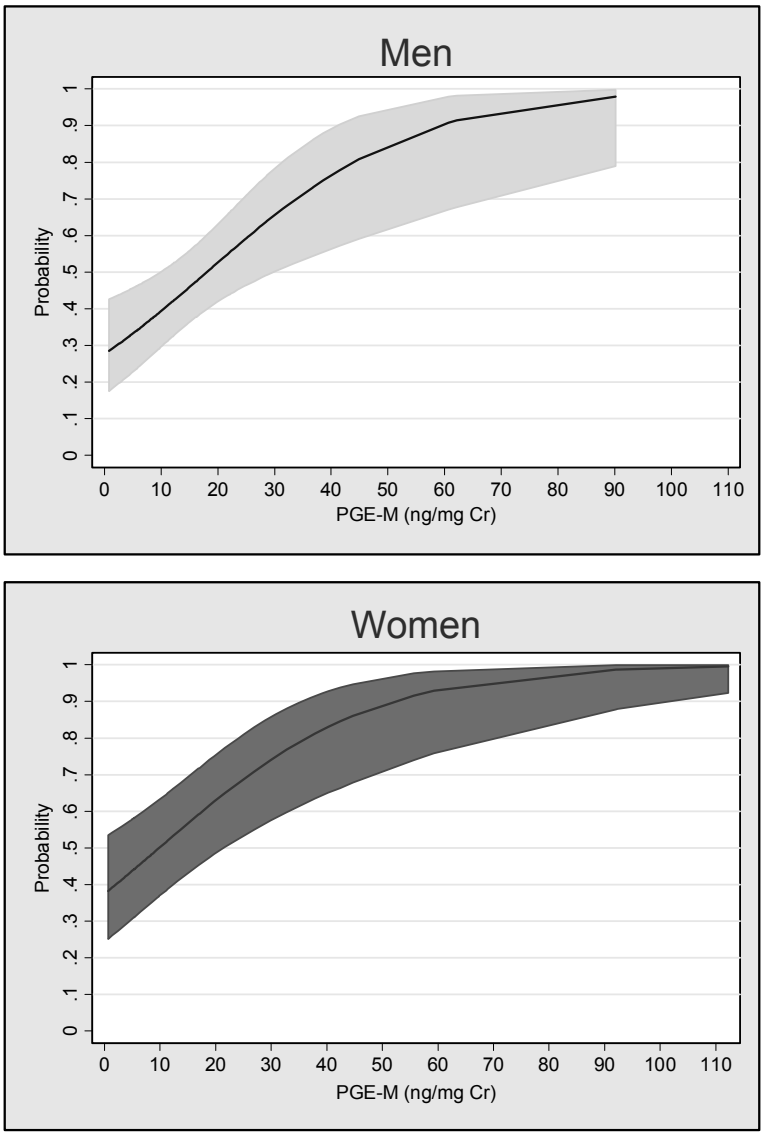
Large versus Multiple Small Polyps. Patients with large (>1cm) polyps trended toward having an elevated urine PGE-M compared to patients with small multiple polyps (<1cm and ≥2 in count, p=0.056). Log transformed median urine PGE-M values and interquartile range (IQR) are indicated by box plot for each group. The inter quartile range of values is represented by the box, with the median logPGE-M value indicated by a line within the box; the bars represent the most extreme values that are not outliers or 1.5 times the size of the IQR, whichever is smaller. Outliers are represented by small circles

Patients with Potential Surgical Disease: Large Adenomas or CRC

Patients with small (< 1 cm, even if multiple) or no polyps had similar median PGE-M values and distributions (median 9.69 [IQR 6.41 – 22.2] versus 7.05 [IQR 2.35 – 24.7], Wilcoxon rank-sum p=0.81). Similarly, patients with CRC or polyps greater than 1.0 cm in size had similar median urine PGE-M values and distributions (median 14.95 [IQR 5.94 – 92.1] versus 18.85 [IQR 11.9 – 25.6], Wilcoxon rank-sum p=0.26). As was the case for all patients with CRC, most of the patients with large polyps in this study underwent surgical resection of their disease since they were not amenable to endoscopic resection. Given these similarities, we grouped patients with large polyps and those with CRC as one group that was considered to be at high risk of having a large polyp or potentially surgical disease and compared their PGE-M levels to those with small or no polyp disease that had a very low risk of having surgical disease. There was a significant difference in PGE-M values between the large polyp/CRC group and the small/no polyp group (median PGE-M 15.1 [IQR 3.68 – 92.1] versus 7.34 [IQR 1.44 – 35.2]; Wilcoxon rank-sum p < 0.0001).

Using this classification scheme, a multiple logistic regression model was created that incorporated gender and PGE-M level. The probability of a patient in our sample population having a large polyp/potential surgical disease increases as urine PGE-M level rises for both genders. At lower PGE-M levels, the discernment between benign and more advanced disease is less clear due to the greater overlap between the distributions of these groups; however, as the PGE-M level increases, the distinction becomes much more apparent (figure 3).

Figure 3.



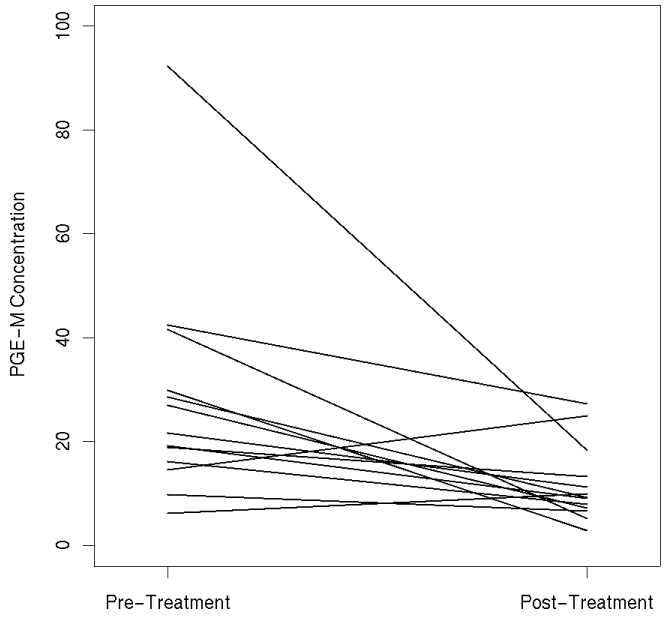
Probability of CRC or Large Adenoma Increases with Urine PGE-M. Multiple logistic regression model using restricted cubic splines demonstrates the probability of having CRC or a large polyp versus a more benign condition (small or no polyps). Both PGE-M level and gender contributed in a significant manner to this model (p=0.0002 and p=0.0006, respectively). The solid line represents the predicted probability obtained from the model; the shaded area represents the 95% confidence interval for the prediction. Probabilities are displayed separately for men and women.

Urine PGE-M and COX-2 Inhibition

Thirteen patients with rectal cancer underwent 5 days of treatment with celecoxib, 400 mg orally twice daily and provided both pre- and post-treatment urine specimens. Paired analysis of these pre- and post-treatment specimens revealed a significant decrease in urine PGE-M after treatment with celecoxib (median PGE-M 21.7 [IQR 16.2 – 29.9] vs. 9.14 [IQR 7.14 – 13.2], signed-rank test p = 0.009). Celecoxib treatment in these patients with rectal cancer suppressed urinary PGE-M to the level of patients with minimal or no detectable colorectal disease (small polyps or polyp-free; figure 4).

Six patients with large polyps were identified to have taken NSAIDs within 48 hours of urine sample collection. Their PGE-M levels were also similar to control levels of PGE-M and therefore these patients were excluded in other comparisons.

Figure 4.



Inhibition of COX-2 in rectal carcinoma. Thirteen patients with a diagnosis of stage I-III rectal carcinoma were treated with a 5 day course of celecoxib 400 mg orally twice a day. Each patient provided pre-treatment and post-treatment specimens, which were analyzed in a paired manner. PGE-m levels were significantly decreased after 5 days of celecoxib treatment (p=0.009). Median urine PGE-M values and interquartile range (IQR) are indicated by the square symbol and bars, respectively, for each time point.

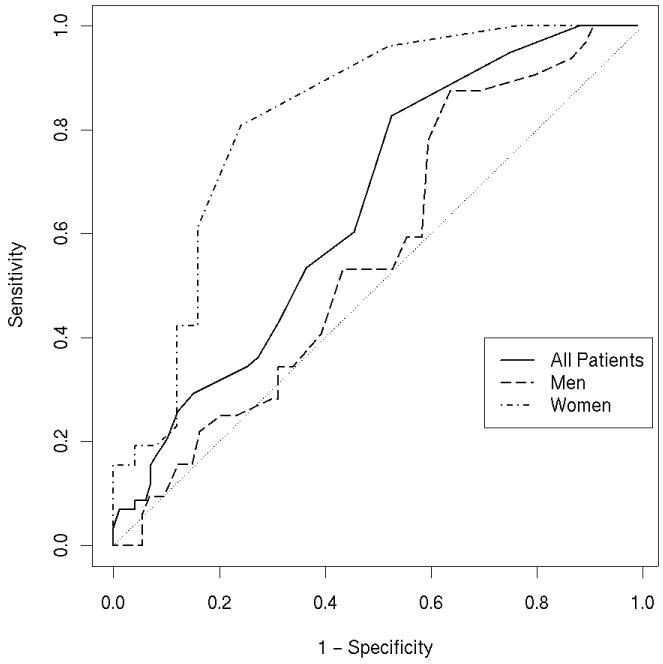
Patients with Crohn's Disease

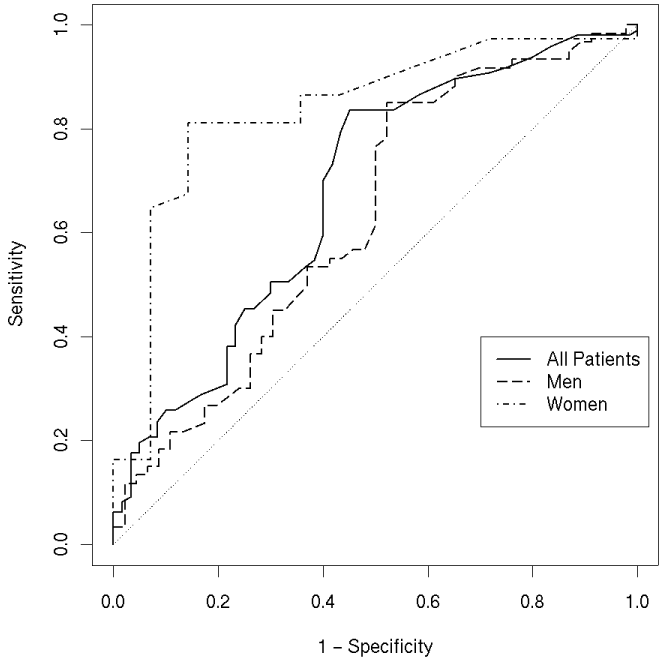
Since COX-2 activity is also induced during inflammatory conditions, we evaluated the status of urine PGE-M levels in a group of patients with a benign gastrointestinal inflammatory condition, Crohn's disease. Urine samples were collected prospectively from a group of 28 consecutive patients who had been diagnosed with Crohn's disease; however, tissue was not uniformly available to perform COX-2 immunohistochemistry. As a group, patients with Crohn's disease had markedly elevated levels of urine PGE-M relative to the other groups that were evaluated (median PGE-M 19.85 [IQR 6.89 – 90.2]). This was significantly different from the small/polyp-free group (Kruskal-Wallis $p = 0.0001$; adjusted rank-sum $p < 0.0001$) and the CRC/Large polyp groups (adjusted rank-sum $p = 0.035$).

Urine PGE-M as a Screening Biomarker

To determine the potential utility of urine PGE-M as a biomarker of colorectal disease, sensitivity and specificity analyses were performed and ROC curves were created. We have shown that two non-pathology related factors impacted PGE-M levels: gender and NSAID use. To that end, ROC curves were created for men, women, and the entire group of patients; likewise, NSAID users were excluded (figure 5).

Figure 5.





Separate ROC analyses were performed, demonstrating sensitivity and specificity at each cutoff value of urine PGE-M for men, women, and all patients combined using PGE-M for: A. potential surgical disease, including CRC or large polyps (median urine PGE-M values for positive individuals

Table 2 summarizes our findings by gender for CRC, as well as the aforementioned potential surgical group (CRC and large polyps). The sensitivity and specificity can be inversely adjusted up or down by modifying the cutoff value selected to indicate a “positive” test. Accordingly as the cut point for a positive test increases, sensitivity approaches 100%, while specificity decreases accordingly.

Table 2. Sensitivity and specificity analysis of urine PGE-M for the detection of CRC versus benign disease (all polyp patients and controls) or CRC/large polyps (potentially surgical disease) versus small/no polyps (non-surgical disease). The area under the curve (AUC) for each receiver-operator curve (ROC) is listed along with the specific cut points that yield the highest correct classification for each group. All analyses exclude NSAID users; combined and gender specific tables are provided.

	CRC vs. Non-Cancer			CRC/Large Polyp vs. Small/No Polyps		
	All	Men	Women	All	Men	Women
AUC	0.64	0.54	0.84	0.70	0.67	0.84
Median PGE-M (Positive)	15.0	14.7	17.0	15.1	15.1	11.4
Median PGE-M (Negative)	11.3	12.3	7.67	7.21	9.59	5.61
Cut point log(PGE-M)	2.07	2.11	2.07	2.04	2.11	2.04
Cut point PGE-M	7.93	19.2	7.23	7.67	8.26	7.67
Sensitivity	90%	88%	92%	88%	92%	83%
Specificity	45%	35%	78%	53%	46%	86%

Discussion

The present study shows that urine PGE-M levels are increased in patients with colorectal carcinomas, large colonic polyps, and Crohn's disease. This is consistent with the fact that 75-85% of colon cancers express COX-2.[55] Accurate quantification of PGE-M requires a single urine specimen and abstinence from COX-2 inhibitors or NSAIDs for 48 hours prior to collection, (although we acknowledge that patients may use these medications surreptitiously despite instructions to the contrary[83]). There is a modest but significant difference in median urine PGE-M levels between healthy men and women that appears to be attributable to gender, independent of age. Although we were unable to demonstrate a correlation between COX-2 expression and PGE-M level, this was likely due to the fact that the majority of tumors express COX-2 to some degree and our sample size of patients with tissue available for IHC was relatively small. Only 6 out of 26 CRC patients and 3 out of 6 large adenoma patients were found to be non-expressers of COX-2; therefore, our sample was underpowered to detect a difference between COX-2 expressing and non-expressing tumors (a sample size of 40 non-expressors and 40 expressors of COX-2 would be required to detect a 5 ng/mg Cr difference in urine PGE-M while achieving an $\alpha=0.05$ with a power=0.80).

Our findings suggest that assessment of urine PGE-M level deserves further investigation as a non-invasive clinical marker for discerning patients who would benefit from colonoscopy for surveillance for colorectal neoplasia. According to the Early Detection Research Network (EDRN), for detection of CRC a candidate biomarker must demonstrate a sensitivity of 40% and specificity of 80% prediction of any stage adenocarcinoma of the colon to be considered for further investigation; for detection of

adenoma(s) of any size, a biomarker must similarly demonstrate a sensitivity of 30% and specificity of 70% to be further investigated.[84] For women in the cohort described, optimal sensitivity and specificity, defined as the cutpoint at which the misclassification rate is lowest, were 92% and 78%, respectively for CRC, while optimal sensitivity and specificity for the detection of potentially surgical neoplasms (large polyps or CRC) were 83% and 86%, respectively. For men, optimal sensitivity and specificity for the detection of CRC were 88% and 35%, respectively, while for potentially surgical neoplasms, optimal sensitivity and specificity were 92% and 46%, respectively. Indeed, it may be that this assay holds more potential for female patients. These data suggest that assessment of urinary PGE-M should be further investigated as a non-invasive test to indicate the need for screening colonoscopy for detection of large adenomas or colorectal carcinoma.

Currently advocated preventative screening measures for CRC in the United States include flexible sigmoidoscopy with double contrast barium enema or colonoscopy and fecal occult blood testing (FOBT).[85] Among asymptomatic patients with an uncomplicated past medical or family history, these tests are not recommended to begin until age 50 for both men and women. Fecal occult blood testing as a part of the regimen is the only currently recommended non-invasive adjunct in screening for colorectal cancer. Appealing features of FOBT are that it is non-invasive and can be performed by patients in their own home; however, problems with compliance (3 consecutive stools must be screened) do exist. Furthermore, the sensitivity and specificity of FOBT for detecting advanced neoplasia are much lower than its more invasive counterparts (i.e. colonoscopy, double contrast barium enema, etc).[86]

Nonetheless, when FOBT was first introduced, its application improved mortality due to CRC by approximately one third according to one randomized trial carried out in Minnesota.[87]

At present, there is no widely accepted, non-invasive screening test for CRC that is easily and completely administered in a clinical setting. Further study in larger populations will be required to determine whether measurement of urinary PGE-M will enhance the detection of occult colorectal carcinomas or adenomas (alone or in combination with FOBT) and allow better selection of patients for more invasive testing such as colonoscopy.

A potential confounding factor in the assessment of urine PGE-M levels as an indicator of colorectal neoplastic disease is the fact that COX-2 levels may be elevated in numerous inflammatory and extracolonic malignant conditions. For example, urinary levels of PGE-M are elevated in patients with advanced lung carcinoma[81] and we have recently found PGE-M to be elevated in pancreatic cancer (unpublished data). However, through larger validation studies, based on a patient's clinical presentation an elevated PGE-M level may potentially serve as an indicator for further clinical evaluation.

In a trial of patients with advanced NSCLC treated at Vanderbilt University using celecoxib and cytotoxic chemotherapy, PGE- M levels were monitored during their treatment course. Those patients who had a decrease in PGE-M levels during the course of treatment had significantly improved survival compared with those patients who had either no change or an increase in urinary urine PGE-M levels.[88] The degree of increase in PGE-M level correlated with the risk of decreased survival as the degree

of decrease in PGE-M level correlated with improved survival. These data suggest that urine PGE-M may serve as a useful biomarker for assessment of intratumoral COX-2 activity and as a prognostic marker of response to treatment in the setting of COX-2 expressing malignancies.

Our results also show that urine PGE-M levels are elevated in patients with Crohn's disease to levels higher than those patients with colorectal carcinoma. Although we did not collect samples after pharmacologic intervention among the Crohn's patients, further studies are necessary to determine whether urine PGE-M may be a useful objective measure of the activity of this disease as well. Furthermore, it will be both interesting and important to determine whether other COX-2 expressing neoplasms or other conditions that cause systemic inflammation are associated with increased urine PGE-M levels.

In the present pre-validation phase of our study of urinary PGE-M, we found that urine PGE-M levels were significantly elevated in patients with CRC and large colorectal polyps. Our findings suggest urine PGE-M levels deserves further investigation as a clinical biomarker of CRC. Further development will be required to determine whether this test can serve as a non-invasive and low cost test in screening normal risk patients for colorectal cancer and significant adenomas or serve as a compliment to FOBT. It is possible that the combination of the two tests could be useful in improving the selection of patients who should undergo colonoscopy. Although the increase urine PGE-M may be a confounding problem in segregating patients with colorectal neoplasia versus those with other types of cancers and inflammatory conditions, additional study with a larger cohort of patients will determine whether elevated urinary PGE-M may be a

useful marker for significant GI pathology or other disease resulting in systemic inflammation.

CHAPTER III

TREATMENT OF RECTAL CANCER WITH CELECOXIB: CHANGES IN GENE EXPRESSION AND CORRELATION WITH URINE PGE_2 METABOLITE LEVELS

Introduction

Cyclooxygenase-2 (COX-2) is an inducible enzyme associated with inflammation and malignancy.[55] COX-2 is expressed in a majority of CRCs and is associated with increased tumor size, invasion, and poor prognosis.[56-59] A large body of literature supports the concept that inhibition of COX-2, either with non-selective (NSAIDs) or selective COX-2 inhibitors (e.g. celecoxib), may offer therapeutic benefit in patients with colorectal neoplasia.[6, 11, 55] Selective COX-2 inhibitors such as celecoxib also inhibit *in-vitro* and *in-vivo* tumor cell growth [66-69], angiogenesis[70-72], invasion and metastasis.[73] Furthermore, epidemiologic studies have shown a benefit to NSAID or aspirin use in preventing or decreasing the mortality from CRC.[1, 61-65] Likewise, celecoxib has been shown to decrease the number and size of colorectal polyps in patients with the familial adenomatous polyposis syndrome (FAP).[6, 7, 55]

In recent years, the characterization of solid tumors using microarray gene expression technologies has proliferated. Gene expression patterns have been used to investigate the biology and clinical prognosis of breast cancer[25-28, 89], head and neck cancer[32], and pancreatic cancer[42] among others. Colorectal carcinoma, has also been characterized using these techniques[16, 46, 90-94] and notably, a gene expression pattern that corresponds to a “poor” or “good” prognosis has been developed for this disease.[47]

Still, while some have studied the effects of COX-2 inhibition on gene expression in CRC cells *in vitro*[95], these effects have not been studied on human CRC tumors *in vivo* to our knowledge.

In this novel study, designed and executed as a prospective clinical trial, we report our findings of changes in gene expression *in vivo* in human CRCs treated with the selective COX-2 inhibitor, celecoxib. Furthermore, we go on to correlate changes in gene expression with a surrogate biomarker of COX-2, PGE-M.

Methods

Patients

Sixteen patients were recruited at the Vanderbilt Ingram Cancer Center at Vanderbilt University Medical Center (VUMC) and the Tennessee Valley Healthcare System Veterans Affairs Medical Center (VAMC) in Nashville, TN between 2003 and 2005. All patients were diagnosed with rectal cancer and clinically staged I-III according to current AJCC guidelines; patients with known stage IV disease were not eligible for enrollment. All protocols and procedures were approved by the Institutional Review Board at Vanderbilt University Medical Center.

Study Protocol

At the time of diagnosis, patients were asked to abstain from using NSAIDs or selective COX-2 inhibitors for 2 weeks prior to undergoing staging endorectal ultrasound (EUS) and pinch biopsy to obtain both diagnostic and study specimens. Participants

then underwent 5 days of treatment with celecoxib (as Celebrex® , Pfizer, Inc.) 400 mg twice daily by mouth. At the conclusion of treatment, patients then underwent a second biopsy to obtain additional tissue for analysis. At both pre- and post-treatment time points, other clinical specimens were obtained as well, including urine and blood.

Tissue Processing

At the time of biopsy, multiple tissue specimens were obtained from the primary rectal tumor. For each patient, one representative pre-treatment specimen was sent to pathology to confirm the diagnosis of adenocarcinoma. The remaining specimens were immediately flash frozen in liquid nitrogen and transported to the laboratory. The biopsy tissue was then stored at -80° C until further use. All specimens were processed for RNA isolation within 2 weeks of receipt.

COX-2 Immunohistochemistry

Immunohistochemistry for COX-2 was carried out on a representative specimen for all tumors using the DAKO EnVision™ visualization kit. Antigen retrieval was carried out using EDTA and high temperature pressure treatment for 15 minutes. The reaction was quenched by incubating with 0.03% hydrogen peroxide and sodium azide solutions for 5 minutes. Incubation with the primary antibody (Oxford Biomedical Research #PG 27 B rabbit polyclonal, dilution 1:200) was performed for 30 minutes. After washing, incubation with the secondary HRP labeled anti-rabbit antibody with a labeled polymer, provided by EnVision™. After development, tissue expression was scored against a seminal vesicle positive control. A gastrointestinal pathologist (MKW) noted the

presence or absence of COX-2 expression as well as the pattern (epithelial or stromal) of expression.

RNA Isolation

Once a diagnosis of carcinoma was made from the tissue sent to pathology, adjacent biopsy specimens were homogenized into lysis buffer. Isolation of RNA was then carried out using the spin-column method (Qiagen RNeasy® kit). RNA was eluted with 10mM Tris/DepC H_2O at pH 8.0 and the quality and quantity of each specimen were estimated using ultraviolet optical density. Those samples with an RNA concentration of 500 ng/µl and a 260nm/280nm absorption ratio of 1.8 – 2.1 were submitted to the Vanderbilt Microarray Shared Resource for further quality control and analysis prior to gene chip hybridization.

Microarray Hybridization

Samples of RNA isolated from tumor specimens were submitted for microarray analysis to the Vanderbilt Microarray Shared Resource (VMSR). Prior to hybridization, RNA quality was determined using the Agilent 2100 bioanalyzer. Using approximately 1 µg of total RNA, electrophorectic spectra were generated for each sample. Using analysis software from the bioanalyzer, an RNA integrity number (RIN) was generated. Only those specimens with RINs greater than 7.0 were carried forward for hybridization. The hybridization reaction was performed using 5µg of total RNA, and generation of cDNA transcripts from RNA templates was by a first strand reaction which included hybridization to T7dt24 oligo using SuperScript II reverse transcriptase with

deoxynucleotides. Subsequently, a second strand reaction which employed E.Coli DNA ligase, E.Coli DNA polymerase I, RNAse H, T4 DNA polymerase, and deoxynucleotides was performed to complete cDNA synthesis. Next, cDNA was purified using a spin column and biotin-labeled cRNA synthesized using biotinylated nucleotides (CTP and UTP) and T7 RNA polymerase. These cRNA transcripts were purified with a spin column, quantitated, fragmented, and hybridized to the array. After hybridization, arrays underwent staining with phycoerythrin-streptavidin and were scanned for signal.

The Affymetrix U133 Plus 2.0 GeneChip® microarray platform was used and is considered to be comprehensive for the human genome; specifically, the probe sets correspond to approximately 33,000 genes. Finally, analysis of microarray results was performed using software included in the Affymetrix GeneChip® Scanner 3000 microarray analysis software suite.

Measurement of Urine PGE-M

Urine specimens were obtained from 13 of patients in the study before and after treatment with celecoxib. These specimens were aliquoted into two 10 mL samples, which were immediately stored frozen at -80^{o} C until final analysis. Samples were submitted for analysis in a randomized order and the personnel performing the analyses were blinded to the clinical and disease status associated with each specimen.

Urine PGE-M was quantified using liquid chromatography and mass spectrometry (LC/MS) by the methods previously reported.[81] Briefly, one ml of urine per patient was titrated to a pH of 3 by addition of 1M HCL and then 0.5 ml of methyloxime solution (1600 mg in 10 ml 1.5 M sodium acetate, pH 5) was added. After one hour, the

methyloxime was diluted with 10 ml H_20 at pH 3 and each sample was applied to a C-18 Sep Pack (after prepping the Sep Pack with 5 ml MeOH and 5 ml H_20 at pH 3). Samples were washed with 10 ml H_20 at pH 3 followed by 10 ml Heptane and then eluted from the Sep Pack with 5 mL ethyl acetate. The PGE-M internal standard (6.2 ng [$^2H_6$] O-methyloxime PGE-M in 10 µl) was added to the sample and the solution was dried under N_2 at 37^o C and reconstituted in 50 µl of LC mobile phase A, consisting of 95% 5 mM NH4oAC, 5% ACN, and 0.1% HoAc. Each sample was placed through a Spin X filter bullet tube by centrifugation and transferred to a mass spectroscopy vial. A TSQ Quantum Triple Quadrupole Mass Spectrometer was used to quantify the amount of PGE-M per sample. Urine creatinine was also measured and values of PGE-M were reported as ng PGE-M/mg creatinine. Data from a time course study indicates that repeated testing of a single sample results in a coefficient of variance of about 15% (for 45 replicate samples).[81]

Results

At the conclusion of this study, 16 patients had been prospectively enrolled and completed treatment; patient demographic data is provided in Table 3. Briefly, the mean age was 60.4 (SD 9.76 years) and the gender break down was 50% male and 50% female. The majority of the patient population was white and most patients had AJCC stage II or III disease at enrollment. All together, RNA from a total of 32 rectal tumors was hybridized for these patients (one pre- and one post-celecoxib treatment for each patient); 26 urine PGE-M values were also available for correlation with our microarray data (pre- and post-celecoxib specimens for 13 of the 16 patients).

Table 3. Demographic and clinical characteristics of patients in the study

Patient	Age	Gender	Race	AJCC Stage
1	53	Female	Black	3
2	67	Male	White	2
3	52	Female	White	2
4	61	Female	White	3
5	69	Female	White	3
6	47	Male	Black	2
7	65	Female	White	2
8	80	Male	White	3
9	50	Male	White	1
10	62	Female	White	2
11	77	Female	White	1
12	66	Female	White	2
13	58	Male	White	2
14	56	Male	White	3
15	49	Male	White	3
16	55	Male	White	1

COX-2 Expression

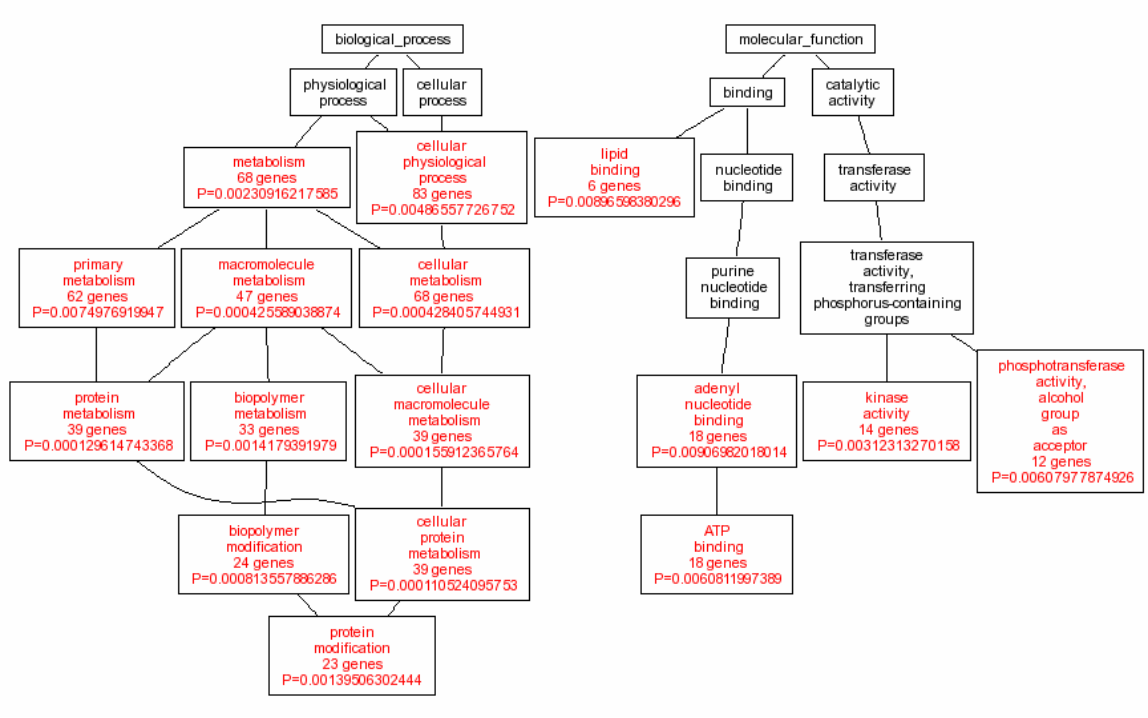
Of the 16 patients in our study, 15 specimens were available to evaluate COX-2 expression by IHC. Eleven out of the remaining 15 tumors expressed COX-2, while 4 tumors did not (data not shown), which was approximately 73% of all specimens assessed. This proportion of COX-2 expressing tumors was found to be consistent with previously published estimates of COX-2 expression in colorectal tumors.[57, 58]

Changes in Gene Expression After Treatment with Celecoxib

After patients underwent the prescribed therapy with celecoxib, microarray data were analyzed using the permutational t-test. From this analysis, 1350 genes were found to be differentially regulated in a significant manner (p-values <0.05). Even after the criteria were tightened (p-values <0.01), a substantial, yet refined list of some 201 genes was left; this included 174 probes that had at least a locus link ID. These data lists were uploaded to WebGestalt (University of Tennessee and Oak Ridge National Laboratory), a web based gene set analysis toolkit that draws from variety of public databases and analysis tools.[96] Pathway information, molecular function, and other genome related queries were then carried out.

Using the refined gene set and Gene Ontology database annotation[97], we were able to view subsets of genes with regard to molecular function, biological process, and cellular component. Using strict criteria (only those categories that included more than 2 genes and had p-values <0.01 based on a hypergeometric test), a classification tree was created to organize our data into the aforementioned categories (figure 6).

Figure 6.



Gene Ontology tree organizing the refined set of genes that changed in rectal tumors after treatment with 5 days of celecoxib. Those with p-values are statistically significant (hypergeometric test)

To evaluate the genes impacted by celecoxib treatment with regard to general biological pathway, we analyzed them with the Kyoto Encyclopedia of Genes and Genomes (KEGG).[98] For more specific pathway analysis genes impacted by celecoxib treatment that had a p-value of <0.05 were analyzed using the Biocarta Pathway project.[99] The results of these analyses are shown in Tables 4 and 5, respectively.

Table 4. KEGG pathway analysis using top significantly changed genes (p<0.01) after celecoxib treatment.

KEGG Pathway	Genes	Entrez Gene IDs	P-value
Purine Metabolism	5	4860, 5167, 5425, 8382, 8833	0.011
Wnt Signaling Pathway	5	1457, 3725, 5579, 56998, 9475	0.008
Focal Adhesion	5	1793, 3725, 5579, 6714, 9475	0.033
Tight Junction	5	1457, 154810, 2017, 5579, 6714	0.004
Pyrimidine Metabolism	3	4860, 5425, 8382	0.044
Butanoate Metabolism	3	157570, 51109, 51400	0.011
Nicotinate and Nicotinamide Metabolism	3	4860, 5167, 55191	0.018

Table 5. Biocarta pathway analysis of genes changed after celecoxib treatment (genes with p<0.05).

Biocarta Pathway	Genes	Entrez Gene IDs	P-value
Signaling of Hepatocyte Growth Factor Receptor	5	1793, 2549, 3725, 5728, 6714	0.028
Rho cell motility signaling pathway	4	10458, 6714, 8395, 9826	0.026
mTOR Signaling Pathway	4	2280, 5164, 5728, 6194	0.022
Nuclear receptors coordinate activities of chromatin remodeling…in carcinoma cells	4	10499, 2962, 6714, 8850	0.009
Skeletal Muscle Hypertrophy…AKT/mTOR Pathway	4	3479, 5164, 5728, 6194	0.029
Role of EGF Receptor Transactivation by GPCRs in Cardiac Hypertrophy	4	183, 1909, 3725, 8038	0.009
p53 Signaling Pathway	3	317, 595, 898	0.049
IGF-1 Receptor and Longevity	3	2688, 3479, 6649	0.018
Role of PPAR-gamma Coactivators in Obesity and Thermogenesis	3	10499, 10891, 6714	0.010
Regulation of PGC-1a	3	10014, 10891, 5465	0.018
The PRC2 Complex Sets Long-term Gene Silencing…Histone Tails	3	2145, 7528, 8726	0.028

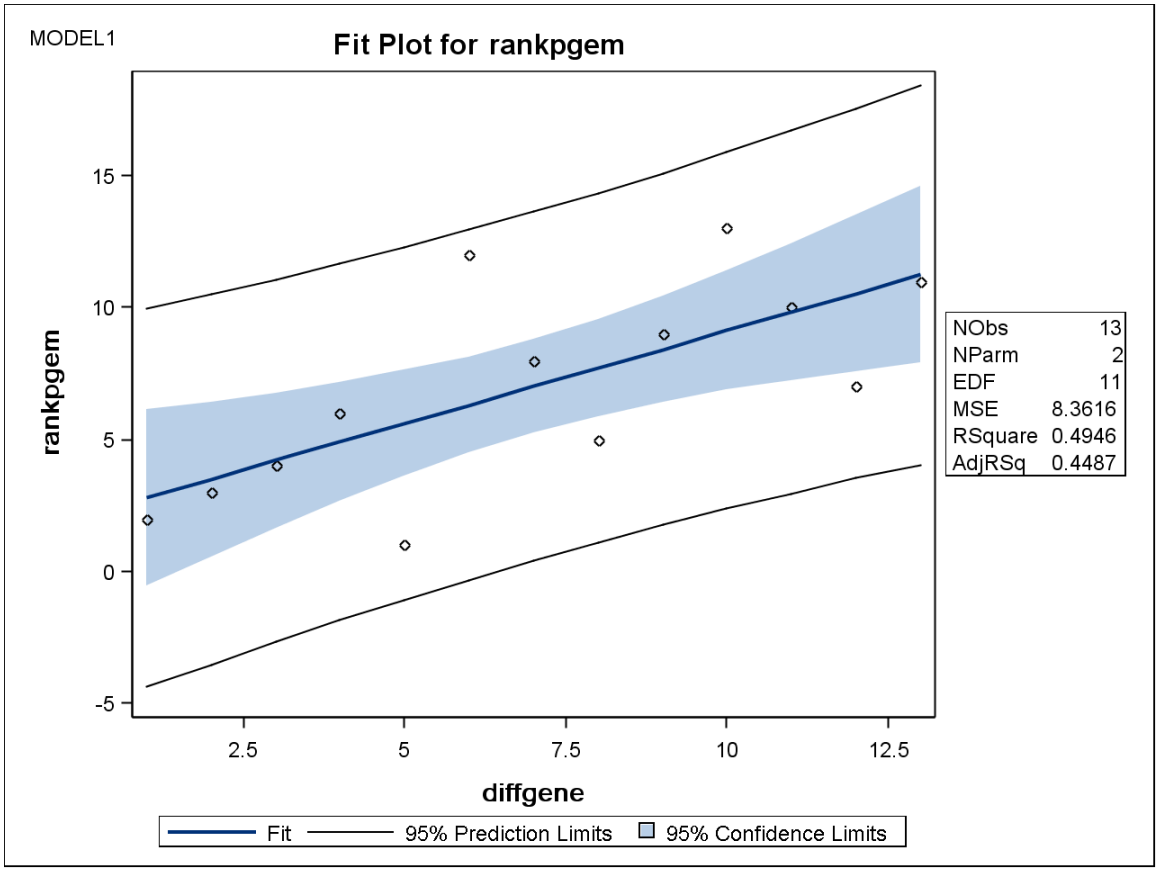
Changes in Urine PGE-M and Gene Expression

To evaluate the relationship between PGE_2 and gene expression, we sought to evaluate possible connections between the two after treatment with celecoxib. A urinary metabolite of PGE_2, PGE-M, was measured before and after celecoxib treatment. We have previously shown this metabolite to be a potential biomarker of colorectal carcinoma.[100] Patients were ranked according to the magnitude of their change in urine PGE-M. These ranks were plotted against changes in expression for each of the 201 genes found to be significantly altered in expression ($p<0.01$) and a regression analyses were performed. Changes in the expression of 13 probes (corresponding to 8 named genes) were found to significantly correlate with changes in urine PGE-M after celecoxib treatment (Table 6). An example, using CTNNBIP1, is shown in figure 7.

Table 6. Probes that showed a significant correlation in fold change versus the change in urine PGE-M after celecoxib treatment.

Affymetrix Probe ID	Gene Symbol	Entrez Gene ID	P-value
219522_at	FJX1	24147	0.029
219324_at	n/a	79159	0.000
241990_at	RHOV	171177	0.024
241903_at	KCTD3	51133	0.029
242444_at	C1QTNF6	114904	0.001
242681_at	CTNNBIP1	56998	0.007
1560181_at	C18orf1	753	0.041
1555778_a_at	POSTN	10631	0.021
57703_at	SENP5	205564	0.035
238542_at	ULBP2	80328	0.001
243825_at	n/a	n/a	0.031
235118_at	n/a	n/a	0.029
230962_at	n/a	n/a	0.007

Figure 7.



Linear regression of the change in urine PGE-M (by rank order) after 5 days of celecoxib treatment versus the fold change in one of the 8 named genes that showed significant correlation with this biomarker. The regression shown here is for beta catenin interacting protein 1 (CTNNBIP1), a negative regulator of Wnt signaling (p=0.007)

Discussion

Inhibition of COX-2 in the treatment of CRC has remained an area of great therapeutic interest. Here, we have presented the results of a prospective clinical trial where we investigated the effects of the selective COX-2 inhibitor, celecoxib, on gene expression in a cohort of patients with rectal carcinoma. To our knowledge, this study is novel in that we sought to evaluate gene expression before and after pharmacologic intervention in the same patients in a prospective clinical setting.

Our analysis of the genes with altered expression after treatment with celecoxib has provided some insight into the biological processes affect by this drug. Using KEGG analysis, we found pathways specific to nucleic acid synthesis and metabolism as well as cell adhesion appear to be affected by celecoxib; likewise, the Wnt signaling pathway sees several of its members negatively regulated after celecoxib treatment. Using Biocarta pathway analysis, we identified members of the HGF and EGF receptor signaling, mTOR, and PPAR-gamma pathways as being differentially impacted after celecoxib treatment. This is significant as many of these pathways have been implicated as being important in the development and progression of CRC.

In addition, we have shown that 13 of the 201 most significantly changed probes correlate with urine levels of PGE-M. This is interesting as it suggests the these genes change in response to interruption of PGE_2 production. Not only does it imply that these genes are affected by the COX-2 specific effects of celecoxib, but that they are specifically influenced by signaling from prostaglandin E_2.

While this present study is small, it was carried out prospectively with a gene chip considered to be comprehensive for the human genome. We have shown that high

quality RNA can be acquired from rectal tumor pinch biopsy specimens; furthermore, using RNA obtained in this manner, microarray technology is feasibly applied in the clinical setting. From the information gleaned in this study, potential targets for therapeutic intervention may be identified that can be combined with celecoxib for maximal effectiveness.

Future studies will be aimed at *in vitro* and *in vivo* validation of these findings. Likewise, trials aimed at evaluating the efficacy of celecoxib in the treatment of CRC, in combination with established surgical, neoadjuvant chemotherapy-radiation, and adjuvant chemotherapy regimens, are ongoing. These studies hold the short-term potential of demonstrating a therapeutic benefit in this disease to use of a drug with a relatively mild toxicity profile. In the long-term, information gathered from these and other gene expression studies may elucidate other targets for intervention using other pharmacologic agents.

CHAPTER IV

SUMMARY AND CONCLUSION

The above body of work has sought to establish a relationship between gene expression patterns and COX-2 activity in CRC. We have also demonstrated that surrogate markers of COX-2 activity (in this case, urinary PGE-M) are feasible markers of colorectal neoplasia and possibly response to therapies directed against COX-2. We have also sought to identify candidate genes and pathways important in the biology of this disease; however, this study is only the beginning. Based on the findings of our experiments, a biological and bioinformatic foundation will be built.

Insight into biological pathways and involved genes will be gained from these studies and expanded toe evaluate higher stage CRC and liver metastases. This in turn can be taken forward for further study in laboratory model systems. From there, long-term survival will become available over the next few years. This will allow a comprehensive analysis of the microarray data in the context of the complete clinicopathological picture. From there gene expression profiles can be modified as it becomes known which patients responded to treatment, recurred, etc. Likewise, new specimens will be collected and prospective application of the gene expression signatures can be undertaken. Ultimately, once confident signatures are developed, they may be used as staging tools (alone or as a component of existing staging systems).

Indeed, COX-2 is a very relevant facilitator of colorectal disease. Clinical trials are proceeding with celecoxib to determine whether it can impact recurrence and survival in CRC. Likewise, we are pursuing urine PGE-M as a biomarker with the assistance of the EDRN. These studies, coupled with our ongoing microarray experiments may lead to a better understanding of how COX-2 affects colorectal disease behavior clinically, as well as the molecular basis for these effects.

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