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Small animal microCT colonography

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Abstract

Microcomputed tomography colonography (mCTC) is a new method for detecting colonic tumors in living animals and estimating their volume, which allows investigators to determine the spontaneous fate of individually annotated tumors as well as their response to chemotherapeutics. This imaging platform was developed using the Min mouse, but is applicable to any murine model of human colorectal cancer. MicroCT is capable of 20 micron resolution, however, 100 microns is sufficient for this application. Scan quality is primarily dependent on animal preparation with the most critical parameters being proper anesthesia, bowel cleansing, and sufficient insufflation. The detection of colonic tumors is possible by both 2D and 3D rendering of image data. Tumor volume is estimated using a semi-automated five-step process which is based on three algorithms within the Amira software package. The estimates are precise, accurate and reproducible enabling changes in volume as small as 16% to be readily observed. Confirmation of mCTC observations by gross examination and histology is sometimes useful in this otherwise non-invasive protocol. Finally, mCTC is compared to other newly developed small animal imaging platforms including microMRI and microoptical colonoscopy. A major advantage of these platforms is that investigators can be perform longitudinal studies, which often have much greater statistical power than traditional cross-sectional studies; consequently, fewer animals are required for testing.

Keywords

Multiple intestinal neoplasia (Min); microCT; microMRI; microoptical colonoscopy; virtual colonography; murine models; colorectal cancer; chemotherapy

Introduction

Human colorectal cancer is a leading cause of cancer deaths among women and men in the United States. About 150,000 new cases were diagnosed and 50,000 deaths occurred in 2008 [1]. A common feature of colorectal cancers is the loss of the *Adenomatous Polyposis Coli* (APC) gene [2]. This gene encodes a protein that regulates the level of β -catenin and consequently is necessary to maintain homeostasis in the intestine [reviewed in 3]. In the absence of APC activity, β -catenin translocates from the cell membrane to the nucleus, where it interacts with other transcription factors to alter gene expression. Patients carrying a germline

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mutation in APC develop hundreds to thousands of colorectal tumors by the third decade of life [4].

Mice carrying the *Min* allele of *Apc* are also predisposed to the development of tumors along the entire length of the intestinal tract [5]. Tumor multiplicity and progression are affected by genetic factors. For example, B6.Min mice develop on average 100 tumors and die by 150 days of age, whereas (SWRxB6)F1.Min hybrids develop on average 15 tumors and live for 370 days [6]. This difference in tumor multiplicity reflects the status of the *Mom1* locus, which encodes the secretory phospholipase A₂ and at least one other modifier of intestinal tumorigenesis [7]. Tumors from B6.Min mice are almost always benign adenomas, whereas tumors from (SWRxB6)F1.Min hybrids are often invasive adenocarcinomas that occasionally metastasize to regional lymph nodes [6]. Presumably, long-lived (SWRxB6)F1.Min develop advanced cancers because time allows the necessary genetic alterations that drive tumor progression. Thus, different strains of the Min mouse model permit the testing of chemopreventive and chemotherapeutic agents.

Intestinal tumorigenesis in the Min mouse is also affected by environmental factors. Nagamine and colleagues found that *Rag2*-deficient Min mice infected with *Helicobacter hepaticus* had a higher incidence of colonic tumors than controls [8]. This effect was observed with other mouse models. Nagamine and colleagues found that *IL10*-deficient mice affected with *Helicobacter hepaticus* and treated with the carcinogen azoxymethane (AOM) developed more colonic tumors than controls [9]. Similarly, Maggio-Price and colleagues found that *Smad3*-deficient mice infected with one or more *Helicobacter* species developed advanced cancers in the cecum but controls did not [10]. The authors determined that *Helicobacter* infection triggered inflammation in the proximal colon. Other bacterial infections also affect intestinal tumorigenesis in Min mice [11]. Thus, an important environmental factor is clearly microbes in the gut.

Intestinal tumorigenesis in Min mice also appears to be affected by exercise. Colbert and colleagues found that voluntary wheel running reduced tumor multiplicity by a small but significant amount [12]. However, the effect of exercise has not been consistently observed. The discrepancy may reflect the overall energy balance; e.g., the Colbert study produced a negative energy balance through restricted feeding and voluntary wheel running, whereas other studies did not because mice were allowed feed *ad libitum*.

The Min mouse has also been widely used for drug studies with over 200 treatments having been tested [reviewed in 13]. Non-steroidal anti-inflammatory drugs were the most potent suppressors of intestinal tumorigenesis [reviewed in 13]. These studies generally had a cross-sectional design. Treated mice and controls were euthanized at a fixed point in time; only a single data point per mouse was collected and the response of any particular tumor to treatment was unknown. For example, Jacoby and colleagues found that piroxicam suppresses intestinal tumorigenesis [14]. Treated mice developed on average 4 tumors in the small intestine, whereas controls developed on average 17 tumors. The development of tumors in the colon also appeared to be suppressed but the difference was not statistically significant. Typically, large numbers of Min mice are needed to determine whether a treatment has an effect on tumorigenesis in the colon because this model develops few colonic tumors. We overcame the limitations of traditional studies with Min mice by developing microCT colonography, which permits individually annotated tumors to be non-invasively monitored over time in living mice [15].

Description of Method

Animal Models

Min mice were bred and housed in the McArdle Laboratory for Cancer Research and scanned in the University of Wisconsin Carbone Cancer Center Small Animal Imaging Facility. All animal protocols were approved by the Institutional Animal Care and Use Committee, following the guidelines set forth by the American Association for Assessment and Accreditation of Laboratory Animal Care.

Anesthesia

Mice were anesthetized with pentobarbital by intraperitoneal injection. The recommended dose is 0.6 mg/g bodyweight which is equivalent to 0.10 ml/g bodyweight for our working solution. We find that it is best to uniformly administer 0.10 ml to all mice and observe their response before increasing the dose to the recommended amount. Min mice, particularly those with advanced disease, are not as robust as wildtype mice and rarely require the full recommended dose.

Using pentobarbital to anesthetize mice admittedly requires some experience before it is consistently safe for the mouse. We have prepared hundreds of mice. For less experienced users, or labs that do not have the requisite DEA license, anesthesia by isoflurane inhalation (1.5%–2.0%) is a good alternative. Our preference for pentobarbital is partially to simplify matters i.e., the mouse is easier to handle during preparation. Our pharmacists prepare a working solution with a final concentration of 6 mg/ml pentobarbital and 10% ethanol; the ethanol suppresses breathing slightly and reduces motion artifact.

Level of anesthesia was monitored by noting breathing rate and tidal volume. Irregular breathing, apnea or gasping may indicate hypoxia. Anesthesia was considered adequate when there was a lack of pain response to pressure on the nociceptors on the plantar side of the hind paws (toe pinch). Animals undergoing anesthesia were given eye lubricant to prevent corneal desiccation.

Mouse Preparation

Mice were maintained on a diet of standard rodent chow, vegetables or the defined diet AIN-93G from Harlan Teklad (Madison, WI). Note that bone present in standard chow does not interfere with scanning; in fact, bright “spots” resulting from bone can help differentiate between fecal pellets and tumors. Approximately 16 hours prior to scanning, water was replaced with a solution of polyethylene glycol (PEG) 3350 (11.0 g/100 ml) and food was removed to facilitate colonic cleansing. Mice seem to prefer cherry-flavored NuLYTELY (Braintree Labs, Braintree, MA), which is cherry or lime flavored over GoLYTELY. This step is similar to that undergone by humans in the clinic with sodium phosphate prior to virtual colonoscopy [16].

Prior to scanning, mice were given an enema consisting of 1–2 ml phosphate-buffered saline (PBS) using a 2 inch gavage needle. PBS was warmed in a microwave to avoid reducing core temperatures of mice. Each mouse was held upright by its nuchal scruff, the gavage needle was inserted up to 3 cm, and PBS was slowly administered and allowed to drain (Figure 1A). The process was repeated until only clear, sediment free PBS drained from the anus. We found that it was important not to allow PBS past distal colonic flexure and to assure that all of it drains properly. In our initial experiments, mice were given glucagon to reduce peristalsis but this drug has no obvious effect on overall image quality so it was eliminated from our protocol.

Each mouse was mounted in the prone position on a 3" × 6" section of cardboard (Figure 1B). The width of the cardboard did not extend outside the CT field of view (FOV), as verified by an anterior-posterior (AP) scout radiograph. The cardboard was covered by absorbent paper with a high-wick top layer and laminated back to absorb urine. Mouse fore and hind limbs were secured with Transpore™ surgical tape. Taping the limbs helped to spread the mouse and facilitate image analysis. Restraint also improved safety for the mouse in case it twitched or came out of anesthesia during the scan.

Good contrast is critical to acquiring high quality images [15]. In our initial studies, the colon was filled with either corn oil or a 2.2% barium sulfate suspension [17]. The tip of a small syringe was coated with surgical lubricant and inserted through the anus. The syringe was secured to the tail with surgical tape and then the colon was filled with up to 2 ml of contrast agent. With this volume, the contrast agent fills the colon, cecum and a portion of the distal small intestine. Corn oil was an effective contrast agent but messy with the animal's fur being soiled and needing to be cleaned; barium was also effective but its concentration needs to be very low to prevent scatter artifact. In our current studies, we use air, which is administered in the same manner as corn oil or barium. However, carbon dioxide might be better. This contrast agent is often used in the clinic because it is more readily absorbed by the colonic mucosa, reducing discomfort and pain [18].

Anesthesia of mice was reassessed before each scan. Adequate air in the colon was the paramount determinant of scan quality for mCTC. To ensure that air had not leaked out the rectum or migrated into the proximal colon or small intestine, the lateral abdominal walls of the mouse were gently and bilaterally compressed in a cranial to caudal motion. This step seemed to dramatically improve air contrast in the distal colon. Note that the entire colon can be visualized with this technique unlike optical colonoscopy with a rigid scope, but the vast majority of colonic tumors in B6.Min mice form in the distal half.

MicroCT

The MicroCAT, MicroCAT II and Inveon (Siemens Preclinical Solutions, Knoxville, TN) have been tested by our group for virtual microCT colonoscopy. Standard microCT protocol was 360 frames at 350 msec exposure per frame without respiratory gating. X-ray tube voltage was 70 kVp and current was 500 uA (900 uA if the Inveon was used). A 0.25 mm aluminum filter was used to increase mean photon energy. A low geometric magnification and large flat-panel detector array of 3072×3072 was used to ensure that the mouse fit in the FOV. The maximum possible FOV was 10 cm (axial) × 10 cm (transaxial). The detector elements were binned by 4 to increase signal to noise ratio (SNR). Detector calibration included light and dark frames. The focal spot size was 50 microns and detector element size was ≤ 33 microns. Resulting isotropic voxel size was approximately 100×100×100 microns, which was more than adequate to resolve polyps over 2 mm³. The image was reconstructed in real-time by a modified Feldkamp cone beam algorithm with a Shepp-Logan filter and appropriate center offset determined prior to scanning. Scan time was approximately 12 minutes; the radiation dose per scan is 0.25 Gy. Higher resolution is possible on both the microCAT II and Inveon systems at the expense of SNR. If SNR is maintained, radiation dose increases with the 4th power of resolution [19]. Thus, increasing the resolution to 50 microns without changing noise would have resulted in a 16-fold increase in radiation exposure to the mouse.

Polyp Detection

Three methods were used to identify polyps from mCTC scans. Readers were blinded to the results of other readers, their own previous results by 2D or 3D rendering, and gross pathologic findings. Scans assessed by 2D rendering were read using the Standard View in Amira, which simultaneously displays axial, coronal and sagittal views (Figure 2). Grayscale window and

level were standardized for all readers. Scans assessed by 3D rendering were read using isosurfaces in Amira, with thresholds set to accentuate air-tissue interface (Figure 2). Fly-throughs were performed by navigating through the colon using the cross-hair tool. Fly-arounds were done with a method similar to Quon and colleagues [20]. Amira was used to render only the back face of isosurfaces.

Sensitivity and specificity were assessed by asking readers to rate their certainty of detection on a 0–5 scale according the grading system established by Pickhardt and colleagues [17], where 5 = definitely a tumor, 4 = probably a tumor, 3 = indeterminate, 2 = probably not a tumor, 1 = definitely not a tumor. Cases in which a finding was not specifically noted were assigned a score of 0. Sensitivity and specificity were calculated as fractions on a per-polyp and per-mouse basis, with varying confidence scores 0–5 as the decision cutoffs. Each point was plotted as 1-Specificity versus Sensitivity. Area under the curve (AUC) was calculated by trapezoidal integration. The sensitivity for detection of tumors with a maximum diameter of 2 mm or more was 93.3%, whereas the sensitivity for detection of tumors with maximum diameter less than 1mm was 40.9%.

Tumors in the small intestine are not resolvable. The morphology of these tumors is “flat” with very little protruding into the lumen and consequently difficult to identify unless tumors become invasive and deform the musculature causing “kinks”.

Segmentation

We have developed a method for mCTC segmentation that is both accurate and precise [15]. The process of estimating tumor volume was rigorously designed to minimize reader subjectivity. Segmentation required that the digital image be partitioned into tumor and non-tumor regions (Figure 3). The first step was to manually outline the tumor in select planes of each orthogonal view. These outlines served as the skeleton for Amira’s “wrap” filter, which is an algorithm based on scattered data interpolation with radial basis functions. This filter was advantageous because the reader was not forced to delineate nebulous boundaries. Instead, the reader chose a small number of 2D boundaries as samples of the entire volume and left interpolation of ambiguous regions to the computer algorithm. The volume was trimmed using a gradient image (Sobel 3D filter) where high-intensity pixels delineate boundaries between tumor and non-tumor regions. Trimming often resulted in satellite segments called “islands” that were not contiguous with the tumor. Small islands of 15 pixels or less were automatically removed but larger islands required verification by the reader before they were removed. The result of gradient trimming was often an underestimate of the apparent volume. Therefore, the final step of the segmentation process was the application of a 3D morphological dilation filter, which expanded the volume by one voxel in every direction. The entire volume segmentation process took 5 to 20 minutes depending on the particular data set. Tumors with an estimated volume as small as 0.8 mm³ are easily resolved and can be followed over time.

Ex Vivo Analysis

Gross pathologic inspection of the excised mouse colon served as the gold standard against which the microCT results were compared. The colon was removed and prepared as described previously (Figure 4) [5]. Photographs were registered to corresponding PET images when applicable. Colonic lesions were observed using a dissecting microscope and carefully cut away from the colon. Samples were sectioned at 5 microns and stained with H&E. The pathology of lesions was determined. Intestines were stored in vials of 70% ethanol in case they might be needed later.

Some animals were euthanized, using CO₂ inhalation in a chamber and confirmed by bilateral pneumothorax, prior to the end of the experiment in accordance with our protocol. In this case

their colons were resected for gross examination as described above. Reasons for early endpoint include cachexia measured by loss of body weight greater than 15%, failure to groom, lethargy, rectal prolapse and severe anemia, which are all associated with the Min model. mCTC did not appear to cause any additional ill effects.

Longitudinal Studies

MicroCT colonography provides a means for non-invasively measuring tumor response to chemotherapy over a period of several weeks. Statistical power in such longitudinal studies depends on tumor response, whereas power in cross-sectional studies depends on raw tumor multiplicity. Longitudinal studies with mCTC may provide up to four times the statistical power of a cross-sectional study, depending on the characteristics of the model [21]. We use a multivariate regression analysis to identify predictors of tumor volume change. In a recent study in which tumors were monitored over a period of three to eight weeks, predictors include sex and initial tumor volume. Similar results were observed with optical colonoscopy, indicating radiation did not have a significant effect.

Concluding Remarks

MicroCT colonography was developed to monitor longitudinally the colonic tumors in Min mice. The estimates of tumor volumes from scans are precise and accurate with changes as small as 16% being readily detectable between two time points. The acquisition of high quality scans depends primarily on proper anesthesia, bowel cleansing and adequate air contrast. Although this imaging platform was developed to monitor colonic tumors in Min mice, microCT colonography can be applied to any murine model of human colorectal cancer. A number of new animal models have been developed in recent years [reviewed in 22]. Such variety is necessary because none of the existing animal models fully recapitulates the human disease [23].

The major criticism of the Min model is that tumors develop primarily in the small intestine rather than the colon [5]. This distribution necessitates analyzing a large number of Min mice in a cross-sectional study to determine whether a treatment is effective against colonic tumors which are the most relevant to human disease. Fewer mice are required for testing in a longitudinal study employing MicroCT colonography or any other imaging platform to monitor colonic tumors before, during, and after treatment, in part because the response of each individually annotated tumor is known. The number of mice needed is also reduced if incidence or multiplicity of colonic tumors is higher. Treatment of Min mice with DSS has both of these effects. Tanaka and colleagues found that incidence increased from 6/19 to 14/14 and average tumor multiplicity increased from 1 to 9 when comparing treated mice to controls [24]. In addition, a variant of the JAX stock of B6.Min, FCCC.Min, has been identified that spontaneously develops more colonic tumors [25]. Thus, the major criticism of the Min model can be largely overcome with MicroCT colonography or by manipulating the model.

Other imaging platforms including microMRI and optical colonoscopy have been developed in recent years. Hensley and colleagues demonstrate that colonic tumors in Min mice can be detected by MRI, using the FCCC.Min variant [26]. Tumor volume was estimated from images using a variation of standard planimetry which is an accepted method of volume determination from MRI and CT data sets [26]. A significant advantage of microMRI and microCT colonography imaging is that estimates of volume of any particular colonic tumor in a living mouse are possible. However, these imaging platforms are quite expensive, and image analysis can be time consuming.

Becker and colleagues demonstrated that inflammation and tumors in the colon of living mice can be monitored by optical colonoscopy [27]. Tumor volume was reported as the percentage

of lumen occluded by a tumor. Subsequently, Hensley and colleagues have developed a method to determine the 2D area of a tumor from optical images [28]. Basically, the area was estimated by comparing the dimensions of the adenoma relative to a reference rod using a novel geometric construction. They demonstrated that area correlated well with volume estimated from MRI images or “wet” weight at necropsy. Optical systems are much less expensive than microCT and microMRI. In addition, optical colonoscopy has other advantages, e.g., tumor biopsies can be taken during the procedure and subsequently analyzed for gene expression by mRNA microarray. A limitation of optical colonoscopy is that only 3–4 cm of distal colon can be viewed with a rigid endoscope.

The ability to monitor an individually annotated tumor in the colon of a living mouse is a major step forward in understanding tumorigenesis and testing drug efficacy. A number of distinct imaging platforms have been developed; each with its own set of advantages and limitations. In our own studies, we envision using a combination of microCT colonography and optical colonoscopy as dictated by the questions being addressed in a particular set of experiments. In addition, we believe that microCT could be used to monitor other tumor types in animal models using the principles outlined here. The detection of tumors must be sensitive and specific; measurements must be precise and ideally accurate.

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Figure 1.
The anesthetized mouse is held in an upright position for administration of an enema (A) and then restrained (B) in preparation for CT scanning.

Figure 2. Tumors can be visualized in 2D and 3D renderings of the data. A single colonic tumor (blue arrow) is evident in a coronal slice (A) and an isosurface (B).

Figure 3.

Estimating tumor volume from microCT images is a semi-automated multiple step process. One or several slices in each orthogonal plane are selected to use as the skeleton for our segmentation volume (A, B). Next a wrap filter is applied (C, D) using an algorithm based on scattered data interpolation with radial basis functions. The volume is trimmed using a gradient image (E, F). Finally, a morphological 3D dilation filter is applied (G, H). Images in the bottom row are 3D renderings of the 2D segmentations in the top row. This image was adapted with permission from Academic Radiology [15].

Figure 4.

Min mice develop tumors along the entire length of the intestinal tract. A mouse was sacrificed at 100 days of age and the intestinal tract was removed. The small intestine was divided into four segments of equal length. The segments were placed on bibulous paper next to the colon, opened longitudinally with a pair of surgical scissors, splayed open, rinsed with PBS, and washed with a copious amount of 70% ethanol. The tissue was fixed in 10% buffered formalin for about 14 hours and then stored in 70% ethanol. Tumor multiplicity varies along the intestinal tract; few tumors develop in the proximal segment of the small intestine nearest the stomach (bottom) and the colon (top), but many develop in the distal segment of the small intestine nearest the cecum (2nd from the top). Black arrows indicate representative adenomas; the white arrow indicates a representative Peyer's patch.