Visualizing superficial human bladder cancer cell growth \textit{in vivo} by green fluorescent protein expression

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There has been no reliable orthotopic model available to visualize the growth of human superficial bladder cancer over time and to evaluate the efficacy of intravesical therapies. We have developed a novel approach to accomplish this task by generating human superficial bladder tumor cells to stably express high levels of green fluorescent protein (GFP) \textit{in vivo}. Superficial bladder tumors were produced in athymic mice by intravesical instillation. In our initial studies tumors were quantitated by image analysis at a single time point, and the results compared to the estimation of the percentage of GFP cells present using flow cytometry after obtaining single cell suspensions of normal and tumor cells in the same bladder. A high correlation between the two methods was seen. Therefore, in subsequent studies, approximately 1 week after the intravesical instillation of the GFP expressing cancer cells a small incision was made to expose the bladder. The anterior, posterior, and lateral images of each bladder were captured to visualize GFP-expressing tumors. The ratio of green fluorescence pixel area, which represented the tumor burden, to the total area of the bladder was then calculated. A similar procedure was performed at 2, 3, and 4 weeks after instillation of the tumor cells. Using this procedure tumor progression over time could be measured in each mouse. By using this approach, it will now be possible to monitor the initial tumor sizes in the bladder of each mouse and then to evaluate the efficacy of various intravesical therapy protocols including intravesical gene therapy alone or in combination with other treatment modalities.


Keywords: bladder cancer; green fluorescent protein; image analysis

Bladder cancer is the fifth most frequent cancer in the United States. More than 50,000 individuals are diagnosed with bladder cancer each year. Between 70\% and 80\% of these cases involve superficial tumor. Such lesions are usually managed with transurethral resection often followed by intravesical immunotherapy with Bacillus Calmette-Guérin (BCG). Although BCG can prolong the length of progression-free survival after the tumor is resected, approximately 50\% of superficial lesions will continue to recur and as many as 30\% will progress to a higher grade and/or stage.\textsuperscript{1} Therefore, new therapeutic approaches, including gene therapy alone or in combination with other modalities of therapy, need to be considered for the treatment of refractory superficial bladder disease.

We recently have developed an intravesical model to produce superficially growing human bladder tumors in athymic mice with high frequency.\textsuperscript{2} In addition, our laboratory in collaboration with others has shown that this model can be used to document efficient intravesical adenoviral-mediated gene transfer when it is combined with the polyamide compound, Syn 3.\textsuperscript{2} Our next challenge was to use this tumor model in vivo to examine the efficacy of various treatments and schedules. However, a sensitive method to evaluate the initial tumor burden in a given mouse and to follow the effectiveness of therapy over time was needed. Such a methodology has now been developed using human bladder cancer cells engineered to stably express the green fluorescent protein (GFP) \textit{in vivo} to visualize the tumor burden over time by intravital imaging.

Materials and methods

\textit{Human bladder cancer cell line}

The human bladder cancer cell line, KU-7, was used for our initial experiments, because tumors form in almost 100\% of the bladders using our procedure for intravesical instillation.\textsuperscript{2} The cells were maintained in Earl’s MEM containing 10\% fetal bovine serum and 50 U/mL of penicillin and streptomycin.

\textit{Generation of stable GFP-expressing clones of KU-7 cells}

A Fugene 6 transfection Kit (Roche, Indianapolis, IN) was used, and the transfection was performed according to the manufacturer’s protocol. The cells were harvested by
trypsin/EDTA 48 hours after transfection and subcultured at a ratio of 1:20 into selective medium containing 500 μg/mL of G418. Stable clones expressing GFP were isolated by trypsin/EDTA with cloning cylinders (Bel-Art Products, Pequannock, NJ). Clone 6 was chosen because of its high level of GFP expression and similar proliferation rate compared to the parental cells (see Fig 1).

Superficial tumor formation by intravesical instillation

The method for producing superficial bladder tumors has been previously described. Briefly, female, athymic (nude) mice, 6–8 weeks of age, were anesthetized with Nembutal 25 mg/kg (IP). The bladders were catheterized with a 24-gauge angiocatheter and washed with phosphate-buffered saline (PBS). Subsequently, 100 μL of 0.25% trypsin in 0.025% EDTA (Gibco-BRL, Carlsbad, CA) was instilled into the bladder for 30 minutes by using a purse-string suture placed around the urethra to occlude it. After 30 minutes, the suture was removed and trypsin was drained from the bladder. The bladder was recatheterized and washed out with PBS. Finally 100 μL of 1×10^7 KU-7/clone 6 cells were instilled into the bladders. Again a purse-string suture was placed to occlude the urethra and the cells were retained for 3 hours. The suture was then removed and the bladder evacuated by spontaneous voiding.

Imaging system for GFP-containing bladder tumors and tumor assessment

At various times after the cells were instilled, a Pfannenstiel incision was made to expose the bladder. The bladder was illuminated either with a modified light source, which detects GFP (Lighttools Research, Encinitas, CA) or with a Leica stereomicroscope equipped with a spot camera (Meyer Instruments, Houston, TX). One hundred microliters of PBS was instilled into the bladder to provide a uniformly similar size for image capture (Fig 2c). To manipulate the orientation

Figure 2 Illustration of bladder exposure for imaging analysis. Shown in (a) is the clean scar (arrow) before the third exposure of the bladder. Subsequently a lower abdominal incision was made (b) and the bladder was exteriorized. Then 100 μL of PBS was instilled into the bladder with a catheter (arrow) to provide a uniformly similar size for image capture (c). A string was sutured on the top of the dome (arrow) to aid in the positioning for each view (d).

Figure 3 Comparison of fluorescence intensity in the bladder to the percentage of GFP-containing cells. Between 10 to 14 days after the KU-7/clone 6 was instilled intravesically, the bladders were removed and illuminated with a modified light source that excites GFP. The amount of green fluorescence seen was initially visualized as none, low, intermediate or high. Single-cell suspensions were then made of all normal bladder and tumor cells present. Subsequently the percentage of GFP-containing cells was counted by flow cytometry. The percentage of GFP cells (those within the M1 gate) correlated well with the degree of fluorescence seen, e.g., no fluorescence (<1%) (a), low fluorescence (8%) (b), intermediate fluorescence (18%) (c), and high fluorescence (42%) (d).

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of the bladder, a suture was placed on the dome (Fig 2d). The bladder was then imaged for the presence of GFP-containing tumor cells. Anterior, posterior, and bilateral images were captured and analyzed using Image ProPlus Version 4.x (Media Cybernetics, Silver Springs, MD).

Flow cytometric analysis

After appropriate images were obtained, the bladder was sterilized resected and placed in ice cold Hank's Balanced Solution (HBS). Subsequently, the bladder was minced, resuspended in 0.25% trypsin–EDTA, and placed on a rocker at 37°C overnight. The following day the bladder was resuspended in 1 mL of PBS. A sample of the solution was inspected under a fluorescent microscope to determine the presence of GFP cells in a single cell suspension. Flow cytometric analysis of bladder tumor cells expressing GFP was carried out as previously described.

Results

Initial quantitation of tumor burden comparing imaging to flow analysis

In our initial studies the GFP-expressing bladder tumors could often be visualized through the skin following

Figure 5 Bladder imaging indicating the presence of several tumors after the intravesical instillation of KU-7/clone 6 bladder tumor cells. The anterior (a), posterior (b), left lateral (c), and right lateral (d) images were captured 2 weeks after injecting the cells. The arrow shows the same tumor in the different views.
Figure 6. Imaging of the tumor growth of KU-7/clone 6 cells. The identical right lateral image of the bladder taken two weeks after the intravesical instillation of KU-7/clone 6 bladder tumor cells (Fig 5d) is shown in (a). The arrow indicates the specific tumor to be analyzed. The same view of the bladder was then taken at 3 weeks (b) and 4 weeks (c), respectively. The ratio of fluorescent area (marker tumor) to the total area of the bladder was calculated with Image-Pro Plus Software to be 0.5% in (a), 4.1% in (b), and 7.3% in (c), clearly showing the progression of the same tumor (arrow) over time.

illumination with a blue light source (Fig 1c). However, the sensitivity of this technique was limited. Subsequently 20 bladders were removed and imaged for GFP 7 to 14 days after the cells were instilled intravesically. The amount of tumor present was scored as low, moderate or high depending on the intensity and area of the green fluorescence. In a few cases no green fluorescence was observed. When single-cell suspensions of the entire bladder were made, which included both normal and tumor cells, the percentage of GFP-containing cells (tumor cells), was similar to the intensity of fluorescence (tumor) seen in the intact bladder (Fig 3). This correlation was even more precise when actual imaging of the tumor was done to determine the percentage of the bladder involved with tumor (Fig 4). Because the initial studies showed a high correlation between the two techniques and because flow analysis requires the sacrifice of the mouse, therefore precluding subsequent evaluation of tumor growth, all the subsequent studies were done using tumor imaging.

To follow intravesical tumor growth in the same bladder over time images were taken initially between 1 to 2 weeks following instillation of the GFP-containing cells and weekly thereafter for four additional weeks by exteriorizing the bladder (Fig 2). All of the initial 10 mice studied were healthy at the termination of the 5-week experiment. There was also no inflammatory nor

Figure 7. Significantly different tumor burden shown by various imaging views. Tumors can be seen well in the left lateral view of the bladder (a) but less readily in anterior and posterior views (b) and (c), respectively, and not at all in the right lateral view (d).
fibrotic reaction surrounding the bladders (Fig 2c and d). Although additional mice have been studied as well as other cell lines only the results from using KU-7 cells are reported here.

Multiple tumors were visualized in the bladder of each mouse, which reflected not only the incidence of tumor take but also the sensitivity of detecting even the smallest tumor by this technique. To avoid missing any tumor present in the bladder, because all tumors readily fluoresced, anterior, posterior, and bilateral images were captured. This approach was facilitated by placing a suture at the top of the dome (Fig 2d). An example of a typical bladder imaged two weeks after initial cell instillation is shown in Figure 5. Because our procedure makes it possible to examine the same tumor over time and because numerous different size tumors are routinely seen, one can also choose a representative tumor to follow in only one view rather than imaging all views (Fig 6). The ratio of the tumor to the total area of the bladder imaged can then be calculated. For example the tumor identified in a right lateral view shown in Figure 6 represented 0.5% of the total bladder area at week 2 (Fig 6a), 4.1% at week 3 (Fig 6b), and 7.3% at week 4 (Fig 6c), demonstrating the growth of the same tumor over time. The results are typical of tumor growth seen in other bladders.

In certain bladders one position gave a significantly better view of the initial tumor burden than other views. This is readily apparent in bladders such as that shown in Figure 7 where the majority of the tumor present is seen in the left lateral view (Fig 7a) and would be the best view to follow over time by imaging to measure tumor growth.

Discussion

Cancer cells that stably express high levels of GFP in vivo have been used in previous studies to image tumor and metastasis. The green fluorescence enables one to visualize the tumor cells in vivo allowing them to be monitored in different sites. We have now developed for the first time an intravesical mouse model to readily image the tumor burden and growth by instillation of bladder cancer cells expressing high levels of GFP followed by fluorescence imaging of the tumors within the bladder over time. Because it was possible to study the growth of individual tumors in the bladder at different times in the same mouse, it will now be possible to document responses to various intravesical therapies, including gene therapy.

The KU-7 cell line was used initially for our studies because it has rapidly produced tumors in almost 100% of the bladders into which it was instilled. Cell clones from additional bladder cancer cell lines that also contain high levels of GFP expression have also been selected. Several of these have also been used in this model system and have provided similar results (unpublished data). They will be used in future studies to examine the effectiveness of various therapies as well.

The imaging system and techniques we developed allow us to readily capture the tumor present in the anterior, posterior, and lateral images by their strong GFP fluorescence. This is a highly sensitive technique that can identify the presence of very small tumors because of the level of fluorescence within the tumor cells and the ease to externalize the bladder for imaging as well as the thinness of the bladder wall. Although GFP levels can decrease over time in some tumors or cell lines we have not found this to be a problem over the 1-month period the tumors have been examined. Using this model, one can determine the tumor burden in each mouse and randomize the mice accordingly to account for differences as well as choose the views that best define the tumor present before the initiation of any treatment.

The conventional methods for evaluating the effect of an intravesical therapeutic agent has been time and labor consuming. First, the mice have to be sacrificed, the bladders need to be inspected under the dissecting microscope, and a large number of bladder tissue sections must be made throughout the entire bladder to evaluate them histologically for the amount of tumor present. Moreover, only one time point can be studied per mouse. Finally, to achieve any statistically significant results, a large number of mice are needed and one could always question whether or not the tumor burden was similar in treated and untreated animals.

In contrast, the system we have developed enables one to examine tumor growth or reduction in the same bladder over time after a specific treatment or schedule and compare this with the typical tumor growth produced by the same cell line in untreated bladders. In addition, because the number and size of the tumors generated in each bladder varies considerably one can also select a representative tumor and follow its growth in one or more views over time to evaluate its response to a specific intravesical therapy (e.g., Fig 6). We are currently using this system in our preclinical gene therapy studies for superficial bladder cancer to evaluate the effect of treatment and to optimize conditions for adenoviral mediated gene transfer before initiating phase I intravesical trials.

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