**TITLE:**

Magnetic Resonance Imaging Assessment of Carcinogen-induced Murine Bladder Tumors

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**SUMMARY:**

Murine bladder tumors are induced with the N-butyl-N-(4-hydroxybutyl) nitrosamine carcinogen (BBN). Bladder tumor generation is heterogeneous; therefore, an accurate assessment of tumor burden is needed before randomization to experimental treatment. Here we present a fast, reliable MRI protocol to assess tumor size and stage.

**ABSTRACT:**

Murine bladder tumor models are critical for the evaluation of new therapeutic options. Bladder tumors induced with the N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) carcinogen are advantageous over cell line-based models because they closely replicate the genomic profiles of human tumors, and, unlike cell models and xenografts, they provide a good opportunity for the study of immunotherapies. However, bladder tumor generation is heterogeneous; therefore, an accurate assessment of tumor burden is needed before randomization to experimental treatment. Described here is a BBN mouse model and protocol to evaluate bladder cancer tumor burden *in vivo* using a fast and reliable magnetic resonance (MR) sequence (true FISP). This method is simple and reliable because, unlike ultrasound, MR is operator-independent and allows for the straightforward post-acquisition image processing and review. Using axial images of the bladder, analysis of regions of interest along the bladder wall and tumor allow for the calculation of bladder wall and tumor area. This measurement correlates with *ex vivo* bladder weight (rs= 0.37, p = 0.009) and tumor stage (p = 0.0003). In conclusion, BBN generates heterogeneous tumors that are ideal for evaluation of immunotherapies, and MRI can quickly and reliably assess tumor burden prior to randomization to experimental treatment arms.

**INTRODUCTION:**

Bladder cancer is the fifth most common cancer overall, responsible for approximately 80,000 new cases and 16,000 deaths in the United States in 20171. After about 30 years without significant advances in the systemic treatment of bladder cancer2, recent anti-PD-1 and anti-PD-L1 checkpoint inhibitor trials have demonstrated exciting and occasionally durable responses in patients with advanced urothelial carcinoma3-5. However, only approximately 20% of patients show an objective response to these treatments, and further studies are needed to expand the effective use of immunotherapy in patients with bladder cancer.

Murine bladder cancer models are critical tools in preclinical evaluation of novel treatments6,7. In order to control for tumor size when randomizing mice to different treatments, tumor burden must be assessed and controlled between treatment groups. Previous studies have used ultrasound or bioluminescence to evaluate orthotopic cell line-based bladder cancer models8-11. However, both techniques present several disadvantages. Ultrasound measurements can be influenced by skills of the operator and lack three-dimensional features and high spatial resolution. Bioluminescence methods can only provide semi-quantitative evaluation of the tumor cells and do not allow for visualization of bladder anatomy and morphology. Furthermore, bioluminescence can only be used with cell line-based models, which express bioluminescent genes in hairless mice or mice with white coats.

Magnetic resonance imaging (MRI), on the other hand, offers unique flexibility in the acquisition of high-resolution anatomical images, exhibiting a broad range of tissue contrast that enables accurate visualization and quantitative assessment of tumor burden without the need to express bioluminescent properties. MR images are more easily reproducible with the appropriate analysis pipelines and guaranteed 3-D visualization of the bladder. The biggest limitations of MRI are the length of time necessary for an examination and associated high costs that limit high throughput assays. However, several studies have shown that MR sequences can provide high-quality diagnostic images that can be used to effectively detect and monitor cell line-based bladder tumors; thus, they may be used for high throughput analysis9,12.

Here, we describe a non-invasive MR-based method to reliably and efficiently characterize carcinogen-induced bladder tumors in mice. To accomplish this, we use a fast imaging with steady state precession MR technique (true FISP), which guarantees short scanning sessions while still providing high quality and high spatial resolution (~100 microns) for the detection and measurement of bladder tumors13. Furthermore, to confirm the accuracy of this non-invasive MRI assay, we describe the correlation between MRI-derived parameters and *ex vivo* bladder weight as well as pathologically-confirmed tumor stage.

**PROTOCOL:**

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Northwestern University.

**1. Induction of tumors with BBN**

1.1 Obtain male C57/BL6 mice, each at least 6 weeks old.

NOTE: Male mice develop bladder cancer more quickly and consistently than female mice14,15.

1.2 Add N-nitrosobutyl(4-hydroxybutyl) amine (BBN) at a dose of 0.05% to the drinking water. Store it in an opaque container and provide it *ad libitum* as drinking water to mice16.

NOTE: Storing the BBN solution in a clear container will degrade the carcinogen17.

1.3 Change the 0.05% BBN water twice per week.

1.4 Monitor the animals by inspecting for signs of distress associated with bladder tumors including hematuria, firm bladder, and masses. Inspect the mice twice per week or in accordance with local IACUC guidelines.

1.5 Expect the tumors to develop between 16 and 24 weeks of exposure18.

**2. MRI setup**

2.1 Perform a subcutaneous injection of sterile saline (0.1–0.2 mL using a 25–27 G needle and 1 mL syringe) 10 min prior to MRI to facilitate bladder filling.

2.2 Anesthetize each mouse with a gas mixture of 100% O2 and isoflurane (2%–4% as necessary). Verify an adequate plane of anesthesia by testing the withdrawal reflex (toe pinch) before proceeding.

2.3 Transfer the mouse to the imaging holder outfitted with a nosecone for delivery of inhaled isoflurane (0.5%–3%).

2.4 Monitor body temperature and respiration using a rectal temperature probe connected to the physiological recording computer.

NOTE: Normal body temperature (36–37 °C) is maintained using the recirculating hot water circuit built into the animal MR holder. Temperature is measured through a rectal sensor and recorded on the physiological monitoring computer using dedicated physiological monitoring software. The same system is used to record the respiration and electrocardiogram signals measured through a pneumatic pillow placed under the rib cage and *via* 3-lead electrocardiogram electrodes. The respiration signal is also used for triggering MRI acquisition and reducing artifacts associated with respiration motion.

**3. MRI image acquisition**

3.1 Utilize a quadrature body coil for excitation.

3.2 Place a 4-channel receiver coil on the lower abdomen of the mouse being scanned to enable optimized detection of signals from the region of interest.

3.3 Initiate automatic adjustments through the integrated imaging software to acquire a tri-axial set of images of the whole mouse body. From this reference set of images, identify the region of interest (in this case, the bladder region).

3.4 Acquire three sets of orthogonal-sliced images along the axial, coronal, and sagittal planes using radiological frames of reference.

3.5 Utilize the true FISP imaging sequence (included as one of the features in the integrated imaging software) with the following MR parameters: TR = 900 msec, TE = 2 ms, FA = 70, 14 averages.

NOTE: This set of parameters allows for rapid imaging with high diagnostic quality, including T1/T2 weighting in <10 min per mouse.

3.6 Spatial resolution and slice thickness are determined by geometric parameters selected by the user through the graphical interface of the integrated imaging platform. This results in a series of slices across the whole bladder of 0.5 mm thickness with an in-plane resolution of 0.148 mm.

**4. MR image analysis**

4.1. Identify the set of slices of 0.5 mm thickness and in-plane resolution of 0.148 mm covering the whole bladder.

4.2. Export to the medical image analysis software by selecting the folder with corresponding images in ANALYZE format.

4.3 Select “representative axial view” at the center of the bladder for quantitative analysis by scrolling through the generated images and identifying a slice at the midpoint of the bladder, which allows for visualization of the bladder wall and lumen.

NOTE: The center slice should be the chosen one with the largest diameter.

4.4 Carefully delineate the region of interest (ROI) by manually tracing the boundaries around the outer edge of the bladder (BLAout) and around the inner lumen (BLAin) of the bladder (see schematic and representative figures in **Figure 2**) in the selected representative axial view.

4.5 Subtract the inner lumen from the outer edge to calculate the surface area of the bladder wall.

BLAwall = BLAout -BLAin

NOTE: The surface area of a control bladder with no tumor is expected to be less than that with a bladder tumor.

**5. Euthanasia and dissection of bladder**

5.1 After 20 weeks of BBN exposure, euthanize the mice using standard operating procedures in accordance with local IACUC guidelines.

5.2 Clean the area of incision with 70% ethanol, then grasp and lift the abdominal wall skin with forceps.

5.3 Make a midline incision from the pubic symphysis to the xiphoid process.

5.4 Sharply incise the peritoneal cavity by grasping with forceps and incising with scissors.

5.5 Identify the bladder, which is located in the midline lower abdomen.

5.6 Identify and cut the median umbilical ligament connecting the dome of the bladder to the umbilicus and abdominal wall.

5.7 Grasp the dome of the bladder with forceps to provide countertraction and dissect the bladder away from surrounding structures, including the seminal vesicles, rectum, and fat.

5.8 Identify the ureters entering the bladder and cut with scissors close to the bladder.

5.9 Lifting the bladder cephalad, cut the urethra with scissors and remove the bladder.

5.10 Immediately weigh the bladder after rinsing it with PBS.

**6. Histologic examination of bladder tissue**

6.1 Fix the bladder tissue in 10% neutral buffered formalin for 36–48 h at room temperature (RT).

6.2 Embed the tissue in paraffin blocks, cut the slides for subsequent examination, and stain the slides with hematoxylin and eosin for microscopic examination as described previously19,20.

6.3 Perform a microscopic examination of the mouse bladder at low (2.5x and 10x) and high (20x and 40x) magnifications, examining for macroscopic lesions, hyperplasia, carcinoma *in situ*, papillomas, papillary tumors, and invasive neoplasms19,21.

**REPRESENTATIVE RESULTS:**

Using the protocol described (**Figure 1**), bladder tumors were induced in C57/B6 male mice. MRI was performed at 16 weeks, and mice were euthanized at 20 weeks. *Ex vivo* bladder weights (BW) for each mouse were recorded. Slides were stained with hematoxylin and eosin, and all histology slides were reviewed for tumor stage.

To analyze the tumor burden using MR, the bladder wall inner lumen (BLAin) was subtracted from the bladder wall outer lumen (BLAout) to calculate the thickness of the bladder wall (BLAwall) (**Figure 2**). Representative true FISP MR images, bladder wall 3-D reconstructions, and pathologic images of a control mouse (i.e., no tumor) are shown in **Figure 3A**–**F,** and a mouse with a large tumor is shown in **Figure 3G**–**L**.

The MRI-derived parameter BLAwall correlates weakly with *ex vivo* BW (rs = 0.37, p = 0.009; **Figure 4**). Examination of the MRI-derived BLAwall parameter and BW data demonstrates an association with tumor stage (Kruskal-Wallis test MRI p = 0.0003, **Figure 5A**; BW p = 0.0006; **Figure 5B**), as well as an association when stratifying pathology by non-muscle-invasive bladder cancer and muscle-invasive bladder cancer (Mann-Whitney U test MRI p = 0.0002, **Figure 5C**; BW p < 0.0001, **Figure 5D**). The performance of BLAwall and BW to determine muscle-invasive bladder cancer is shown in **Figure 5E**. The area under the curve (AUC) for BLAwall (AUC = 0.81, 95% CI 0.68–093) is statistically similar to AUC for BW (AUC = 0.89, 95% CI 0.80–0.98; p = 0.30).

**FIGURE AND TABLE LEGENDS:**

**Figure 1:** **Schema for bladder tumor induction with BBN and timing of MRI and euthanasia**. BBN is administered *ad libitum* at a concentration of 0.05% in drinking water. Mice undergo MRI at 16 weeks. Mice are euthanized at 20 weeks and bladders of each are examined with immunohistochemistry.

**Figure 2: Schematic graphical depiction of method to obtain BLAwall and representative MR image with corresponding outlines.** Using intensity of MRI images, the outer wall of a bladder was identified and an outline was drawn in red (BLAout). The hyperintense bladder lumen was outlined in green (BLAin), and the corresponding bladder lumen area was obtained. Subtraction of these two quantities yielded the BLAwall parameter, which corresponds to the light gray disk in the graphical image.

**Figure 3:** **Representative true FISP MR images, bladder wall 3-D reconstructions, and pathologic images of a control mouse (i.e., no tumor)** **(A**–**F)** **and a mouse with a large tumor** **(G**–**L)**. **(A)** Representative MR image of a mouse with no tumor. **(B)** Segmentation of bladder wall area (BLAwall), outlined in red, defined as the area between the bladder lumen (BLAin) and outer bladder wall (BLAout). **(C)** 3-D rendering of the bladder wall from a control mouse, generated by defining BLAwall at every slice through the bladder. Green arrows illustrate the bladder on a 2-D image translated to 3-D rendering. **(D)** 3-D rendering of a cut-out of BLAwall from a control mouse. **(E)** Low power (2.5x) and **(F)** high power (10x) images of the same mouse bladder. **(G)** Representative MR image of a mouse with a large tumor. **(H)** Segmentation of bladder wall area (BLAwall), outlined in red, defined as the area between the bladder lumen (BLAin) and outer bladder wall (BLAout). **(I)** 3-D rendering of the bladder wall of a mouse with a large tumor. **(J)** 3-D rendering of a cut-out of the bladder of a mouse with a large tumor, generated by defining BLAwall at every slice through the bladder. Green arrows illustrate the bladder on a 2-D image translated to 3-D rendering. **(K)** Low power (2.5x) and **(L)** high power (10x) images of the same mouse bladder.

**Figure 4:** **Spearman correlation between the MRI-derived BLAwall and final bladder weight.**

**Figure 5: Comparisons of pathologic stage and MRI-derived parameter BLAwall in 47 mice. (A)** Comparison of all pathologic stages and MRI BLAwall (Kruskal-Wallis test).**(B)** Comparison of all pathologic stages and bladder weight (Kruskal-Wallis test). **(C)** Comparison of non-muscle-invasive bladder cancer (stage ≤T1) and muscle-invasive bladder cancer (stage ≥T2) with MRI BLAwall (Mann-Whitney U test). **(D)** Comparison of non-muscle-invasive bladder cancer (stage ≤T1) and muscle-invasive bladder cancer (stage ≥T2) with bladder weight (Mann-Whitney U test). **(E)** ROC curve of the MRI-derived bladder area and final bladder weight in determining muscle-invasive bladder cancer (stage ≥T2). The listed p-value is the difference between the two AUCs.

**DISCUSSION:**

Accurate imaging of tumor models is necessary for appropriate pre-euthanasia staging and animal randomization prior to initiation of experimental treatment. Using the procedure presented here, we demonstrate methodology to (1) generate bladder tumors using the BBN carcinogen and (2) stratify bladder tumor burden through the use of MR. An MR-derived area measurement (BLAwall) correlates significantly with *ex vivo* bladder weight and is associated with pathologic tumor stage.

By adopting a rapid imaging approach with short acquisition times at high spatial resolution (true FISP) and high diagnostic quality, we can conduct high throughput assays of mice at intermediate stages of tumor development, prior to treatment randomization. Our report is consistent with prior reports of MR imaging of cell line-based tumor implants9,12 and confirms its potential as a tool to optimize large subject number drug studies.

In this MRI protocol, it is critical to image the mouse with a full bladder to obtain high quality images and delineate the differences between the tumor and bladder lumen. We find that injecting each mouse with saline 10 minutes before imaging allows for adequate imaging of the bladder. Further critical steps include reliable triggering of MRI acquisition using the respiration signal detected with a pneumatic pillow placed under the mouse rib cage and acquisition of an adequate number of MR slices that enables coverage of the whole bladder.

Other options for imaging development and progression of murine bladder tumors include ultrasound8 and bioluminescence10,11. Micro-ultrasound imaging of implanted MBT-2 cells detected tumors in 15 mice, 13 of which were histologically confirmed to have tumors8. Ultrasound volume correlated significantly with stereoscopic volume of tumor, but tumor weight and stage were not investigated8. Bioluminescence has been used to accurately monitor cell line-based tumor implants, but it cannot be used to monitor carcinogen-induced cancers without transplanting carcinogen-derived tumors from one mouse to another. The ability to accurately monitor carcinogen-induced cancers is critical, as these models have several advantages over cell line models. Cell line-based models are genetically homogenous and derived from tumors that have already evaded immunosurveillance, and implanted tumors grow rapidly without a chronic inflammatory microenvironment22. The BBN model has been used successfully for over 30 years, and it remains a critical model for the understanding of bladder cancer development and treatment23-25. Furthermore, the BBN model demonstrates mutational and gene expression profiles similar to human bladder cancer, while still retaining the intact immune system to allow for the study of potential immunotherapeutic agents26,27.

Availability of dedicated small animal MRIs as shared resources at multiple institutions makes this techniques advantageous and practical for basic research and screening of novel therapies. However, there are some limitations. Mice were imaged only at one timepoint, not continuously during the development of tumors. However, based on our statistical results, we suggest that the single timepoint value is able to accurately stratify mice into groups by tumor size and stage, and it represents an ideal, non-invasive parameter to classify and assign subjects to different groups. Multiple tumor stages were generated using BBN, ranging from Ta to T4. However, these may be stratified (as suggested in **Figure 5C-D**) as muscle-invasive (T2 or greater) and non-muscle invasive (T1 or less), as this is standard management in human bladder cancer28.

Another potential limitation is that the BLAwall parameter was derived using a single slice through each bladder and not all available slices covering it. These criteria were chosen to reduce analysis pipeline requirements (i.e., requirement of drawing multiple ROIs across multiple slices) and were deemed sufficient for a fast, quantitative assay. More complex volumetric analysis can be conducted on the subjects (i.e., shown for illustrative purposes in **Figure 3**) but would inevitably require more effort and costs. Automated image processing algorithms can be used for automatic delineation of bladder region; however, these methods suffer from intrinsic variability of bladder shape and size among individual mice and require significant testing and validation prior to reliable adoption in a preclinical study29.

Qualitative assessment of volumetric data suggest that this single slice method is sufficient for this type of assay. However, it is possible that more advanced assays may require this additional data/image processing step. From the acquisition point of view, there are several additional scans that could be acquired, which may further increase the ability to predict progression of tumors while also revealing more subtle tumor microenvironment changes. These additional techniques include dynamic contrast enhanced MRI, diffusion weighted MRI, and other sequences30 that enable a comprehensive, multi-parametric characterization of the bladder wall. However, consideration of cost and efficiency led us to confine our assay to the one described in this protocol.

In conclusion, we describe the methodology for T1/T2-weighted rapid imaging MR sequences (true FISP) to acquire multi-slice images covering the entire mouse bladder. We demonstrate that these images can be used to determine the extent of tumor in a carcinogen-based model of murine bladder cancer. MRI data correlates with bladder tissue weights and is associated with tumor stage. These results support the use of this fast and reliable MRI assay to stratify mice prior to experimental treatment randomization.

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**DISCLOSURES:**

The authors have nothing to disclose.

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