GFP image analysis in the mouse orthotopic bladder cancer model

MARCO A. DE VELASCO[1], MOTOYOSHI TANAKA[1], SATOSHI ANAI[3],
ATSUSHI TOMIOKA[3], KAZUTO NISHIO[2] and HIROTSUGU UEMURA[1]

Departments of [1]Urology and [2]Genome Biology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka; [3]Department of Urology, Nara Medical University, 840 Shijo-cho, Kashihara, Nara, Japan

Abstract. Precise and objective measurements of tumor response have yet to be standardized in the mouse orthotopic bladder cancer model. In this study, we used image analysis and green fluorescent protein (GFP) to objectively measure tumor size in response to chemotherapy. KU-7 human bladder cancer cells transfected with GFP were intravesically inoculated into 8-week-old female nude mice. Fourteen days after tumor cell inoculation, the mice were assigned into a control (PBS) group or a doxorubicin (conc. 1.0 mg/ml) treatment group and received a single instillation of treatment. Fourteen days after treatment, the bladders were surgically exposed and fluorescent images were captured and later analyzed using image analysis. Bladders were processed for histological examination. Tumor incidence determined by GFP expression and histology was 100 and 80%, respectively, in the doxorubicin treatment group. A 9-fold (histology) vs. 12-fold (GFP expression) difference in tumor regression measured by tumor area ($P<0.05$) and a 5-fold (histology) vs. 9-fold (GFP expression) difference in tumor regression measured by the percent of tumor area in the bladder ($P<0.001$) were observed in the doxorubicin treatment group. Our findings suggest that using image analysis provides a precise, sensitive and objective means to measure tumor growth and treatment response in the mouse orthotopic bladder cancer model in lieu of histological methods. Consequently, the number of mice required in an experiment can be reduced since tissue samples are not needed for histology, thus making tissue samples readily available for additional assays in both a labor-effective and cost-effective manner.

Introduction

In the United States alone, urinary bladder cancer accounts for over 60,000 new cases per year and in Japan, over 16,000 new cases are reported annually (1,2). In Japan and western countries over 90% of the bladder carcinomas are urothelial carcinomas (3). Transurethral resection in combination with intravesical Bacille Calmette-Guérin (BCG) or chemotherapy are the mainstay for treatment of these lesions; however, 5- and 10-year recurrence rates of over 60 and 90%, respectively, pose a major challenge in the treatment and eradication of disease (3,4). These factors combined necessitate further evaluation of new treatment strategies.

Orthotopic tumor models provide researchers with an invaluable research tool, as tumor cells are able to grow in a native environment that more closely resembles its natural state. The mouse orthotopic bladder cancer model has been used for over 30 years, recently this model was modified by using human bladder cancer cells transfected with green fluorescent protein (GFP) implanted transurethally for *in vivo* visualization of tumor growth (5-7). GFP, a spontaneously fluorescent protein that absorbs UV-blue light and emits green fluorescence, has been shown to be suitable in monitoring and visualizing tumors *in vivo* (8). Additionally, tracking cells that stably express GFP *in vivo* allows for a rapid and more precise method for detecting tumor cells than the more traditional histological methods (9). Orthotopic bladder cancer models have already been used successfully to measure the treatment efficacy in chemotherapy, immunotherapy, gene therapy, and chemoprevention models (10-13). This system provides an ideal setting for testing chemotherapeutic agents *in vivo*; however, the challenge to accurately, practically, and objectively measure tumor response remains. Traditional means to measure tumor response have either been qualitative (rather than quantitative) or subjective. The use of computer assisted image analysis provides a solution to this problem in that it allows for a means to measure data in an unbiased manner. Measurement data can be spatially calibrated and since the manner of analysis is standardized, the results are consistently reproduced.

Correspondence to: Dr Motoyoshi Tanaka, Department of Urology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan
E-mail: mtanaka@med.kindai.ac.jp

Abbreviations: BCG, Bacille Calmette-Guérin; CCD, charge-coupled device; Dox, doxorubicin; EDTA, ethylenediamine tetra-acetic acid; GFP, green fluorescent protein; H&E, hematoxylin and eosin; PBS, phosphate-buffered saline; PSA, prostate specific antigen; ROI, region of interest

We propose a model that allows the use of image analysis software to accurately measure the growth and response of orthotopically implanted GFP-labeled human bladder cancer cells to intravesical drug treatment. For this purpose, we used doxorubicin (Dox), an anthracycline antibiotic commonly used in maintenance therapy for superficial bladder cancer (4).

Materials and methods

Bladder cancer cell line and transfection. KU-7, a human bladder cancer cell line derived from a superficial papillary tumor was used for our experiments (14). KU-7 cells were maintained in Gibco DMEM (Dulbecco's modified Eagle's medium) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum in a 37˚C incubator with 5% CO_2. Stable GFP clones were generated as previously described (7). GFP expression was confirmed under a fluorescence microscope.

Orthotopic tumor implantation and intravesical treatment. Prior to bladder instillation, the cells were trypsinized and re-suspended in serum-free DMEM at a concentration of $1x10^8$ cells/ml. A method for the orthotopic instillation of tumor cells has been described (7). Briefly, eleven 8-week-old female athymic nude mice were anesthetized by intra-peritoneal injection with Nembutal (Dainippon Sumitomo Pharma, Osaka, Japan). A 24-gauge catheter was inserted transurethrally into the bladder (Fig. 1A) and washed with 200 μl of PBS (phosphate-buffered saline). The bladder was emptied and 100 μl of 0.2% trypsin in 0.02 EDTA (Invitrogen, Carlsbad, CA) was infused and retained for 20 min. A 100-μl KU-7/GFP cell suspension containing $1x10^7$ cells was instilled into the bladder thereafter. The urethra was then ligated for 2-3 h with a purse-string suture to prevent leakage of tumor cells. Fourteen days after cell implantation, the animals were assigned to a control (PBS) (n=6) or doxorubicin (doxorubicin hydrochloride) (Kyowa Hakko Co. Ltd., Tokyo, Japan) (conc. 1.0 mg/ml) (n=5) treatment group and received a single intravesical treatment instillation that was retained for 1 h by purse-string suture.

In vivo fluorescence analysis of mouse bladders. Twenty-eight days following the instillation of tumor cells, the mice were again anesthetized as previously described and the bladders were surgically exposed. The whole mouse was placed under a Leica MZFL (FLVO III) dissecting stereo-microscope equipped with a fluorescent light source with a GFP2 filter and a CCD camera (Leica Microsystems, Heerbrugg, Switzerland). The exposed bladders were examined under fluorescence for the presence of tumors and digital images were captured under both fluorescent and incandescent light (Fig. 1B-D). The mice were then sacrificed and bladders were removed then fixed in 10% neutral buffered formalin.

Histological examination. The bladders were fixed overnight in 10% neutral buffered formalin and then transferred to 70% ethanol. Bladders were then sliced transversally in half and examined under a stereomicroscope. Tumor presence was confirmed and representative images were captured. The samples were then embedded in paraffin, step-sectioned and stained with hematoxylin and eosin (H&E). Tumors were verified under a light microscope and representative digital images were recorded for subsequent image analysis.

Image analysis. Image analysis was performed with ImageJ public domain software available through the National Institutes of Health (Bethesda, MD; available at http://rsb.info.nih.gov/ij/). All images were spatially calibrated for area measurements. Fig. 2 demonstrates an example of the process used to analyze and measure the bladders and tumors. Images of the processed bladders were captured from H&E stained slides and both the bladder and tumors were traced individually and identified by creating a ROI (region of interest) for each. The area was then calculated for each of the selected ROIs. Multiple step sections were taken from the embedded bladders and sections containing the largest tumor size were used for the area calculations. In cases with multiple tumors, lesions were identified and measured individually, however, the measurement data from multiple tumors in an individual bladder was pooled and the area is represented as a single area measurement.

Statistical analysis. Data were statistically analyzed using the Student's t-test and differences were considered to be significant at $P<0.05$. For comparisons between histological analysis and GFP expression, regression analysis was performed. Statistical analysis was carried out using SigmaStat 3.5 (Systat Software, Inc., Jose, CA).

Results

Tumor incidence. We first measured tumor incidence for the implanted KU-7/GFP cancer cells. To measure tumor incidence by GFP expression, surgically exposed bladders were viewed under blue fluorescent light. Bladders without tumors failed to demonstrate any fluorescence; on the other hand, bladders with tumors expressing GFP were easily identified by green fluorescence. Tumor appearance ranged from small individual nests of cells to multiple clusters and/or large masses (Fig. 3). Tumor incidence is defined as the percentage of mice with visible tumors and was determined by direct observation of positive GFP cells within the bladder *in vivo* and/or the presence of tumor cells in the bladder by histological examination. Tumor incidence measured by GFP expression was 100% for all mice in the control and doxorubicin groups. Tumor incidence measured by histological analysis was 100% for the control group but 80% for the doxorubicin treatment group. Histological analysis for tumor incidence failed to reveal microscopic lesions that were clearly detectable by GFP fluorescence.

Measurements of tumor area. Next, we confirmed that cells emitting the green fluorescence were indeed the KU-7/GFP inoculated. To accomplish this, we examined the H&E stained paraffin sections of bladder tumors and compared them with the *in vivo* GFP image and the image of the transversally sliced gross bladders. Once confirmed, we proceeded to measure the bladders and tumors. Tumor size measurements are represented by tumor area and tumor area percent and were calculated by histological analysis and GFP expression. Tumor area is defined a the area of positive GFP emitting regions of green
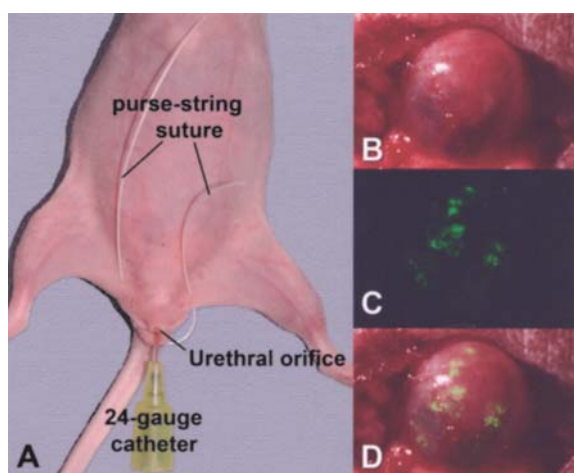
Figure 1. The anatomical view of the nude mouse during the urinary catheterization procedure (A). *In vivo* digital images were obtained by first making a transverse incision across the lower abdomen to expose the bladder. The exposed bladder was filled with PBS an image was acquired using a tungsten light source (B). The bladder was then exposed to a 470-nm wavelength blue light and an image was captured through a 530-nm wavelength emission filter (C). The two images are merged together for the purpose of demonstrating the anatomical location of GFP-labeled tumor cells within the bladder *in vivo* (D).
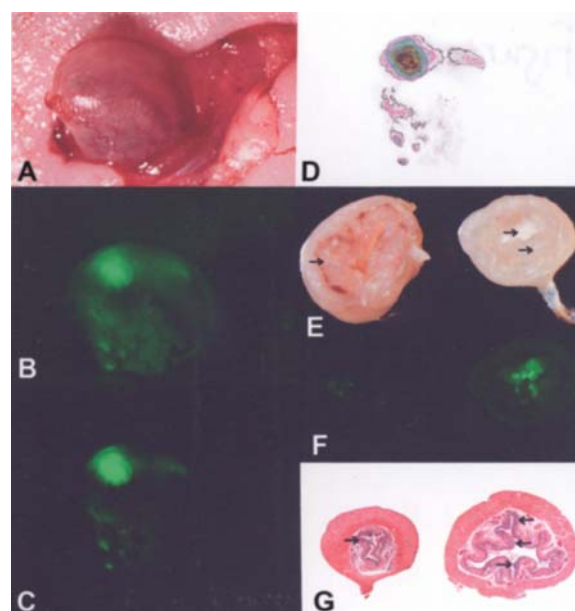

Figure 2. Bladder images were captured under tungsten light (A) and fluorescent light (B) *in vivo*. The raw unmanipulated image was then adjusted and corrected to remove non-specific fluorescence (C). Non-specific fluorescence was subtracted from the background by adjusting contrast and brightness and selecting the appropriate maximum and minimum light and dark set-levels for each individual image. The corrected image was converted to a 16-bit inverted grayscale image, and a mask using color-coded contour lines corresponding to light intensity (corresponding to GFP expression) was applied to identify the tumor (D). Tumor tissue (arrows) was confirmed with the gross bladder (E-F) and the corresponding H&E stained section (G).

fluorescent light in the images captured *in vivo* or regions of tumor bounded by the ROI in selected histological slides. All area measurements are represented by mm^2. To avoid possible differences that may have occurred by having bladders expanded to different diameters, we calculated the tumor area percent. Tumor area percent represents the percent of bladder area comprised of tumor. Regression analysis determined the R^2 values for tumor area and tumor area percent to be 0.281 and 0.692, respectively (Fig. 4). Doxorubicin efficacy against

tumor growth resulted in a 9- and 12-fold decrease of tumor area when measured by histological analysis and GFP expression, respectively (Fig. 5A). Similarly, a 5- and 9.25-fold difference in tumor growth was noted when
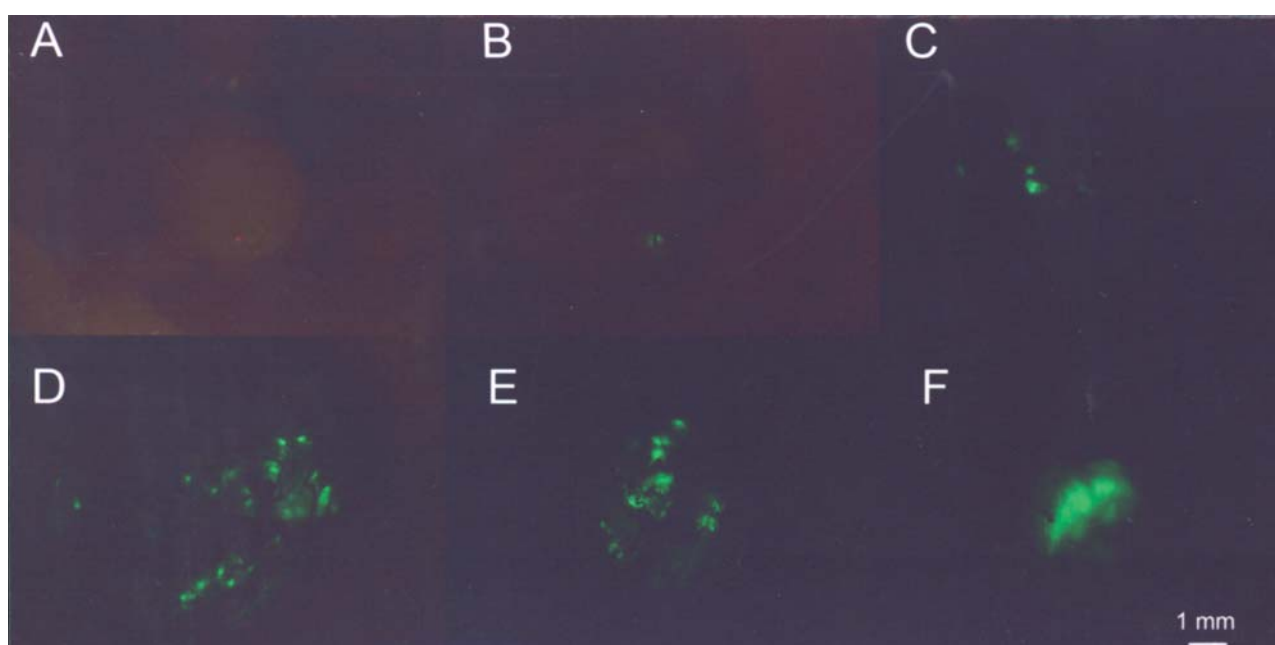

Figure 3. Representative images of bladders taken under a fluorescent light source demonstrate the presence of KU-7/GFP cells by the emission of green fluorescent light. No tumor is present in (A) and as small microscopic lesion is visible (B). Tumors may appear as multiple tumor clusters of varying sizes (C-E) or as a large mass (F).
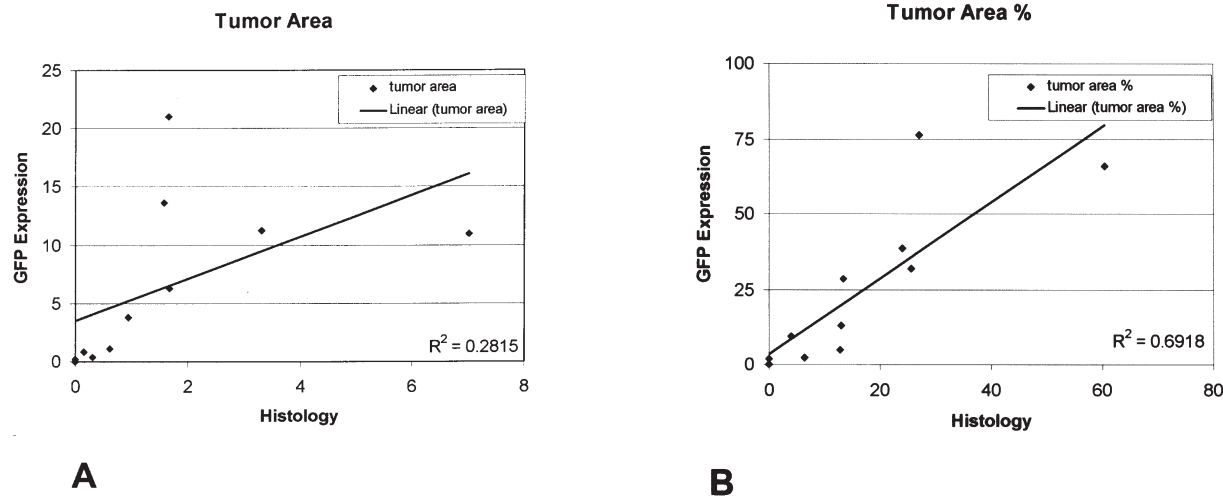
Figure 4. Correlation of tumor area (A) and tumor area percent (B) for all mice bearing KU-7/GFP orthotopic bladder tumors (n=10). GFP expression was plotted against histology examination.
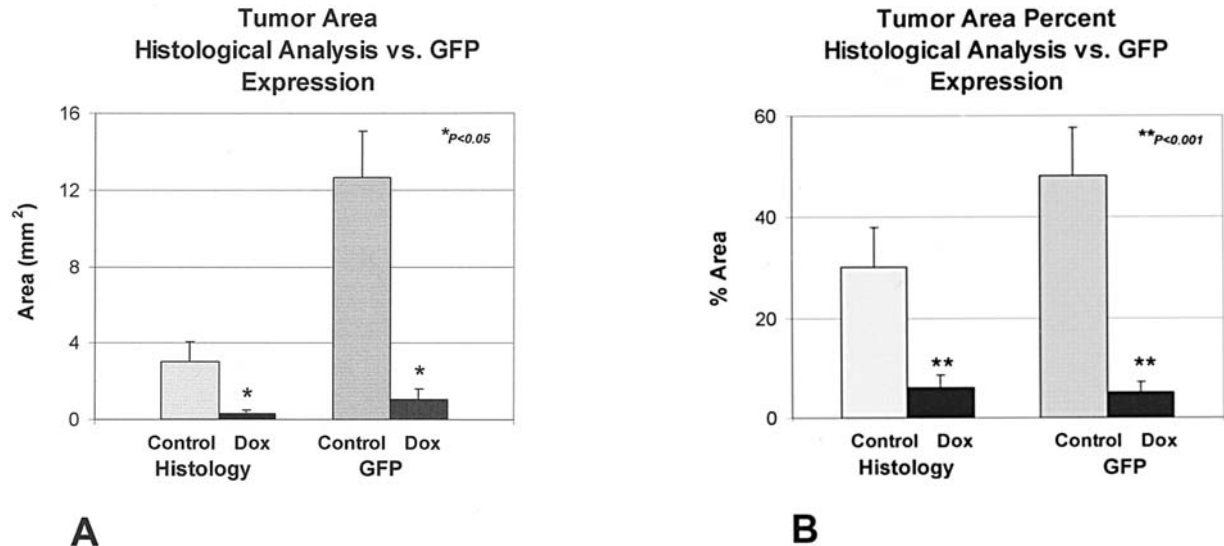

Figure 5. Comparison of tumor response to doxorubicin graphed as tumor area (A) and tumor area percent (B) by GFP expression and histological analysis. *, **P-values versus control, Student's t-test analysis.

measuring tumor response as a percent of the total bladder area as measured by histological analysis and GFP expression, respectively (Fig. 5B).

Although correlation analysis does not demonstrate a strong correlation between tumor sizes measured by GFP and histological analysis, reproducibility and sensitivity were greater by using GFP analysis alone. Similar results were obtained by repeating similar experiments at varying time points (data not shown).

Discussion

The orthotopic bladder cancer model has proven to be one of the most effective models to study tumor biology, particularly in studies involving intravesical therapy. This is due to the fact that tumors are directly exposed to the chemotherapeutic agents in their natural environment (15). Unlike ectopic xenograft models that grow easily visible subcutaneous tumors,

bladder orthotopic models grow tumors internally that cannot be visualized grossly *in vivo* and thus renders a challenge to confirm tumor incidence, growth and response. Traditional methods to determine tumor burden in the orthotopic bladder cancer model consist of weighing the bladder and histological analysis (16). However, sensitivity and objectivity in measuring tumor burden is limited due to variability of bladder and tumor size, especially in the case of microscopic disease. Use of GFP-labeled bladder cancer cells in the orthotopic bladder cancer model provides an added means to qualitatively identify such tumors *in vivo*, however, precise and objective quantitative methods have yet to be developed.

The focus of this experiment is to establish a method to measure tumor size in an objective and efficient manner. We compared sensitivity, objectivity and efficiency in measuring tumor response after chemotherapeutic treatment with doxorubicin against the more traditional histological evaluation method. Our results clearly elucidate some of the benefits

gained by using this method versus the more traditional histological method, namely sensitivity and objectivity. This technique allows for a more sensitive and faster method to detect tumor presence in the bladder, especially in cases involving microscopic lesions. Use of image analysis provides not only an objective solution for the interpretation of visual data; it also allows us to establish a workflow in a convenient and efficient manner (17). Our data suggest that measuring the amount of GFP fluorescence emission is sufficient to determine tumor burden and calculate tumor response to treatment.

In traditional methods, tumor burden is calculated by histological examination (i.e., H&E stained slides) usually examining a single plane of the tumor, thus giving us only a snapshot that may or may not be accurate. Large tumors pose a problem in that they are irregular which may lead to bias when searching for the best representative section to analyze and quantify. Furthermore, small tumors present a different problem in that they may be too small to accurately capture in a thin section and may therefore lead to inaccuracies as they may be missed during routine histological procedures. Another complication is that we attempt to examine a two-dimensional object and interpret it as a three-dimensional structure. These differences are supported by our correlation analysis data of tumor size measurements by GFP emission and histology confirming that these are in fact two separate parameters.

An alternate method used to determine tumor burden in the orthotopic bladder cancer model is weighing the bladder; however, this only compares the bladder of one mouse versus that of other mice (18). The problem with this method is that it does not provide any information pertaining to the location of the lesion or the number of lesions involved. Another method used by some investigators is to measure urine PSA (prostate specific antigen) levels generated by tumors of genetically modified cancer cells to produce PSA (19). An advantage to this method is that it allows for a non-invasive method for the determination of tumor growth, however, it fails to provide any insight into the morphological dynamics including the number of tumors present or tumor distribution and location in the bladder. Use of bioluminescent particles, such as firefly luciferase, coupled with image analysis allow for accurate *in vivo* measurements of labeled tumors (20,21). The drawback to this method is the investment involved as it requires specialized dedicated imaging equipment for imaging small animals.

Our results demonstrate that using computer assisted image analysis to measure GFP expression, as a parameter for the determination tumor response to therapy in the mouse orthotopic bladder model, serves as a powerful and objective tool proving to be more sensitive than histology. Additionally, using this method allows the investigator to conduct more labor-effective and cost-effective experiments with minimal investment. Furthermore, this methodology is not limited to the orthotopic bladder cancer model as it can be applied to other tumor models requiring precise objective quantitative analysis of tumor size.

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