Longitudinal Assessment of Colonic Tumor Fate in Mice by Computed Tomography and Optical Colonoscopy

Benjamin Y. Durkee, PhD
Department of Medical Physics
University of Wisconsin – Madison, Madison, Wisconsin

Kazuhiko Shinki, PhD
Department of Radiology
University of Wisconsin – Madison, Madison, Wisconsin

Michael A. Newton, PhD
Department of Biostatistics and Medical Informatics and Department of Statistics
University of Wisconsin – Madison, Madison, Wisconsin

Caitlin E. Iverson, BS
McArdle Laboratory for Cancer Research – Department of Oncology
University of Wisconsin – Madison, Madison, Wisconsin

Jamey P. Weichert, PhD
Department of Medical Physics and Department of Radiology
University of Wisconsin – Madison, Madison, Wisconsin

William F. Dove, PhD
McArdle Laboratory for Cancer Research – Department of Oncology and Laboratory of Genetics
University of Wisconsin – Madison, Madison, Wisconsin

Richard B. Halberg, PhD
McArdle Laboratory for Cancer Research – Department of Oncology
University of Wisconsin – Madison, Madison, Wisconsin
Current address: Department of Medicine, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin

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Correspondence to:
William F. Dove
McArdle Laboratory for Cancer Research
1400 University Avenue
Madison, Wisconsin 53706
Phone: 608-262-4977; Fax: 608-262-2824
dove@oncology.wisc.edu
Abstract

Rationale and Objectives: The purpose of this study was to evaluate the relative merits of microCT colonography (mCTC) and optical colonoscopy (OC) for longitudinal studies of colonic tumors in mice. Materials and Methods: Colonic tumors in mice carrying the Min allele of Apc were followed over a period of several weeks using microCT colonography (mCTC) and optical colonoscopy (OC). A total of 146 colonic tumors were monitored: 62 in 32 untreated Min mice, 53 in 43 Min mice treated with 5-fluorouracil (5-FU), and 31 in 17 Min mice treated with piroxicam. Results: Colonic tumors in Min mice had three different spontaneous fates: 29 grew, 24 remained static, and 9 regressed. Treating Min mice with 5-FU increased the percentage of regressing tumors from 15% to 58%. The response was dependent in part on the initial size of the tumor. By contrast, treating Min mice with piroxicam did not alter colonic tumor fate. Conclusions: mCTC and OC can be used to determine the spontaneous fates of colonic tumors in mice and to document their individual responses to treatment. The ability to follow individually annotated colonic tumors reduces the number of mice needed for testing.

Keywords: mouse models of human colorectal cancer; microCT colonography; optical colonoscopy; longitudinal studies; tumor fate mapping
Introduction

Murine models of human colorectal cancer afford investigators the opportunity to rapidly test newly developed treatments. The \textit{Apc}^{\text{Min}\text{+}} (Min) mouse carries a nonsense mutation in codon 850 of the \textit{Adenomatous polyposis coli} (\textit{Apc}) gene and develops benign adenomas along the entire length of the intestinal tract (1, 2). This model has been used over the past 18 years to test at least 269 treatments, including dietary supplements, NSAIDs, and exercise (3). For example, Jacoby and his colleagues exposed 15 Min mice to 100 ppm piroxicam via the diet for 42 days (4). Tumor multiplicity in a 4 cm segment of the distal small intestine was reduced from 10 ± 2 to 2 ± 1. This protective effect of piroxicam in the small intestine has been confirmed in several independent studies (5-7).

A limitation of genetic mouse models for human colon cancer is that tumors develop primarily in the small intestine instead of the colon (8). The low multiplicity of colonic tumors leads to the need for large experimental cohorts of mice for statistical significance in studies with a traditional cross-sectional design. This limitation can be overcome by longitudinally monitoring individually annotated colonic tumors in mice, where the statistical power depends on the ability to monitor the tumor response rather than on tumor multiplicity. \textit{In vivo} imaging modalities such as MRI, mCTC, and OC may provide complementary tools to monitor longitudinally the biology and therapeutics of colonic tumors (9-12).

Hensley, Chang, and Clapper demonstrated that colonic tumors in Min mice with a maximum diameter of at least 1.5 mm can be consistently identified by MRI (9). Tumor volume was estimated from images using a novel approach to planimetry in 3D data sets. The estimates correlated with wet weight as determined after excision at necropsy. Similarly, Pickhardt, Halberg, and colleagues have demonstrated that colonic tumors in the mouse with a maximum diameter greater than 2 mm can be readily detected by mCTC, with a sensitivity of 93% and specificity of 98% (11). In addition, estimates of tumor volume from mCTC images are both precise and accurate, enabling changes as small as 16% to be detected by mCTC with great confidence (13). Becker and his colleagues also demonstrated that colonic tumors in the mouse are readily visualized by OC (14). Tumor size was estimated as the portion of the intestinal lumen occluded by the tumor. Recently, Hensley and colleagues have improved these estimates of tumor size from optical images using a measuring probe as reference. These estimates are correlated with the 3D measurements made by MRI (Pearson coefficient 0.85) and with wet weight at necropsy (Pearson coefficient 0.94) (10).

In this study, the relative merits of mCTC and OC for longitudinal studies of the spontaneous fates of colonic tumors in the Min mouse and the response to drugs including 5-FU and piroxicam are evaluated.
Methods

Mice. All animal studies were conducted under protocols approved by the Institutional Animal Care and Use Committee at our institution, following the guidelines of the American Association for the Assessment and Accreditation of Laboratory Animal Care. C57BL6/J (B6) and B6 Tyr<sup>2c/2c</sup> mice carrying the Min allele of Apc were used because this model has been widely used for drug testing (3). All mice were maintained on a defined diet AIN93G (Harlan Teklad, Madison, WI) and water ad libitum, except as explicitly stated for treated mice.

Treatment with 5-FU or piroxicam. Mice were treated with either 5-FU (n=53) or piroxicam (n=17) (Figure 1). Treatment with 5-FU consisted of daily bolus doses (40 mg/kg i.p.) for two-week cycles of 5 days on followed by 9 days off. Cycles continued until the mouse was euthanized when moribund or at the end of the treatment regime. Either mCTC or OC was performed at the end of each cycle. Piroxicam was given at 100 ppm in the AIN93G diet for up to six weeks. Either mCTC was performed every other week or OC was performed every week.

Untreated mice (n=32) served as controls. The extent of longitudinal monitoring of tumors in these mice varied: mCTC was performed multiple times over a period of three to eight weeks on five of these mice (Figures 2 and 3), mCTC was performed only twice over a period of two to four weeks on seven mice (Figure 3), and OC was performed multiple times over a period of 6 weeks on 20 mice (Figure 4A).

MicroCT Colonography. Mice were prepared and scanned using Siemens microCAT II or Inveon (Knoxville, TN) as described previously (13), except that the x-ray tube voltage was 70 kVp with a current of 500-900 μA depending on the particular scanner. The isotropic voxel size was 100x100x100 microns. Higher resolution is possible on both the microCAT II and Inveon systems. However, increasing the resolution to 50 microns from 100 microns without changing the signal-to-noise ratio would require a 16-fold increase in radiation dose to the mouse. Each scan took approximately 10 minutes to complete. Resulting image quality was assessed using our standard good-fair-poor grading system (13). Reader precision correlates with image quality, which is dependent on air contrast, motion artifact, tumor shape, tumor margin definition and the presence of residual fecal material (13). Poor quality images were discarded and the scan was immediately repeated after the colon was flushed with warm PBS and insufflated a second time. Tumor volumes were estimated using a semi-automated five-step process that is both accurate and precise (13). Tumor regression and growth were defined by a 16% or more change in tumor volume, which corresponds to scoring at the 95% confidence level (13). Stasis is scored as a change of less than 16%. Tumor multiplicities in the colons of Min animals are low, permitting each tumor to be individually annotated by its distance from the anus.

Multiple Regression Analysis. Predictors of tumor growth and regression were determined using multiple linear regression analysis. Change in tumor volume dV/dt was modeled as dV/dt=μ+ε, where μ is a linear combination of contributions from various predictors, including a treatment/control indicator, sex, initial tumor volume, age, and change in mouse weight, and where ε is an error term. Tumor volume and age of the mouse were lag variables, meaning they were taken at the previous observation. Separate regression models were constructed for the 5-FU and piroxicam treatments. In each case, the Akaike Information Criteria (16) was used to eliminate predictors that gave a negligible contribution to μ. Bootstrapping was used to obtain standard errors, confidence intervals, and p-values (17). Considering possible correlation of measurements within individuals, bootstrapping was performed at the animal level with mice that were randomly selected from the dataset with replacement. From these individuals, the regression model was re-estimated and the process was repeated 1,000 times. Confidence
intervals were constructed using the bootstrap percentile method. $P$-values were calculated from a normal approximation using the bootstrap mean and standard deviation of each coefficient.

**Optical Colonoscopy.** OC using the Coloview system was performed by one operator (RBH) with 18 months of experience using a procedure described previously (12). Mice were anesthetized using isoflurane and immobilized on a surgical platform. The colon was flushed with warm PBS. A small 1.5 mm rigid endoscope in an operating sheath was inserted through the anus into the colon. The colon was insufflated with air using a small pump and photographs were obtained as the endoscope was withdrawn. This procedure took about 5 minutes on average. Tumor scoring was done according to the protocol established by Becker and his colleagues, grading size relative to the cross-sectional area of the colonic lumen (12). The colon reached maximal distention during each procedure because a small reduction in air flow resulted in partial collapse; consequently the cross-sectional area was relatively constant over time. Tumor behavior from week to week was assessed as growth, stasis, or regression.

**Treatment with DSS plus piroxicam.** The combined effect of DSS and piroxicam was tested by comparing treated Min mice (n=17) and controls (n=19). (These mice are not included in Figure 1 because they were not monitored by mCTC or OC). Weanlings received drinking water containing 4% DSS for 4 days, standard water for 17 days, and then water with 4% DSS for another 4 days as described previously (15). Two weeks after the second cycle of DSS, 100 ppm piroxicam was added to the AIN93G diet (Harlan Teklad, Madison, WI). Some mice (n=7) were euthanized at 150 days of age, and others (n=10) were allowed to age until moribund. The intestinal tract was removed, cut longitudinally, rinsed with PBS and then 70% ethanol, fixed in 10% buffered formalin overnight, and stored in 70% ethanol. Tumor multiplicity was scored under an Olympus dissecting microscope at 10x magnification.

**Power.** A statistical model similar to Ware (1985) was developed to relate the power of a prospective longitudinal study to that of a conventional cross-sectional study to detect an effect of a treatment (18). Suppose that $X_{g,t,i}$ denotes a measured response (e.g. tumor volume) on mouse $i$ at time $t$ and in some treatment group $g$. When the response is suitably scaled, it is naturally decomposed as, $X_{g,t,i} = \mu_{g,t} + \alpha_{t,i} + \epsilon_{g,t,i}$, where $\mu_{g,t}$ is the population average response for animals at that time under that treatment, $\alpha_{t,i}$ is a random animal effect representing the deviation that the well-measured response, $\mu_{g,t} + \alpha_{t,i}$, differs from the population average for animal $i$ at time $t$, and finally $\epsilon_{g,t,i}$ is a random measurement error. The case of two time points $(1,2)$ and two treatment groups $(a,b)$ is most simple to analyze, and allows us to compare two designs for detecting a non-zero treatment effect $\theta = (\mu_{b,2} - \mu_{a,2}) - (\mu_{b,1} - \mu_{a,1})$. A balanced longitudinal design in which a total of $T_L$ animals are measured at two times has the same power as a balanced cross-sectional design (two time points) in which a total of $T_C$ animals are measured once each, where $T_L = (1/2) T_C((1 + (1 - \rho)\kappa)/(1 + \kappa))$. Here $\kappa$ is the ratio of the animal-specific variance of $\alpha_{t,i}$ to the technical measurement variance of $\epsilon_{g,t,i}$, which is liable to be large for many response types, and $\rho$ is the correlation between effects $\alpha_{t,i}$ taken on the same animal at different times. The case of low technical noise (large $\kappa$ ) reveals that $T_L \leq T_C$, i.e., the total number of animals required in the longitudinal design never exceeds the total in the cross sectional design. Furthermore, with positive $\rho$, the total number of measurements in longitudinal ($2T_L$) is less than the total number for cross sectional designs ($T_C$).
Results

The range of tumor fates in control mice. Colonic tumors in Min mice were observed by mCTC to have three distinct spontaneous fates: growth, stasis, and regression. A group of untreated mice bearing tumors with an initial volume less than 5 mm$^3$ were imaged several more times over a period of three to eight weeks. For the majority of tumors (7/10) in these controls, the volume increased significantly over time (Figures 2A and 2B; Wilcoxon Rank Sum Test, $p<0.05$). However, the increase in volume was rarely linear. In several cases, growth was followed by spontaneous regression. Notably, one tumor apparently regressed completely.

Tumor regression in Min mice does not appear to be a consequence of the radiation exposure involved in mCTC. Colonic tumors in Min mice were also followed over a period of six weeks by OC; many (17/43) grew, most (22/43) remained static, and a few (4/43) regressed (Figure 4). Thus, the distinct spontaneous fates of colonic tumors in Min mice can be observed by either mCTC or OC.

Response of colonic tumors to 5-FU. Colonic tumors in Min mice were monitored by mCTC before, during, and after treatment with 5-FU. The majority of colonic tumors with an initial volume of more than 5 mm$^3$ in mice treated with at least a single cycle of 5-FU (12/16) regressed (Figure 3). By contrast, the response of smaller colonic tumors was much less dramatic. Some tumors with an initial volume of less than 5 mm$^3$ in mice treated with at least one cycle of 5-FU (7/18) regressed, but the majority (10/18) actually grew (Figure 3). Statistical modeling indicated that sex, initial tumor volume, and treatment with 5-FU were significant predictors of tumor fate, but age and body mass were not (Table 1). Interestingly, among tumors that regressed, the first cycle of 5-FU had a significantly greater effect than subsequent cycles of 5-FU ($p < 0.001$).

Colonic tumors in Min mice were also monitored by OC before and after a single cycle of 5-FU (Figure 4B). The majority (12/19) shrank dramatically after treatment and many displayed areas of discoloration and hemorrhaging. Again, responsive tumors tended to be larger in size, occluding more than half the lumen before treatment, while resistant tumors tended to be smaller.

Resistance of colonic tumors to piroxicam. Treatment with piroxicam does not affect colonic tumors in Min mice. A total of 11 colonic tumors in treated mice were followed by mCTC (Figure 3). Statistical modeling indicated that piroxicam was not a significant predictor of colonic tumor fate ($p = 0.248$). An additional 20 colonic tumors in treated mice were followed by OC (Figure 4C). The relative frequencies of growing versus regressing tumors were similar in treated compared to control mice. Further, histological examination of five colonic tumors from treated mice revealed no signs of regression (data not shown). However, an effect of piroxicam on tumors in the small intestine of Min mice was confirmed. Treated mice developed 15 ± 18 tumors in the small intestine, whereas control mice developed 117 ± 28. This observation ensures that the drug was being delivered effectively in our study. Because piroxicam was delivered systemically in the diet, the failure of colonic tumors to respond while tumors in the small intestine are responsive presents an interesting conundrum for further investigation.

DSS-piroxicam treatment of Min mice alters tumor distribution and leads to enhanced longevity. The regional distribution of tumors and longevity in the Min mouse model may be significantly altered by treatment with a combination of agents. Min mice were first treated with DSS to increase the multiplicity of colonic tumors (15) and then piroxicam. Treated mice
developed 31 ± 21 tumors in the small intestine and 10 ± 6 in the colon, compared to controls with 122 ± 45 and 1 ± 1, respectively. This reduction in the multiplicity of small intestinal tumors presumably caused the increased longevity of these treated mice: they lived 131 ± 24 days, whereas controls lived 100 ± 18 days ($p = 6 \times 10^{-6}$, Wilcoxon Rank Sum Test). Five of the mice being treated with piroxicam were alive at 200 days of age, despite carrying multiple colonic tumors, as evidenced by optical colonoscopy.
Discussion

This study has explored the relative merits of mCTC and OC for longitudinal studies. Colonic tumors in both control and treated Min mice show any of three distinct fates: growth, stasis, and regression. This spectrum of fates mirrors that of human colorectal cancers (19). Not surprisingly, changes in tumor size were rarely linear, for example, growth was often followed by stasis or even regression (Figure 2B). The dynamic nature of tumor fate underscores an advantage of multiple measurements over an extended period of time as compared to a single measurement at a fixed endpoint. Several factors affected tumor fate in Min mice including sex of the mouse, treatment with 5-FU, and initial tumor volume.

Humans and murine models exhibit a sex bias in neoplasia of the colon. Two recent epidemiological studies indicated that males are at higher risk for developing colorectal cancer than females in human populations (20,21). This difference may reflect either the protective effect of female hormones, or sensitization by male hormones, or a combination of the two. The rate of colon cancer was reduced by 37% in postmenopausal women given estrogen and progestin relative to postmenopausal women given a placebo (22). In the present study, colonic tumors in Min males were significantly larger than those in Min females. Similarly, gender effects have been observed in other murine models of human colorectal cancer. For example in the Pirc rat, which carries a knockout allele of the \textit{Apc} gene, on the F344 background males develop on average 14 colonic tumors, versus 7 in females (23). Thus, the effect of sex hormones on neoplasia in the colon appears to be conserved across species.

The response of colonic tumors in mammals to 5-FU is heterogeneous. This drug acts principally by inhibiting the reductive methylation of deoxyuridine monophosphate to thymidine monophosphate. A recent meta-analysis determined the efficacy of 5-FU in the clinic (24). A total of 1390 patients with advanced colorectal cancer were randomized into 19 trials; tumor response was only 11%. Further, a review based on the results from several independent studies reported that tumor response to 5-FU ranged from 3 to 43% and that responding tumors inevitably develop resistance to the drug (25). In our study, the majority of tumors (22/34) in Min mice responded to a single cycle of 5-FU, as monitored longitudinally by mCTC and OC. Larger tumors regressed, whereas smaller tumors grew more slowly (Figures 2, 3 and 4; Table 1). Interestingly, a single cycle of 5-FU was more effective than multiple cycles, indicating that many tumors acquire resistance. OC affords us the opportunity to biopsy tumors before and during treatment. The tissue collected can then be analyzed at a molecular level to discriminate prospectively between tumors with different fates and responses.

mCTC and OC are complementary imaging modalities for the longitudinal study of colonic tumors in mice. Both are useful in objectively estimating change in tumor size in serial observations. The advantage of mCTC over optical endoscopy in the accuracy and precision of the measurement of tumor sizes in animals (13) continues to hold. The recent improvements in OC reported by Hensley and colleagues give only two-dimensional estimates (10). Detecting tumor size change with certainty is an essential first step to testing the efficacy of new chemotherapeutics. mCTC also affords users the ability to examine tumors in the proximal region of the colon that are inaccessible by OC because the proximal tumors are simply beyond the reach of the rigid endoscope due to the curvature of the colon or because a more distal tumor completely blocks access through the lumen.

OC has several distinct advantages over mCTC in terms of efficiency. OC requires about 5 minutes per mouse, whereas mCTC takes 10 minutes for scanning and an additional 10 minutes for volume measurement. The OC system is significantly less costly and consequently
more readily available. Further, the OC system is portable, whereas microCT requires a dedicated space. Finally, OC has investigative advantages. The mucosa can be stained with methylene blue to allow scoring of very small lesions and better characterization of tumor morphology (14,26). Morphologic changes are apparent during regression (Figure 4B) and progression (27). Flat adenomatous polyps may progress directly to invasive carcinoma without passing through a polypoid stage (27); this type of neoplastic transformation would be obvious by OC but not mCTC. Biopsies can be taken and analyzed either histologically or molecularly with yields of total RNA ranging from 100 to 400 ng (RBH and WFD, in progress).

Risks to the mouse are minimal but real for each modality. Both require a similar preparation including anesthesia (arguably the biggest risk to moribund mice), and flushing the colon with saline. Bowel perforation is possible with OC, although the overall risk is relatively small for experienced users. Lethal or noxious effects of x-rays are possible but unlikely in microCT; rodents have long been known to be resistant to ionizing radiation (28). It is important to ask whether tumor stasis and regression are caused by the radiologic doses used in the documentation of tumor fate. Note that the same spectrum of tumor fates can be observed by optical colonoscopy in the absence of ionizing radiation.

The power of a longitudinal study design implemented with mCTC and OC can be compared to that of a cross-sectional study design. Theoretical analysis reveals that for a given power, a longitudinal study design requires fewer animals than a cross-sectional study design to detect an effect of treatment (see Methods). Further, the number of measurements in a longitudinal study design is reduced if a positive correlation exists between successive volume measurements. For example, for the correlation index of 0.39 that is observed for the control data in Figure 3, the number of scans (mCTC) or visits (OC) required for a given statistical power is reduced by a factor of 3.

Two limitations of this study need to be acknowledged. The first is that tumors were monitored over short period of time. B6.Min mice have a massive tumor burden, particularly in the small intestine. Consequently, they become severely anemic and typically die by 100 days of age. The second limitation is that the tumors were most likely benign adenomas rather than advanced cancers. These limitations can be overcome by refining the Min model or by developing better murine models of human colorectal cancer. In this study, treating B6.Min mice with a combination of piroxicam and DSS reduced the overall tumor burden and changed the ratio of tumors between the small intestine and the colon, favoring the latter. This created a situation that is more relevant to human disease, and increased the lifespan of the animals. Intestinal tumors in long-lived Min mice often become invasive adenocarcinomas that occasionally metastasize to regional lymph nodes (29). These refinements should improve the use of Min and other mouse models for identifying effective chemopreventive and chemotherapeutic agents.

In summary, mCTC and OC can be used to determine the spontaneous fates of colonic tumors in mice and to document their individual responses to treatment. The ability to follow individually annotated colonic tumors reduces the number of mice needed for testing.
Reference List


### Table 1. Several factors affect tumor volume in treated mice and controls.

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<th>Upper</th>
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<td>-0.004</td>
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<td>-0.143</td>
<td>0.016</td>
<td>0.121</td>
</tr>
</tbody>
</table>

*a This table includes only results from the regression model constructed for the 5-FU treatment.

*b A negative value indicates that the factor correlated with a decrease in tumor volume.

*c Initial volume is the volume of the tumor at Day 0. Some tumors were significantly larger than others. Large tumors were more likely than small tumors to regress spontaneously or in response to treatment.

*d Tumors were larger on average in males than females.
Figure Legends

Figure 1. The experimental designs of the longitudinal studies are represented schematically with the number of mice, the number of tumors, and tumor fates shown for all groups of mice.

Figure 2. The fates of colonic tumors in Min mice were assessed non-invasively by mCTC. Min controls were scanned at least three separate times between 50 and 105 days of age. (A) The volume of each individually annotated tumor was estimated from images collected during a visit (volume of tumor indicated by arrow = 9.73 mm³). (B) Colonic tumors in Min mice exhibit three different spontaneous fates: growth, stasis, and regression.

Figure 3. Tumor fate is affected by a single cycle of 5-FU or multiple cycles of 5-FU, but not piroxicam. Mice were scanned before treatment (Day 0) and immediately prior to sacrifice. Tumor fate following treatment with 5-FU was affected by initial volume: <5 mm³ (left) and > 5 mm³ (right). Regression (dotted lines) and growth (solid lines) are defined by a 16% (95% confidence level) or more decrease or increase in tumor volume, respectively. In the panel for controls with initial volume > 5 mm³, two of the lines for regressing tumors overlap; there were 4 regressing tumors in this set.

Figure 4. The fates of colonic tumors in Min mice were assessed by OC. (A) A tumor in an untreated mouse grew significantly in 3 weeks. (B) A large colonic tumor (white arrow) regressed dramatically following a single cycle of 5-FU treatment, but a smaller one (black arrow) did not. Another regressing tumor had an opaque mass at the center of the lesion (red arrow). (C) A colonic tumor did not respond to a 3-week treatment with piroxicam.
12 mice were untreated

Tumor fates:
12/19 (63%) growth
2/19 (11%) stasis
5/19 (26%) regression

7 mice were scanned for 3-8 weeks by mCTC

Tumor fates:
5/11 (45%) growth
3/11 (27%) stasis
3/11 (27%) regression

13 mice were treated with piroxicam

Tumor fates:
3/15 (20%) growth
2/15 (13%) stasis
10/15 (67%) regression

13 mice were treated with one cycle of 5-FU

Tumor fates:
9/19 (47%) growth
1/19 (5%) stasis
9/19 (47%) regression

47 mice were scoped weekly by OC

20 mice were untreated

Tumor fates:
17/43 (40%) growth
22/43 (51%) stasis
4/43 (9%) regression

10 mice were treated with piroxicam

Tumor fates:
9/20 (45%) growth
10/20 (50%) stasis
1/20 (5%) regression

17 mice were treated with one cycle of 5-FU

Tumor fates:
1/19 (5%) growth
6/19 (32%) stasis
12/19 (63%) regression

13 mice were treated with multiple cycles of 5-FU

Tumor fates:
3/15 (20%) growth
2/15 (13%) stasis
10/15 (67%) regression

13 mice were treated with one cycle of 5-FU

Tumor fates:
9/19 (47%) growth
1/19 (5%) stasis
9/19 (47%) regression

Tumor fates:
3/11 (27%) growth
3/11 (27%) regression
4/11 (36%) regression

Tumor fates:
7/19 (37%) growth
5/19 (26%) stasis
7/19 (37%) regression

17 mice were treated with multiple cycles of 5-FU

Tumor fates:
6/19 (32%) growth
12/19 (63%) regression

10 mice were treated with piroxicam

Tumor fates:
9/20 (45%) growth
10/20 (50%) stasis
1/20 (5%) regression

13 mice were treated with one cycle of 5-FU

Tumor fates:
9/19 (47%) growth
1/19 (5%) stasis
9/19 (47%) regression

10 mice were treated with piroxicam

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1/20 (5%) regression

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3/15 (20%) growth
2/15 (13%) stasis
10/15 (67%) regression
A

Mouse age (days)

Mouse 1 (1 tumor)
Mouse 2 (2 tumors)
Mouse 3 (2 tumors)
Mouse 4 (2 tumors)
Mouse 5 (3 tumors)

B

Tumor volume (mm$^3$)

Mouse 1 (1 tumor)
Mouse 2 (2 tumors)
Mouse 3 (2 tumors)
Mouse 4 (2 tumors)
Mouse 5 (3 tumors)

Mouse age (days)
Initial volume < 5 mm³

Control (no treatment)

5-FU (1 cycle)

5-FU (≥ 2 cycles)

Piroxicam

Initial volume > 5 mm³

Figure 3