Murine orthotopic model for the assessment of chemoradiotherapeutic interventions in rectal cancer

Sergio Huerta, Xiaohuan Gao and Debabrata Saha

A murine orthotopic model for the study of colon cancer has been described earlier. However, for the study of rectal cancer, three issues remain: (i) the relative sensitivity of the implanted tumors to ionizing radiation (IR); (ii) the location of the tumor for the delivery of external beam IR; and (iii) the assessment of a given modality over time before necropsy. In this protocol, we have modified an orthotopic model for colon cancer described earlier for the specific assessment of chemoradiation in rectal cancer by (i) cecal transplantation of tumors with a known response to IR; (ii) securing the tumor to the lateral abdominal wall with a permanent suture for the administration of IR; and (iii) transfection of cells with luciferase before tumor implantation for the assessment of the chemoradiotherapeutic interventions over time by bioluminescence imaging before the end on the study. This technique allows targeted delivery of IR in an intraperitoneal tumor. Imaging throughout the course of the treatment is possible such that the timing of chemoradiation can be determined and permits comparison between groups before the end of the treatment. This model represents a modified technique that allows the assessment of chemoradiotherapeutic interventions in rectal cancer.
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Departments of Surgery and Radiation Oncology, University of Texas Southwestern Medical Center, Dallas, Texas, USA

Correspondence to Dr Sergio Huerta, MD, FACS, Department of Surgery and Radiation Oncology, University of Texas Southwestern Medical Center, 4500 S. Lancaster Road (112), Dallas, Texas 75126, USA
Tel: +1 214 857 1800; fax: +1 214 462 4893;
e-mail: Sergio.Huerta@UTSouthwesten.edu

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Introduction

Although there are many similarities between the colon and the rectum in terms of their biology, histology, and genetic alterations leading to carcinogenesis, rectal cancer differs in the management, which emanates in large by the administration of chemoradiation in patients with stage II/III rectal cancer compared with individuals affected by colon cancer. However, the response to preoperative chemoradiotherapeutic interventions in patients with rectal cancer is heterogeneous and unpredictable [1]. Modalities to identify preoperative markers for a response to ionizing radiation (IR) are urgently needed. In addition, novel radiosensitizers are required for patients with tumors that are resistant to conventional interventions.

An orthotopic model for the study of colon cancer has been described earlier by Tseng *et al.* [2]. However, the study of rectal cancer might require the delivery of external beam radiation to assess the tumor throughout the treatment period for determining the optimal timing for the administration of chemoradiosensitizers and external beam radiation. We have earlier used a xenograft model of rectal cancer such that the tumor can be visualized for the administration of targeted external beam radiation and to assess the response of the various radiosensitizers [3,4]. Nevertheless, the subcutaneous nature of these tumors limits their translatability to human rectal cancer as the delivery of the radiosensitizing agents might not mimic the effect of having a tumor in the subcutaneous tissue compared with the intraperitoneal cavity.

In this report, we have overcome these limitations by establishing cecal orthotopic tumors with colorectal cancer cells with a known response to IR, transfecting the cells with luciferase before implantation, and securing the cecum to the abdominal wall with a permanent suture for the delivery of external beam radiation.

Methods

Cell culture

We have characterized several cancer cell lines earlier in terms of a response to IR [3]. Our earlier experimental protocol showed the following response to IR: the most radioresistant cell line was the HT-29 (HTB-38) clone, followed by DLD-1 (CCL-221), Scott and White (SW)-480, SW620 (colon adenocarcinoma CCL-228 passage number: 96 and colon adenocarcinoma, lymph node metastasis CCL-227 passage number: 83, respectively), and SW837 (CCL-235). HCT-116 (CCL-247) cells were the most radiosensitive clones. All of these cells were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). The cells were grown and handled as described earlier [3,4].

For this orthotopic model, we elected to study the most radioresistant cell line in nude mice to allow comparison with earlier studies. Thus, HT-29 cells were selected for

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this study. HT-29 cells were maintained at 37°C in ATCC-formulated Leibovitz's L-15 medium supplemented with 10% heat-inactivated fetal bovine serum, and 1% (v/v) L-glutamine.

Cell labeling with luciferase and bioluminescence imaging

HT-29 cells were transfected with a lentivirus-expressing luciferase. Bioluminescence imaging of tumor-bearing mice was done using the in-vivo imaging system (IVIS), Lumina System (Xenogen, Alameda, California, USA) as described earlier [5,6]. The images were acquired and analyzed using the Living Image data acquisition software (Xenogen). Before imaging, the mice were anesthetized in an acrylic chamber with isoflurane (Halocarbon, North Augusta, South Carolina, USA) and a total volume of 200 µl D-luciferin (the substrate) solution (450 mg/kg in PBS, Gold BioTechnology, St Louis, Missouri, USA) was administered subcutaneously in the neck region of each mouse. A digital overlay of a pseudocolor image on a grayscale image represents the spatial distribution of the detected photons emerging from active luciferase within the mouse. Bioluminescence imaging signals were quantified by measuring the photon flux within a region of interest using the Living Image software package.

Orthotopic model

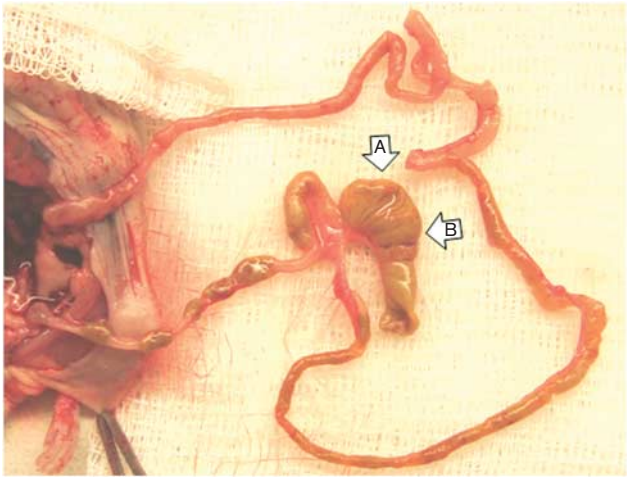
Approval for this protocol was obtained from The Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. All animal experiments were carried out in accordance with the institutional guidelines for animal welfare and maintained under clean room conditions in sterile rodent microisolator cages.

Gross inspection of the murine gastrointestinal tract showed that the cecum was the most desirable location for tumor implantation (Fig. 1). The implantation of the tumor into the cecum has been described earlier by Tseng *et al.* [2] by two techniques: (i) implantation of cells directly into the cecum and (ii) development of a xenograft in one mouse (Fig. 2a) followed by the transplantation of a small piece into the cecum of a second nude mouse. Of these techniques, we found the latter to provide more reliable results and it was the one adopted in this protocol (Fig. 3a and b).

Six-week-old nude mice ($n = 4$) received sterile rodent chow and water *ad libitum*. For tumor growth experiments, HT-29 cells (1×10^6) were injected subcutaneously with a 27-gauge needle into the right flank region of 6-week-old nude mouse, for the development of a xenograft for transplantation into a second mouse. The HT-29 cells had been labeled (luciferase-transfected) before injecting them for tumor formation as described above.

Once the animal designed to develop the xenograft had a palpable tumor (400 mm^3), it was killed by cervical dislocation on the same day that the tumor implantation was to take place. The tumor was resected and placed in PBS solution. The tumor was divided into small equal pieces (50 mm^3). These tumors were implanted into the cecum of 6-week-old nude mice ($n = 3$; Fig. 3) as described by Tseng *et al.* [2]. The technique described by Tseng *et al.* [2] is depicted in Fig. 3 with an important modification. For this protocol, we secured the cecum with a permanent suture to the abdominal wall as shown in (Fig. 3d–i). A mark shown by the permanent suture showed the location of the delivery of external beam radiation for exclusively targeting the tumor.

Fig. 1



The murine gastrointestinal tract. Arrow (A) shows the location of the cecum most appropriate for tumor implantation. Arrow (B) shows the most appropriate location for securing the cecum to the abdominal wall.

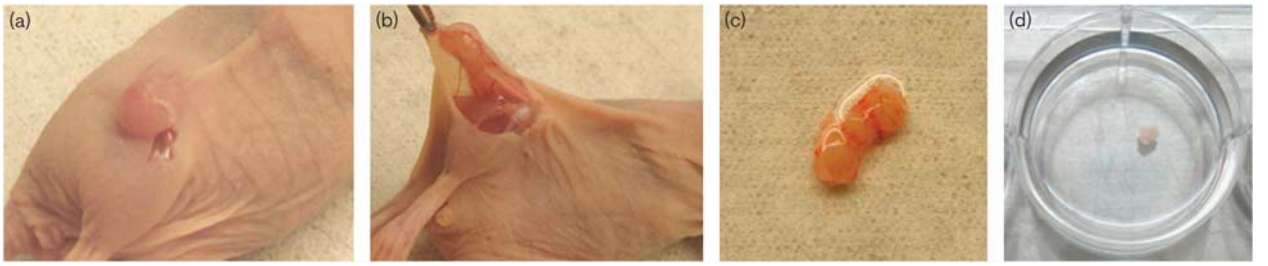
After tumor implantation, the animals were imaged once a week. Once imaging detected tumor development (7 days after implantation) (Fig. 4a), these mice were subjected to external beam radiation [2Gy every day x 5 (total 10Gy)] as described earlier [3,4]. For this experiment, these mice were irradiated using an an X-RAD 320 irradiator (Precision X-Ray, Inc., North Branford, CT) with a variable collimator to generate a single adjustable collimated dorso-ventral beam of X-rays at a dose rate of 1Gy/min. Lead blocks were used to shield the nontumor parts of the mice. The dose of IR was selected based on earlier studies that had assessed the most effective dose of IR based on small fractionated doses [3,4]. Tumor growth was assessed over 5 weeks after IR by weekly imaging.

Results

Assessment of tumor over time

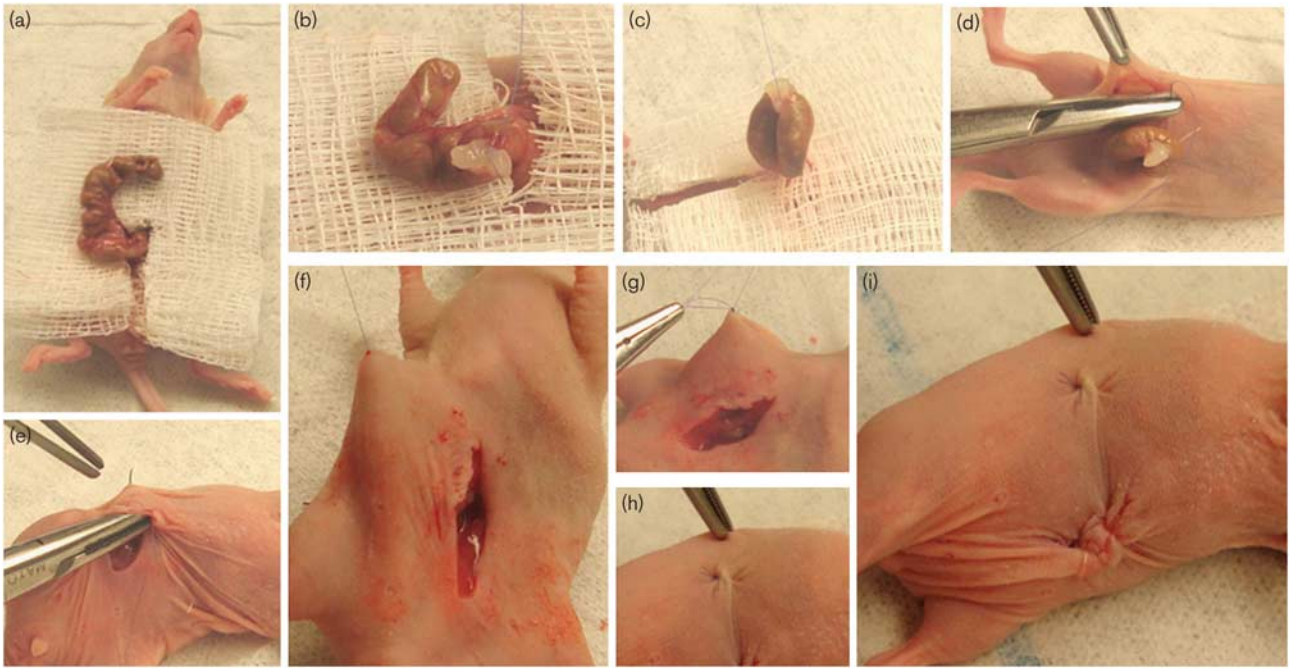
Transfection of the HT-29 cells before tumor implantation with luciferase, reliably allowed the visualization of

Fig. 2



(a) Development of tumor xenograft with colorectal cancer HT-29 cells. (b) The tumor was resected from the subcutaneous tissue and (c) placed in PBS and then divided into small pieces for tumor implantation into the cecum (d).

Fig. 3



(a) Once the tumor xenograft had been resected a second mouse was prepared and the cecum exposed, (b and c) the tumor was implanted into the cecum, and (d–g) the cecum was secured to the abdominal wall. (h and i) The permanent suture also served as a mark for the delivery of ionizing radiation.

the tumor by the IVIS Lumina Imaging System. Figure 4 shows the growth of the tumor over 5 weeks: at week 1 (a), week 3 (b), and week 5 (c).

Delivery of external beam ionizing radiation

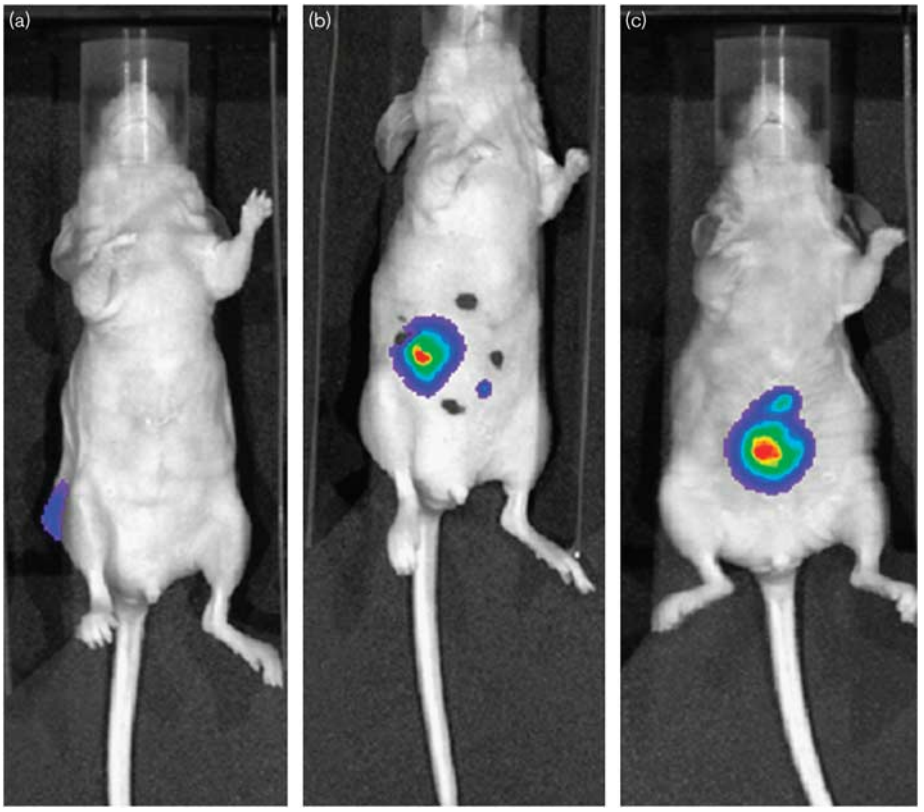
External beam radiation was administered by targeting the tumor, while shielding the rest of the mouse. This was possible because of securing of the cecum to the abdominal wall with a permanent suture. Figure 4a shows tumor growth, but the tumor is not palpable at this stage. Having secured the cecum to the abdominal wall and marked the site with a suture, the delivery of IR was then possible. No bowel obstruction was noted to occur in these mice after these interventions.

Excision of the tumor at the end of the treatment

At the end of the treatment, the tumor was palpable and visible on the abdominal wall (Fig 5a; arrow). Intraperitoneal inspection showed that the cecum wall adhered well to the abdominal wall and the suture remained in place (Fig. 5b; arrow). The tumor was in the predicted location on the cecum (Fig. 5c; arrow). No gross adhesions resulting from radiation injury to the small bowel were noted in this short follow-up (Fig. 5d).

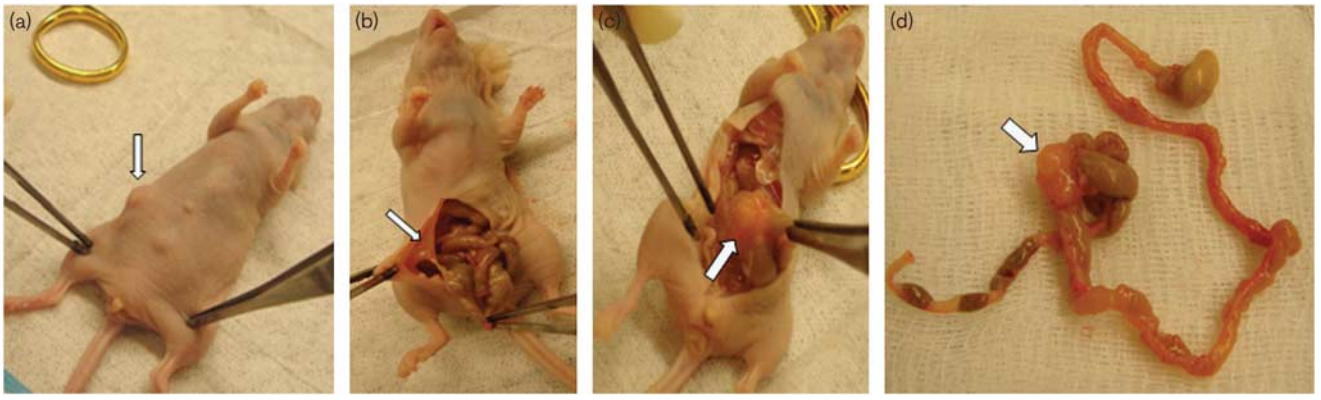
At the end of the treatment, the liver and the lungs were resected for the examination of metastatic disease. No metastases were noted by the implantation of HT-29 tumors. No peritoneal tumor disease occurred.

Fig. 4



Bioluminescence in-vivo imaging. (a) Tumor growth was detected 1 week after IR. (b) Tumor growth continued 3 weeks after implantation and (c) at the end of the treatment.

Fig. 5



Examination of a mouse at the end of treatment. The tumor is visible and palpable on the abdominal wall (a, arrow). The permanent suture remained in place securing the cecum to the abdominal wall (b, arrow). (c) shows the examination of the tumor on the cecum. The small bowel showed no radiation-induced injury and the tumor was limited to the cecum (d, arrow).

Discussion

The current in-vivo models of the study of colorectal cancer include (i) the Apc^{min} mouse; (ii) the azoxymethane (AOM)-induced colon cancer model in rats; (iii) xenograft models implanted into immunocompromised mice; (iv) an orthotopic model with cecal implantation of human colon cancer or tumor or cells; and (v) intrarectal/transanal injection of cancer cells.

The Apc^{min} is a mouse model of intestinal carcinogenesis originally derived from a germ-line mutation induced in founder animals by ethylnitrosourea treatment [7]. These mice develop multiple intestinal neoplasias throughout the intestinal tract within a few weeks after birth. These mice, however, develop polyps and not invasive cancer and are, therefore, more appropriate for chemopreventive studies [8–10].

The AOM-induced colorectal cancer model is produced in 8-week-old F344 rats by the injection with AOM. Colon tumors and malignant cancers develop with an incidence of 70% on the average of one to two tumors per animal after 40 weeks of treatment. This model is limited, in that the rats primarily develop aberrant crypts and are used for chemopreventive studies [11]. In addition, the use of the carcinogen, AOM, is of concern for investigators.

Human cancer cells (including colorectal cancer cells) have been successfully implanted into the subcutaneous tissues of immunocompromised mice. Xenografts develop after the inoculation of cells. These models have been used to evaluate several chemoradiotherapeutic interventions [3,4]. However, the subcutaneous location of these xenografts limits their translatability to human disease. Another limitation is the delivery of radiosensitizing drugs as the chemotherapeutic agents delivered intravenously or intraperitoneally might not reach the subcutaneous xenograft compared with tumors located at the site of interest.

Two models specific for rectal cancer have been described earlier. Intrarectal injection of cancer cells into the rectal mucosa for the assessment of metastasis from the rectum has been described earlier [12]. Murine B16 melanoma cell lines created skin and para-aortic metastases. Murine colon carcinoma, MCA38 cells, and human carcinoma, LS174T cells, produced large rectal tumors, but no metastases. This was a model that studied the behavior of local and metastatic disease, but there was no ability to provide local radiotherapeutic interventions.

Similarly, a second report documented successful rectal tumor growth after submucosal injection of murine colon cancer (CT26) cells in the distal posterior rectum of BALB/c mice. In this setting, the delivery of targeted IR was not described [13].

In 2007, Tseng *et al.* [2] described and murine orthotopic model for colon cancer. By the implantation of tumors into the cecum with the desired cell xenograft, the effects of the treatment can be evaluated in this model, which are closer to the environment of the rectal tumors compared with the xenografts. In this protocol, we have modified this technique for the study of rectal cancer to address specific issues related to this disease compared with colon cancer: (i) an array of cells with a known response to IR for tumor development, (ii) implantation of the tumor with a predictable location for the delivery of external beam IR exclusively targeting the tumor, and (iii) the ability to follow a response to treatment and possible metastatic disease over time before necropsy.

As we have reported elsewhere, seven colon cancer cells have a wide range of response to IR in the following order from the most radioresistant to the most radiosensitive: HT-29, DLD-1, SW480, SW620, SW837, and HCT-116. Thus, tumors can be developed in mice with a varying degree of resistance to IR, similar to the phenomenon that occurs in human rectal cancers, in which some patients achieve complete pathological response, whereas others do not respond to the same form of treatment [1]. In addition, our group and other investigators have earlier reported specific molecular phenotypic differences in these cells, which might be used as targets for the development of radiosensitizing modalities. For instance, HCT-116 cells are wild type for p53, whereas HT-29 cells bear mutations of both of the alleles of the p53 gene.

An important limitation in the study of rectal cancer of the model described by Tseng *et al.* [2] for the study of colon cancer was the delivery of external beam radiation as placing the cecum in the peritoneum will inevitably be covered with small bowel. In this scenario, it is difficult to provide a similar and uniform form of IR. Of the several possibilities examined, including excluding the bowel with a piece of proline mesh, securing the cecum to the abdominal wall was the most rapid, reliable, and reproducible approach. Using this technique, the same suture marked the lateral abdominal wall for targeted radiation. We did not experience any cases of bowel obstruction with this technique and there was no gross observable IR injury to the bowel at the end of the treatment.

For rectal cancer, it is important to determine when the tumor is effectively growing at the desired site, such that chemoradiosensitizing modalities might be initiated. Without knowing the viability of the tumor in the site, it is impossible to determine at the end of the treatment whether the effect was the response of the drug leading to a pathologically complete response or whether the initial xenograft was not properly implanted. This limitation was overcome in this protocol by the IVIS. In this protocol, we used the Bioluminescence Imaging System as described earlier [5,6]. Bioluminescence is a chemiluminescent reaction involving a direct conversion of the energy of the biochemical phenomenon into light energy. The reaction involves an enzyme (luciferase), which is the biological catalyst that accelerates and controls the rate of chemical reactions in cells, photons, and ATP, and a substrate (luciferin), which is a specific molecule that undergoes a chemical change when affixed by an enzyme and oxygen, which acts as a catalyst. The combination of luciferin and luciferase in the presence of ATP and oxygen provides

photons that can be imaged and analyzed on an imaging system [6,14]. This technique allowed us to detect the tumor for the initiation of external beam radiation therapy.

We recognize the preliminary nature of this report, but the technique allows for a wide array of applications. For instance, by securing the cecum to the abdominal wall a piece of human tumor can be placed in the cecum to test the radiochemotherapeutic agents in mice with human tumors. Further, by labeling the cells with luciferase and by having cells with a known response to IR, the timing for the delivery of IR with various radiosensitizers can be assessed.

With this model, we show that an orthotopic model of rectal cancer can be established in tumors with a varying degree of resistance to IR, targeted external beam radiation can be applied directly to the tumor, and the effect of intervention can be followed over time by an in-vivo imaging system.

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