

Video Article

# Using Micro-computed Tomography for the Assessment of Tumor Development and Follow-up of Response to Treatment in a Mouse Model of Lung Cancer

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## Abstract

Lung cancer is the most lethal cancer in the world. Intensive research is ongoing worldwide to identify new therapies for lung cancer. Several mouse models of lung cancer are being used to study the mechanism of cancer development and to experiment with various therapeutic strategies. However, the absence of a real-time technique to identify the development of tumor nodules in mice lungs and to monitor the changes in their size in response to various experimental and therapeutic interventions hampers the ability to obtain an accurate description of the course of the disease and its timely response to treatments. In this study, a method using a micro-computed tomography (CT) scanner for the detection of the development of lung tumors in a mouse model of lung adenocarcinoma is described. Next, we show that monthly follow-up with micro-CT can identify dynamic changes in the lung tumor, such as the appearance of additional nodules, increase in the size of previously detected nodules, and decrease in the size or complete resolution of nodules in response to treatment. Finally, the accuracy of this real-time assessment method was confirmed with end-point histological quantification. This technique paves the way for planning and conducting more complex experiments on lung cancer animal models, and it enables us to better understand the mechanisms of carcinogenesis and the effects of different treatment modalities while saving time and resources.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/53904/>

## Introduction

Lung cancer is the leading cause of cancer death around the world<sup>1</sup>. Research into the prevention, early detection, and treatment of lung cancer is ongoing in many research centers throughout the world<sup>2,3</sup>. Several animal models for lung cancer have been developed, and they have proven useful in studying the mechanisms of lung carcinogenesis and cell of origin, in determining the presence of cancer stem cells, and in examining various novel therapeutic strategies<sup>4</sup>. Earlier models relied on carcinogen-induced tumor initiation in sensitive strains of mice<sup>5</sup>. The development of knockout and transgenic mouse models in which lung cancer arises as a result of specifically manipulated genetic lesions has substantially improved our ability to control tumor induction and mimic several aspects of human lung cancer<sup>4</sup>. However, a major challenge in the use of lung cancer animal models is the absence of a real-time method to accurately identify and monitor the onset and development of tumors in mouse lungs and to document any later change in their sizes, such as their continued growth or reduction in response to treatments. This has forced researchers to resort to several time, effort, and resource-consuming techniques to identify the tumors and to evaluate their experimental results. The presence of inherent inter-mouse variation in response to tumor induction requires the use of large numbers of animals in each experimental group to reduce data variability. The inability to assess the tumor growth or response to treatment in real-time has forced researchers to blindly euthanize mice at multiple time-points in prolonged experimental protocols to guarantee that they will collect the right data, resulting in the waste of resources from the samples collected at time points that are either too early or too late.

In the present study, a method to exploit a small-animal micro-computed tomography (micro-CT) scanner to detect and follow-up lung tumors in living mice is introduced. We used our recently described *Sftpc-rtTA* and *Tre-Fgf9-ires-eGfp* double-transgenic (DT) mice that rapidly develop lung adenocarcinoma following induction with doxycycline<sup>6,7</sup>. The use of micro-CT enables us to (among other things) exclude mice with aberrant lung abnormalities before induction, confirm development of tumor nodules in the lung after induction, and observe changes in tumor nodules in response to experimental treatments. End-point euthanasia of mice and histological assessment confirmed the accuracy of the real-time assessment conducted with micro-CT. We believe that this technique will pave the way for conducting better-planned experiments using lung cancer animal models while saving valuable resources, shortening observation time and increasing the accuracy and understanding of results.

## Protocol

Animal experiments were approved by the Institutional Animal Care and Use Committee of Keio University.

Note: In this study, we used the *Stipcr-rtTA* and *Tre-Fgf9-ires-eGfp* DT mice in which lung adenocarcinoma rapidly develops after induction by feeding chow containing doxycycline<sup>6,7</sup>. However, all assessment procedures can be applied to other lung cancer mouse models.

### 1. Experiment Outline:

1. Identify the status of the lungs at the baseline:
  1. Before tumor induction, when the DT mice are 8 - 12 weeks old, perform the first micro-CT scan (see sections 2 and 3 below). This serves as the lung baseline scan, confirms the absence of spontaneously developed nodules caused by a leaky transgene, and document the absence of any existing lung pathology before tumor induction.
2. Initiate tumor induction:
  1. Switch the DT mice from regular chow to doxycycline chow to induce Fibroblast Growth Factor (*FGF*)9 expression in the alveolar cells and to initiate tumor development. Give doxycycline chow (200 ppm) ad libitum.
3. Confirm the development of tumor nodules in mouse lungs:
  1. Perform a micro-CT scan to identify the development of tumor nodules compared to the pre-induction scan.
4. Assess response to treatment:
  1. To test the ability of the micro-CT to detect changes in tumor nodules in response to treatment, administer the FGF Receptor (FGFR) inhibitor AZD4547, then perform additional micro-CT scans after 5 and 10 weeks.
5. End-point evaluation:
  1. Euthanize all treatment and control mice and process tissues for histological evaluation (see section 6 below).

### 2. Preparing Mice for Micro-CT Image Acquisition:

1. Turn on the micro-CT scanner and computer.
2. Click on the software named "R\_m CT2", then click on "warm up".
3. Remove the sample bed upon which the mouse will be placed from the micro-CT chamber. Wrap the bed with plastic wrap.
4. Set the anesthesia induction box by adding isoflurane to the anesthesia vaporizer up to the marked level. Set the isoflurane flow rate at 3 L/min.
5. Open the oxygen tank to start the oxygen flow into the induction box and set the flow rate at 1 L/min.
6. Place the mouse in the anesthesia induction box and confirm that it is deeply anesthetized by the absence of spontaneous movement and in response to a skin pinch (a gentle pinch of a small fold of skin, which does not cause tissue damage or skin break).
7. Apply an ocular lubricant to prevent corneal dryness during anesthesia.
8. Open the micro-CT chamber and place the mouse with the dorsal side up on the sample bed. Hold the head of the mouse and pull the body downward from the lower limbs in order to stretch and straighten the body symmetrically.
9. Turn off the anesthesia flow to the induction box and turn it on towards the tube that connects to the micro-CT chamber.
10. Place the anesthesia tube into the mouse's nose to administer continuous anesthetic.
11. In order to fix the mouse in position; wrap the mouse and the sample bed with plastic wrap.  
Note: It is critical to keep the mouse under deep anesthesia and wrapped to the sample bed because any slight movement or twist of its body during the scan will result in hazy images and difficulty in interpretation.
12. Close the micro-CT chamber.
13. Adjust the micro-CT system to 90 kV and 160  $\mu$ A and the scan time to 4.5 min. Set the image range to 24  $\times$  19 mm and the voxel size to 50  $\times$  50  $\times$  50  $\mu$ m. Use the synchronous mode for heartbeats.
14. Make a new folder in the "Database" in order to save new images in that folder.
15. Start the scan.
16. After completion of the scan, move the mouse into an empty cage and observe it until it regains consciousness. Do not put it with other mice until it has fully recovered from the effects of the anesthesia.

### 3. Pre-induction Micro-CT Image Visualization and Analysis:

1. To visualize the micro-CT images, download the free ImageJ software from the following web site: <http://imagej.nih.gov/ij/>. Note: Every micro-CT data file is a stack of approximately 500 TIF Files (.tif). Other image viewing software can also be used.
2. Open the serial micro-CT images files and scroll through all of the images of each mouse from the neck to the abdomen or vice versa.
3. Using scans of naïve wild-type mice, identify the density of different areas and normal anatomical structures in the chest based on knowledge of mouse anatomy (see **Figure 1A**).
  1. Hold the cursor with the computer mouse and scroll up, starting from the whitish abdominal viscera and diaphragm, through the chest and up to the neck.
  2. Identify the bony chest cage landmarks (sternum in the front, vertebrae in the back and ribs on the sides).
  3. Identify the heart in the front of the chest and the major blood vessels near the heart and in the mediastinum.

4. Observe the trachea lumen (as a small dark circle at the level of the neck and upper chest), which bifurcates into the right and left main bronchi then continue to branch into smaller and smaller bronchi. Note that each bronchus is closely associated with two or three blood vessels (**Figure 1A**).
4. Start examining the scans of un-induced DT mice and identify the presence of any abnormalities.
  1. Exclude mice with abnormal pre-induction lung shadows (e.g., nodules, emphysematous bullae, etc.) from any further experiments. (**Figure 1C-E, G, H**).

## 4. Tumor Induction:

1. To induce tumor development in experimental mice that showed normal lung scans, switch their food from regular chow to doxycycline-containing chow (200 ppm).

## 5. Follow-up scans:

1. After 10 weeks, perform a second micro-CT scan of all mice to confirm the development of tumor nodules in their lungs (see **Figure 2**).
2. Split mice into two groups. Administer the FGFR blocker AZD4547 (125 µg/kg/day via a gastric tube for 6 days/week for 10 weeks) to one group and a placebo to the other control group for 10 more weeks.
3. Follow up the changes in the nodular shadows by performing a third scan 4 - 5 weeks later.
4. At the end of 10 weeks of treatment, perform a fourth scan.
5. Euthanize all mice with CO<sub>2</sub> inhalation or with intraperitoneal injection of 0.1 mg/200 µl of pentobarbital.
6. To identify the dynamic changes in the tumor nodules after induction and in response to treatment, identify similar positions within the serial scan images of the same mouse at two or more different time-points then check for the appearance/disappearance of any abnormal shadows (**Figure 3**).
7. To facilitate the identification of the same plane in the same mouse at different time-points, try to associate the plane of interest with anatomical landmark structures within the mouse chest.
  1. Use landmarks such as the trachea, its bifurcation, the right and left main bronchi, aorta, diaphragm, and large blood vessels.  
Note: Chest bones, including the thoracic vertebrae, ribs, and sternum are less useful as positioning landmarks because of common minor tilts in mouse body alignment on the sample bed, which increase the possibility of misinterpretation of the scan position. Similarly, the image serial number within the scan file is unreliable for identifying the same position over time because of the change in mouse body length from one time-point to another. Failure or inaccuracy in identifying the same plane at different time points may result in false positive/negative interpretation of findings.

## 6. Mouse Euthanasia and Lung Collection:

1. Euthanize mice with CO<sub>2</sub> inhalation or with intraperitoneal injection of 0.1 mg/200 µl of pentobarbital.
2. Expose the abdominal viscera by cutting longitudinally through the abdominal wall. Bleed the mouse to reduce the volume of blood in the lungs by dissecting the abdominal aorta.
3. Slit the diaphragm with fine scissors; this will result in loss of the negative pressure from the chest cavity, thus collapsing the lungs. Expose the lungs and heart by cutting and removing parts of the ribs of the anterior chest wall. Clean the frontal part of the neck by cutting the skin and soft tissues to expose the trachea.
4. Cut away the heart and thymus gland. Insert forceps behind the trachea to separate it from the esophagus.
5. Cannulate the trachea with a G24 cannula then secure it in place by tightening a thread around the inserted part.
6. Inflate and fix the lung using ice-cold 4% paraformaldehyde (PFA) through the tracheal cannula using a 25 cm column. Detach the cannula and tighten the thread to prevent PFA leakage then cut the upper trachea off its attachment to the larynx.
7. Pull the trachea from the suture thread and dissect it from its attachment, and continue downward to remove it with the lung *en-block*. Insert it into a 15 ml tube containing 5 ml of 4% PFA. Leave the lungs in PFA O/N to ensure complete tissue penetration and fixation, then process the tissue into a standard paraffin block<sup>8</sup>.
8. Cut paraffin blocks into 6 µm-thick slices on a microtome and stain with hematoxylin and eosin using standard techniques.

## 7. Histological Evaluation:

Note: Although the use of a "slide scanner" for digital histological evaluation is described here, the use of regular microscopes and visual histological evaluation for assessment is also possible.

1. Turn on the slide scanner instrument and computer.
2. Click on the "NDP scan" software.
3. Select the scan mode "Batch of slides" and "Semi-Auto Mode" for scanning a series of slides.  
Note: The robotic arm that picks up the slides during sequential scanning is very sensitive to any irregularities on the edges of the glass slides.
4. Palpate the edges of all glass slides before loading them into the machine. If there is any protrusion of the cover glass or dried mounting medium, clean it off with a cutter or a scalpel.
5. Load the slides into a slide cassette. Open the specimen hatch and slide the cassette into position "one". Close the door.
6. On the computer software, give short descriptive names to all slides in their corresponding position in the cassette then click the "OK" button.
7. Select the profile mode: "Brightfield".
8. Click on "Start Batch" to start the provisional scanning.

Note: Once the machine has finished scanning all the slides, the software will automatically detect areas with tissues on all the slides and suggest it as a region of interest.

9. If needed, redefine the region of interest by holding down the left mouse button and pulling the region border.

Note: Defining excessively large areas of the slides as the regions of interest will result in a much longer scan time.

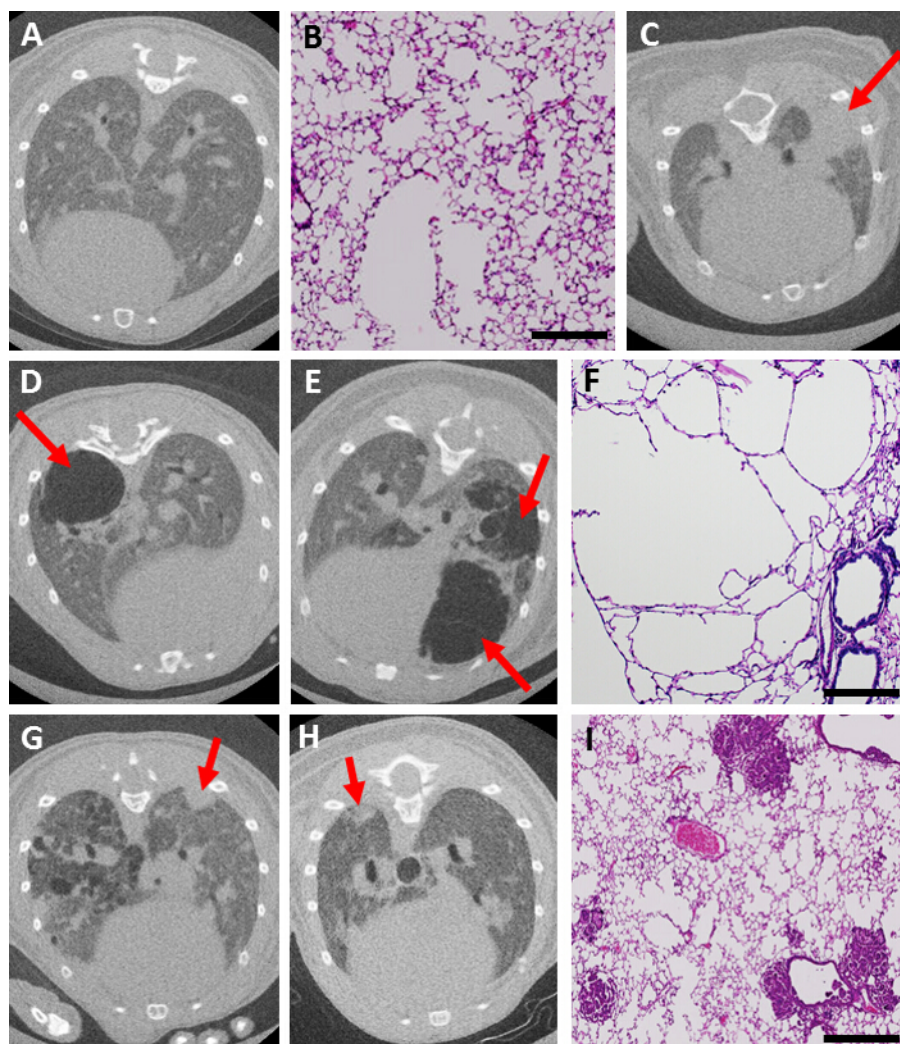
10. Once satisfied with the regions of interests on all of the slides, click on "Scan" to start scanning all slides.

Note: The scanned files can be observed digitally in low and high resolution, and images can be exported as JPEG files.

## Representative Results

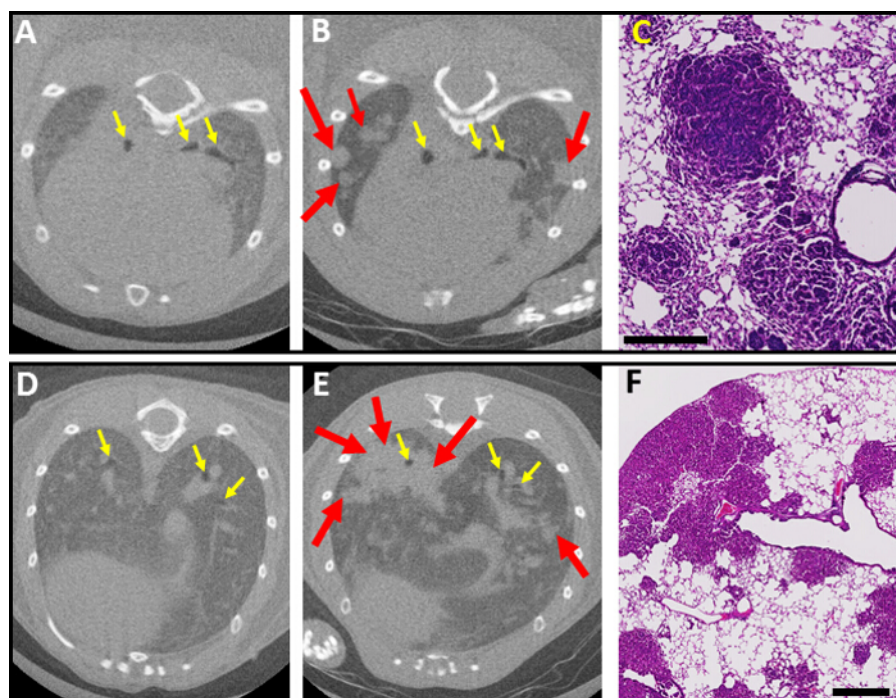
Identification of mice with lung abnormalities was performed at baseline. Before tumor induction, when the DT mice were 8 - 12 weeks of age, the lungs of all mice were scanned with micro-CT. Surprisingly, approximately 50% of mice showed abnormalities that forced us to deem them unsuitable for inclusion in the subsequent study. These abnormalities were nodule-like shadows, large single or multiple small emphysematous bullae and/or lobar atelectasis (**Figure 1A, C, D-E, G-H**). Then, the *FGF9* transgene and tumor induction was activated. Only mice that showed normal lung scans (without abnormalities) were switched from regular chow to doxycycline chow to induce *FGF9* expression in alveolar cells and subsequent tumor development. To confirm the development of tumor nodules in mouse lungs after ten weeks from the initiation of tumor induction, follow up micro-CT scans were performed. These revealed the development of multiple nodules of variable sizes in all mice (**Figure 2**, compare **A** to **B** and **D** to **E**). Micro-CT scans were performed to assess the response to treatment. These revealed that mice that were administered the FGFR inhibitor AZD4547 for 10 weeks indeed exhibited disappearance or reduction in the size of nodules that were visible in the pre-treatment scans (**Figure 3**, compare pre-, post-induction and post-treatments, **A-C**, **E-G** and **I-K**). On the other hand, control mice that were not given the FGFR inhibitor and were given placebo instead showed no change, increase in nodules size and appearance of new nodules (**Figure 3**, **M-O**); confirming that the decrease in size observed in the treatment group is a genuine effect of the therapeutic intervention.

End-point histological evaluation of mouse lungs from the different time-points confirmed the accuracy of the findings detected with micro-CT scanning. Naïve mice before doxycycline induction: Mice judged to have clear lungs on micro-CT scans showed normal lung histology. Mice that showed abnormalities on the scan had various histological changes including bullae and nodules (**Figure 1B, F, I**). Mice after 10 - 12 weeks of induction with doxycycline: Multiple adenocarcinoma nodules with variable sizes and positions were observed in all animals (**Figure 2C, F**). Mice after 10 - 12 weeks of doxycycline induction followed by 10 - 12 weeks of an FGFR blocker: Nodules were much fewer in number and smaller (**Figure 3D, H, L**) compared to those observed post-induction. Mice after 10 - 12 weeks of doxycycline induction followed by 10 - 12 weeks of placebo: Multiple large nodules are visible (**Figure 3P**) similar to these seen at the post-induction time point.

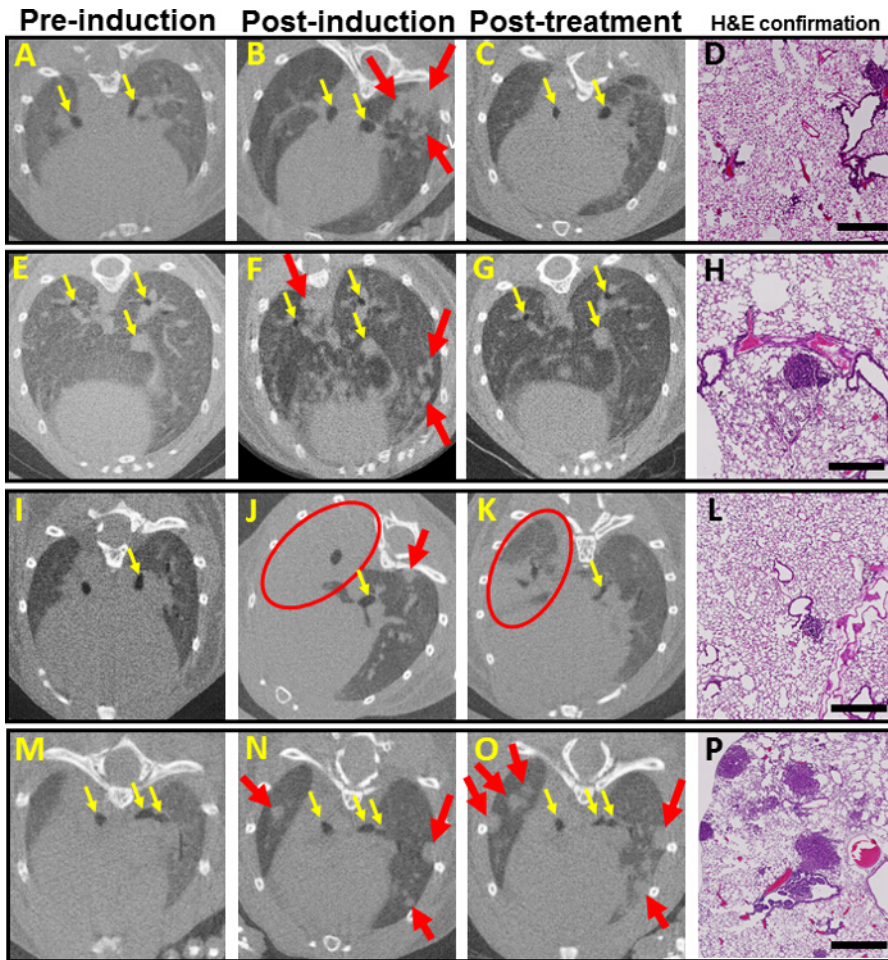


**Figure 1. Micro-CT Identifies Pre-induction Lung Abnormalities.** All mice were examined with micro-CT before the initiation of doxycycline induction. When lung abnormalities were detected, mice were euthanized for histological confirmation. Representative micro-CT scan images for (A) a normal lung; (C) lungs with an atelectatic area; (D-E) lungs with emphysematous bullae and (G-H) lungs with an abnormal nodular shadow. Histological evaluation confirmed the accuracy of the micro-CT findings: (B) normal lung histology; (F) multiple emphysematous areas; and (I) multiple tumor nodules. Scale bar: 200  $\mu$ m. [Please click here to view a larger version of this figure.](#)





**Figure 2. Micro-CT Identifies the Development of Tumor Nodules after Induction.** Mice that showed a clean pre-induction scan were fed doxycycline chow for 10 weeks then re-evaluated with micro-CT, after which they were euthanized for histological evaluation. Representative micro-CT scan images from two different mice show the appearance of multiple nodular shadows 10 weeks after the start of doxycycline chow. **(A, D)** Pre-induction clean lung, **(B, E)** Same-position scan images showing development of multiple nodules (red arrows). Small yellow arrows point to the anatomical landmarks used to identify the same position at different time points. **(C, F)** Nodules seen in the micro-CT scan were confirmed in the lung histological sections. Scale bar: 200  $\mu$ m. [Please click here to view a larger version of this figure.](#)



**Figure 3. Micro-CT Identifies Changes In Tumor Nodules in Response to Treatment.** Mice that showed clean pre-induction scans were fed doxycycline chow for 10 weeks, and then a post-induction micro-CT was performed. Next, they were administered the FGFR inhibitor AZD4547 for 10 more weeks and re-evaluated with micro-CT (post-treatment). Control mice were administered placebo instead of the FGFR inhibitor to clarify the actual effect of the therapeutic intervention. Finally, the mice were euthanized for histological evaluation. Representative micro-CT scan images from four different mice. **(A, E, I, M)** Pre-induction scans confirmed that mice had no abnormalities. **(B, F, J, N)** Scans from the same mice after they were induced with doxycycline chow for 10 weeks; all showed multiple nodules (red arrows, the red oval encircles an area that had been completely shadowed by nodules). **(C, G, K)** Scans from the same mice after an additional 10 weeks of treatment with the FGFR inhibitor showing complete disappearance or marked reduction in the size of nodules. **(O)** Scan from a control mouse to represent the control group that was administered a placebo instead of the FGFR inhibitor showing increase in tumor nodules' size and number. Small yellow arrows point to the anatomical landmarks used to identify the same position at different time points. **(D, H, L)** Histological sections confirmed the marked reduction in nodule size and number (compare to **Figure 2 C and F**). **(P)** Shows the presence of multiple tumor nodules when placebo was administered. Scale bar: 200  $\mu$ m. [Please click here to view a larger version of this figure.](#)

## Discussion

The micro-CT-based method described here for the real-time identification of lung abnormalities and monitoring of the development of tumor nodules and the response to treatment in lung cancer animal models will enable scientists who are conducting lung cancer-related experiments to plan more accurate and efficient experiments while saving time and resources. We have previously used MRI for the same purpose<sup>6</sup>. The clarity of the scan and threshold for the detection of lung nodules with MRI were inferior to those with the micro-CT scans described in this study<sup>6</sup>.

Previous studies that used similar lung adenocarcinoma mice models (with different genetic backgrounds and tumor induction methods) obviously had several disadvantages that could have been avoided or improved had they used micro-CT scanning<sup>9,10</sup>. They had no means to identify any pre-induction lung abnormalities or nodules, thus risking confounding their results by the inclusion of unsuitable animals. They also had no means to confirm the development of tumors after induction or after propagating cancer cells into wild-type mice (to induce secondary tumors), so they had to wait for 4-8 months then blindly euthanize the mice for the histological identification of tumors. When these models were used to assess the therapeutic potential of a drug, researchers had to allocate several groups to be euthanized in a chronological sequence to detect the effect of treatments on tumor nodules with histology and to be able to identify the time point of maximal effect<sup>11</sup>.

One limitation of our method, however, is the appearance of hazy shadows that obscure the detection of nodules in the lungs when some foreign substances are injected intratracheally<sup>7</sup> (data not shown). Some mice seemed to develop a prolonged inflammatory reaction to the injected

substance and the shadow of this reaction and accompanying interstitial edema masked the existing nodules. In such cases, MRI can be tried as an alternative.

Recently, other imaging modalities have been introduced to assess various pathologies in small animals; like bioluminescence imaging<sup>12</sup> and positron emission tomography (PET) scan<sup>13</sup>. To our knowledge, these imaging systems have not been used yet to evaluate lung tumors in mice models so comparison of detection efficiency and image clarity with small animal MRI and micro-CT images is not yet possible. The availability and cost of these machines are a limiting factor to their widespread use.

We detected lung abnormalities in approximately 50% of mice at the pre-induction screening and accordingly these mice were excluded from further experiments. This high incidence of abnormalities might be a result of a "leaky" FGF9 transgene in some animals resulting in the development of tumor nodules even before they are induced<sup>14</sup>. Indeed, roughly half of the excluded animals when examined histologically showed the presence of multiple small nodules. These could also be caused by an aberrant effect of the inserted transgene on the neighboring genes in the mouse genome<sup>14</sup>. The development of these sort of abnormalities are by no means specific to the *Sftpc-rtTA* and *Tre-Fgf9-ires-eGfp* double-transgenic mice and thus other lung cancer models might be suffering from similar situations. This high incidence of abnormality highlights even-more the importance of pre-inclusion screening of mice using micro-CT. Naïve wild type mice never showed any abnormal shadows on micro-CT (**Figure 1A**).

In conclusion, we have described a method that exploits a small animal micro-CT machine for the accurate detection and monitoring of the development of tumor nodules in the lungs of an adenocarcinoma mouse model. By using it to monitor changes in nodules over time, more accurate data can be collected, and a cost and time-efficient time course can be adapted.

## Disclosures

The authors declare that they have no competing financial interests.

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