Abstract. Background: Despite receiving post-operative 5-fluorouracil (5-FU)-based chemotherapy, approximately 50% of patients with stage IIIC colon cancer experience recurrence. Currently, no molecular signature can predict response to 5-FU. Materials and Methods: Mouse models of colon cancer have been developed and characterized. Individual tumors in these mice can be longitudinally monitored and assessed to identify differences between those that are responsive and those that are resistant to therapy. Gene expression was analyzed in serial biopsies that were collected before and after treatment with 5-FU. Colon tumors had heterogeneous responses to treatment with 5-FU. Microarray analysis of pretreatment biopsies revealed that Hp1bp3, a gene encoding heterochromatin protein 1 binding protein 3, was differentially expressed between sensitive and resistant tumors. Conclusion: Using mouse models of human colorectal cancer, Hp1bp3 was identified as a candidate marker of intrinsic 5-FU resistance and may represent a potential biomarker for patient stratification or a target of clinical importance.

Colon cancer is the third most common cancer in the world, and many patients are diagnosed in later stages when cure is less likely. Following surgery, patients with stage IIIC disease have a 5-year overall survival rate of approximately 53% despite adjuvant chemotherapy, because tumors often recur (1). Treatment with 5-FU, an anti-metabolite drug, is commonly used in adjuvant treatment of colon cancer. Patient-specific alterations in the genome and gene expression contribute to heterogeneity in response to chemotherapeutic treatments. Identification of predictors of response in patients prior to treatment would allow clinicians to make educated treatment decisions, so patients whose cancer is predicted to be unresponsive to 5-FU-based chemotherapy need not be exposed to unnecessary side-effects and high cost.

Drug resistance remains a major obstacle in the treatment of many cancer types. There exist two classifications of resistance to chemotherapy. Intrinsic, also known as de novo, resistance exists in a tumor prior to any treatment or chemotherapy. Intrinsic resistance is an initial marker that can guide the first line of treatment. Acquired resistance occurs when cells gain selective advantage during treatment. Acquired resistance is important for treating and understanding recurrent disease. Genetic, epigenetic, and microenvironmental alterations have been implicated in different chemotherapy responses. Resistance can be caused by a variety of mechanisms including alterations of targets, errors in death or survival pathways, DNA damage repair mechanisms, and increased drug efflux (2, 3).

Various studies have attempted to identify predictive markers of response to chemotherapy in colon cancer. For example, metastatic colon cancer can be treated with cetuximab, an anti-epidermal growth factor receptor targeted therapy. KRAS-mutant tumors are associated with cetuximab resistance and have worse prognosis than those with wild-type KRAS.

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type KRAS (4). However, there are no correlations between mutations and tumor responsiveness to 5-FU currently identified. In vitro and retrospective studies have attempted to identify potential predictors of 5-FU response using differences in gene expression. While a number of genes have been associated with resistance to therapy, none have been validated clinically. High expression of a Fanconi anemia protein, FANCJ, correlates to poor response in MLH1-positive tumors and cells treated under multiple 5-FU-based regimens (5). ABCB5, an ATP binding cassette, is overexpressed in therapy-resistant rectal adenocarcinomas following 5-FU-based chemoradiation treatment (6). Markers of sensitivity have also been identified. Low-level amplification of c-Myc occurs in only one-third of patients and correlates with an increase in disease-free survival in patients treated with 5-FU and levamisole in the adjuvant setting (7). WNT pathway misregulation can in turn cause c-Myc overexpression; c-Myc expression can then lead to apoptosis through p21 repression, resulting in 5-FU sensitivity (7). Predictive markers are beginning to be identified especially for particular patient populations, but more work still needs to be done (8, 9).

Clinically useful markers of responsiveness and resistance to treatment have not been established due to a variety of factors. First, removal of primary tumors prior to chemotherapy hinders the identification of markers that are intrinsic to the tumor. Second, surrogate end-point markers, such as overall survival following surgical removal and adjuvant chemotherapy, may not yield sufficiently robust stratification categories for gene signature validation. Third, patient populations within and across many studies have a variety of disease sub-types and treatments, and this variability can prevent further meta-analysis across previously published work. Lastly, changes in gene expression identified in previously treated tumors or cells may identify resistance that is acquired as opposed to intrinsic, which may limit clinical usefulness to patients with metastatic disease when considering adjuvant chemotherapy.

Mouse models can help elucidate many of the challenges associated with the analysis of human cancers. Colon tumor development, growth, and progression in (SWRxB6) F1ApcMin/+ mice treated with dextran sodium sulfate (DSS), herein referred to as DSS-treated F1.Min, are well characterized and can facilitate the identification of important markers in colon tumorigenesis (10, 11). Tumors readily occur in the distal colon and, after an early period of growth, often become static in size over time. The long lifespan of the F1.Min mice is advantageous for a detailed understanding of tumor growth characteristics prior to and following treatment with 5-FU. Definitive end-points for the categorization of sensitivity and resistance that are critical for finding differential markers can be established from in vivo longitudinal imaging. Mouse models can minimize variability since all mice are genetically identical, tumors are initiated in the same manner, and lifestyle differences are minimal.

We used DSS-treated F1.Min to identify molecular markers that predict whether or not a tumor will progress from a benign to malignant state. A 68-gene molecular signature differentiated tumors that remained static from those that grew and progressed (11). Of the 68 genes, five have already been linked to colon cancer and two, Muc2 and Tff2, have been shown to categorize human colorectal cancers into sub-types (12). The levels of Muc2 and Tff2 were higher in tumors that remained static than those that grew and progressed in mice. Similarly, patients with Stage III colorectal cancers with high levels of Muc2 and Tff2 can be treated with surgery and watchful waiting, as the cancers tend to be less aggressive. Thus, our mouse model is likely to provide important insights into tumorigenesis and treatment response in humans. In this study, we used this mouse model to identify markers that predict response or resistance to 5-FU-based chemotherapy.

Materials and Methods

Mouse husbandry and genotyping. All animals were studied in the Wisconsin Institute for Medical Research (Madison, WI, USA) vivarium under protocols approved by the Institutional Animal Care and Use Committee at the University of Wisconsin (Madison, WI, USA) following American Association for the Assessment and Accreditation of Laboratory Animal Care guidelines. All mice were housed in climate-controlled rooms with 12-hlight/dark cycles and given food and water ad libitum except during treatment, as described below. Mice were generated by crossing SWR females (SWR/J; The Jackson Laboratory, Bar Harbor, ME, USA; Stock Number 00689) with C57BL/6 ApcMin/+males (C57BL/6J-ApcMin/J; The Jackson Laboratory; Stock Number 002020). F1.Min hybrids were identified by genotyping at Apc using a previously described PCR assay (13).

Dextran sodium treatment. At or within one week of weaning (24-35 days of age), F1.Min mice were treated with 2 cycles of dextran sodium sulfate (average molecular weight: 500,000; Thermo-Fisher Scientific, Pittsburgh, PA, USA) to increase distal colonic tumor multiplicity as described previously (11). None of the mice, which were monitored throughout treatment, experienced substantial weight loss, loose stool, or gross or occult bleeding which would have precluded further treatment.

Colonoscopy surveillance protocol. Surveillance for colon tumors began four weeks following the final DSS treatment. The distal 4 cm of the colon were visualized once a month using the Karl Storz Coloview system (Tuttlingen, Germany), as previously described (14, 15). The mice were anesthetized using inhaled isoflurane. Phosphate buffered saline enemas were given until the distal colon was clear of fecal material. The colon was insufflated by air delivery through a compressed air pump attached to the operating sheath of the colonoscope. The colonic mucosa was inspected during withdrawal, and digital video of the mucosa was captured.
recorded. Still images were collected of each tumor as encountered. To standardize images for comparison of discrete tumors over time, the colon was maximally insufflated and the colonoscope was positioned so that the entire tumor could be seen with the base of the tumor just at the edge of the screen. Individual tumors were monitored monthly until they became static in size. In vivo tumor sizes were calculated as percent of lumen occluded in 2D images using ImageJ, as previously described (16); such sizes correlated to ex vivo weight, r²=75.6. Tumors that lost more than 26% of their size were considered to be shrinking, whereas those that exhibited a size increase over 13% were considered growing. Tumors that changed between 13% and −13% were classified as static.

5-FU treatment. Twenty-three mice with tumors that exhibited stasis for at least four weeks were selected for treatment with 5-FU. A total of 24 tumors were monitored; 1 mouse had 2 readily distinguishable tumors. These mice were given AIN93G defined diet (Harlan, Madison, WI, USA) for 1 week prior to and throughout 5-FU treatment, because standard rodent chow has components that could potentially alter response. Three days prior to 5-FU treatment, tumor biopsies were obtained using 3 French biopsy forceps that fit in the working channel of the operating sheath housing the colonoscope. Two biopsies were taken per tumor; one for storage in RNALater (Qiagen, Valencia, CA, USA) and the other for fixation in 10% formalin. Daily bolus doses of 5-FU at 40 mg/kg were given intraperitoneally for one cycle of 5-FU treatment or earlier if moribund.

Tissue sample preparation. After one day, formalin-fixed tissue was placed in 70% ethanol until paraffin embedding. Histological analysis was performed on hematoxylin and eosin-stained slides. Tissue in RNALater was stored at −80°C until sample preparation using the SimplyRNA and Maxwell 3000 system (Promega, Madison, WI, USA). Samples were disrupted in buffer with a Kontes Pellet Grinder (Kimple & Chase, Vineland, NJ, USA). A Nanodrop DU-800 (Thermo Scientific, Waltham, MA, USA) was used to quantify yield and assess purity.

Gene expression analysis. Affymetrix Mouse Gene 2.0 ST GeneChips (Affymetrix, Santa Clara, CA, USA) were used for microarray analysis. Hybridization and scanning of the chips were performed by the Gene Expression Center at the University of Wisconsin Biotechnology Center according to a scaled-down, standard Affymetrix protocol. Chips were normalized by robust multiarray averaging using the XPS system20. The two-group EBarrays method with the LNNMV model was used to identify differentially expressed genes (17).

Reverse transcription and qPCR. RNA quantification was performed on a Nano-Drop DU-800 (Thermo-Fisher). cDNA was generated by reverse transcription of 50 ng total RNA, which had been stored at −80°C, according to the manufacturer’s recommendation (ImProm II Reverse Transcription System, Promega). cDNA was stored at −20°C. Untreated tumor cDNA was similarly generated from samples of 8 colon tumors. Control cDNA was generated from Mouse Total Colon RNA (Clontech Laboratories, Mountain View, CA, USA).

Commercially available primer and hydrolysis probe sequences were chosen using the Taqman Assay Search tool (Invitrogen, Thermo-Fisher). The hydrolysis probes: Hp1bp3 (m00802807_m1), Xpo7 (Mm01315615_m1), Myc (Mm00457804_m1), and Tbp (Mm00446973_m1) contained fluorescein amidite as a reporter molecule. Reactions were done in triplicate for each sample. Assays were performed in a volume of 20 µL including Taqman Gene Expression Master Mix (Applied Biosystems, Thermo-Fisher Scientific), primer/hydrolysis probe sets, and 1.5 ng of RNA. A CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) was used with the cycling conditions: 2:00 at 50°C, 10:00 at 95°C followed by 45 cycles of 0:15 at 95°C and 1:00 at 60°C. All values were calculated using the CFX Manager software (Bio-Rad, Hercules, CA, USA).

Results were analyzed using the threshold cycle (CT) measure for relative quantification of gene expression. Values were normalized to an endogenous reference (Tbp) and relative expression was compared to a normalized CT value obtained from Mouse Total Colon control cDNA and subsequently expressed as 2−ΔΔCT. If multiple plates were used, analysis was performed using the gene study program in CFX Manager software using Mouse Total Colon control cDNA to normalize interplate variability. Wilcoxon rank sum and Fisher’s exact tests were performed on qPCR data using Mstat software (Madison, WI, USA).

Results
Colon tumors in DSS-treated F1 Min mice have heterogeneous responses to 5-FU chemotherapy. DSS-treated F1 Min mice received an intraperitoneal injection of 5-FU once daily for five consecutive days. Characteristics of these mice are shown in Table I. Treatment began when tumors had maintained a stable size for at least four weeks, knowing that the tumors were likely to remain stable over time, based on our previous study (11). At first injection, mice were on average 254.3±42.3 days of age. Tumor response was categorized based on changes in tumor size following treatment, which was estimated based on the percentage of lumen occluded by the tumor, as previously described (16). Owing to limitations in our ability to measure changes in size in vivo, tumors were stratified into 2 classes (Figure 1). Several tumors were sensitive (9/24; 38%) with size changes that ranged from −30.1 to −66% (median=−33.2). An equal number (9/24; 38%) exhibited resistance with size changes ranging from −8.8 to 847.

<table>
<thead>
<tr>
<th>5-FU response category</th>
<th>Number of tumors</th>
<th>Age of mouse at necropsy, mean±SD (days)</th>
<th>Gender</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistant¹</td>
<td>9</td>
<td>248±23</td>
<td>2</td>
</tr>
<tr>
<td>Sensitive¹</td>
<td>9</td>
<td>277±57</td>
<td>4</td>
</tr>
<tr>
<td>Unable to categorize¹</td>
<td>6</td>
<td>280±42</td>
<td>3</td>
</tr>
</tbody>
</table>

¹One male mouse had two tumors analyzed, one sensitive and one unable to be categorized. Both tumors are counted herein, thus the number of total mice treated (n=23) is discordant with the total tumors in this table (n=24).

Table I. Characteristics of F1 Min mice treated with DSS and 5-FU.
11.8% (median=−4.1%). The remaining tumors (6/24; 25%) could not be categorized. Upon analysis of H&E-stained histological sections by a board-certified veterinary pathologist, all tumors were determined to be adenomas.

Overexpression of Hp1bp3 in pre-treatment biopsies in tumors resistant to 5-FU. Microarray analysis was performed on biopsies taken prior to treatment. A total of 13 probes were initially identified as differentially expressed among six tumors resistant to 5-FU.
resistant tumors and four sensitive tumors. Two of these corresponded to known genes, Heterochromatin protein 1 binding protein 3 (Hp1Bp3) and Exportin 7 (Xpo7). qPCR analysis of these tumors and the additional resistant and sensitive tumors validated overexpression of Hp1bp3 (Figure 2; p<0.001, Wilcoxon rank sum, two-sided). Differential expression of Xpo7 between sensitive and resistant tumors was not significant upon further analysis. False positives such as this were expected because the limited number of samples resulted in a false discovery rate of 50%.

Expression of Hp1bp3 in colon tumors from DSS-treated mice. To determine the range and pattern of inherent expression of Hp1bp3 in colon tumors from DSS-treated F1.Min, an additional 8 colon tumors from DSS-treated F1.Min controls were combined with the 18 tumors analyzed prior to treatment. Colon tumors from DSS-treated F1.Min (n=26) have variable Hp1bp3 expression; exhibiting a range of 0.56-2.30 (median=1.16) fold change over control normal epithelium (Figure 3). Using values of above and below the median to define two categories of expression (higher and lower), 9/9 sensitive tumors were considered low and 8/9 resistant tumors were considered high. Only 1 resistant tumor was discordant under this dichotomy, having expression levels of 1.04-fold change over normal. The dichotomy identified by an intrinsic difference in Hp1bp3 expression levels was able to predict sensitive and resistant tumors (Fisher’s exact test, p<0.001). Thus, the inherent heterogeneity of Hp1bp3 expression among colon tumors from DSS-treated F1.Min could be useful in the pretreatment prediction of 5-FU response.

Increase of Hp1bp3 expression following 5-FU treatment in responsive tumors. No tumors exhibited complete resolution following 5-FU treatment. We examined the expression of Hp1bp3 in the tumor tissue that remained following treatment. Remaining tumor tissue exhibited overexpression of Hp1bp3 (1.22±0.25-fold change over normal, n=12). There was no longer a significant difference between previous sensitive and resistant tumors (Wilcoxon rank sum test, p=0.44), indicative of the protective effect of high Hp1bp3 expression in certain cell populations within tumors that were sensitive to 5-FU. Surviving tumor tissue remaining in sensitive tumors was resistant to 5-FU treatment and had similar expression levels of Hp1bp3 to intrinsically resistant tumors.

Tumor sensitivity is independent of pre-treatment c-Myc expression. Previous studies have shown that colon tumors with increased c-Myc expression were sensitive to 5-FU and those with low levels of c-Myc amplification were associated with a significant increase in disease-free survival (7). Pre-treatment biopsies were analyzed to test whether c-Myc levels could identify sensitive tumors in DSS-treated F1.Min mice. On average, these tumors had low c-Myc expression compared to normal colon epithelium (0.86±0.27-fold change over the control, n=13). There was no significant difference (Wilcoxon rank sum test, p=0.47) in c-Myc levels between sensitive and resistant tumors (0.80±0.23 versus 0.92±0.32-fold change; Figure 4). Fundamental differences in the underlying biology might not allow for the comparison of human clinical end-points (e.g. disease-free survival), in vitro cell death analysis, and tumor response in DSS-treated F1.Min.

Discussion

The parameters set to define tumor sensitivity or resistance to treatment can be variable among in vivo systems. By understanding the overall growth characteristics of tumors in the DSS-treated F1.Min mouse model, we were able to definitively categorize tumor fates and reduce variability caused by incorrect or vague definitions of response. For example, a previously growing tumor that exhibited stasis in size following treatment might be categorized as sensitive. Conversely, a static tumor remaining static throughout treatment would be considered resistant. Stasis would describe both tumors, despite their difference in response. Many studies using in vitro approaches may not recapitulate the complex anatomical and physiological processes involved in response. Our defined end-points, the use of the DSS-treated F1.Min mouse model, and the discovery approach using the pre-treatment transcriptome resulted in the identification of a novel marker of intrinsic 5-FU resistance in colon tumors, Hp1bp3.

Early molecular changes in a tumor might dictate response to 5-FU-based chemotherapy. Other investigators recently proposed the Big Bang model of tumorigenesis, which postulates that critical molecular changes arise as colon tumors are first forming and consequently some tumors might be “born to be bad” (18). Consistent with this notion, we demonstrated that changes in gene expression that occur very early during tumorigenesis can predict whether or not a tumor could progress from a benign to malignant state (11). Moreover, we demonstrated that some early colon adenomas in mice are intrinsically resistant to 5-FU (19). Therefore, we reasoned biomarkers for treatment response could be identified by comparing gene expression between colon adenomas that are resistant to 5-FU and those that are sensitive. Hp1bp3 was expressed at a higher level in resistant tumors than sensitive tumors. This gene encodes a protein involved in chromatin structure and organization that binds directly to HP1 and linker DNA. Tight binding of HP1BP3 protein to linker DNA at the entry/exit site of nucleosomal DNA involves a long N-terminal extension, globular core, and
were relatively small. The effect of HP1BP3 on chromatin also receiving an injection. The tumors that formed in these mice lacking HP1BP3 produced tumors in only half the mice received the cells viability. A431 cancer cells produced tumors in all mice that condensation during hypoxia. This effect increases tumor cell spectroscopy. One protein cluster included HP1BP3. Functional A431 cancer cells under hypoxic conditions utilizing mass redundancy of other H1 knockout mice (24, 25). Taken together, HP1BP3 clearly has novel and non-

HP1bp3 is required for vitality and growth (23). First, Garfinkel and colleagues demonstrated that Hp1bp3 has two splice variants. One of the two variants is expressed in a wide variety of tissues including the brain, intestine, and lung. Second, they demonstrated that the expression of 383 genes was altered in HeLa cancer cells lacking HP1BP3. The effect on transcription was moderate with the level changing by 1.4- to 2-fold. In addition, the affected cells appear to be cell-type specific. Finally, they demonstrated HP1BP3-deficient neonates either die within 24 h of birth or fail to thrive being significantly smaller even as adult mice than wild-type littersmates. This observation was surprising given the normal phenotype of other H1 knockout mice (24, 25). Taken together, HP1BP3 clearly has novel and non-redundant physiological roles.

Hp1bp3 has been linked to tumorigenesis (26). Dutta and colleagues identified proteins associated with chromatin in A431 cancer cells under hypoxic conditions utilizing mass spectroscopy. One protein cluster included HP1BP3. Functional studies revealed that this protein mediates chromatin condensation during hypoxia. This effect increases tumor cell viability. A431 cancer cells produced tumors in all mice that received the cells via subcutaneous injection. In contrast, A431 lacking HP1BP3 produced tumors in only half the mice receiving an injection. The tumors that formed in these mice were relatively small. The effect of HP1BP3 on chromatin also appears to confer chemo-resistance. The IC_{50} of doxorubicin decreased from 670 ng/ml to 503 ng/ml when comparing A431 cells to those lacking HP1BP3. Thus, HP1BP3 promotes tumorigenesis in certain biological contexts.

It is unknown whether Hp1bp3 has a direct role in pathogenesis of colon cancer. Expression in tumors is variable; therefore, HP1BP3 is not necessary for colon tumors to persist. However, differences in expression of Hp1bp3 might be indicative of global patterns and changes in the genome and transcriptome. Methylation of Hp1bp3 has been identified from patient blood samples as a biomarker for predicting postpartum depression risk. The differential expression is believed to be a marker for global estrogen-related epigenetic changes in the hippocampus (27). The differential expression in mouse colon tumors may also be indicative of broad changes within the genome and nucleus including the state of genomic organization, transcription, epigenetic regulation, and replication. This regulation may not influence c-Myc expression, but may affect other genes of interest. However, since no tumors in this study exhibited complete resolution, and overexpression of Hp1bp3 was observed in tissue that survived 5-FU treatment, these changes to the nucleus and genome may be controlled by more local mechanisms. Further understanding of the mechanisms of HP1BP3 and its involvement with altering the transcriptome, as well as a better understanding of the intratumoral heterogeneity, will be necessary.

Characterization of Hp1bp3 in human colon cancers in The Human Protein Atlas revealed that 5/12 (42%) tumors had medium staining, 5/12 (42%) had low staining, and 2/12 (17%) had no staining (28). Similar Hp1bp3 expression variability was also observed in F1.Min colon tumors prior to treatment, 42% at or below normal levels and 58% above. The human studies were not coupled to outcomes; therefore, further studies in human tumors and cell lines are needed to confirm whether different levels of Hp1bp3 expression can be used in the clinic to stratify patients that may be resistant to 5-FU-based chemotherapy.

Mouse models coupled with state-of-the-art imaging are likely to be powerful tools for the identification of biomarkers and predictors of tumor response to chemotherapy. We identified one potentially useful marker in this initial study. A much larger study with even better mouse models is likely to reveal more potential predictors of 5-FU response and further characterization may identify pathways involved in Hp1bp3 function. We recently published a report demonstrating that colon cancers with mutations in multiple drivers in mice are highly aggressive and often metastasize to the liver (29).

In conclusion, we identified Hp1bp3 overexpression as a potential novel marker for intrinsic 5-FU resistance. Longitudinal monitoring of colon tumors in DSS-treated F1.Min mice provided a better understanding of tumor behavior prior to and following treatment. Using size parameters of resistance and sensitivity derived from the natural tendency of these tumors to become static in size, we discovered the differential expression of Hp1bp3 using microarray and qPCR technologies. Hp1bp3 has variable expression in colon tumors from DSS-treated F1.Min mice. Tumors with high expression prior to 5-FU treatment remained unchanged, whereas those with lower expression shrank. All tumor tissue that remained following treatment, these changes to the nucleus and genome may be controlled by more local mechanisms. Further understanding of the mechanisms of HP1BP3 and its involvement with altering the transcriptome, as well as a better understanding of the intratumoral heterogeneity, will be necessary.

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Competing Interests

The Authors declare that they have no competing interests.

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