Abstract. Malignant glioma is the most common type of primary central nervous system cancer. Gliomas are very difficult to completely resect due to their invasiveness. In the present study, we compared fluorescence-guided and standard bright-light resection of a human glioma orthotopically implanted in nude mice. U87 human glioma cells, expressing red fluorescent protein (RFP), were injected stereotactically into the nude mouse brain through a craniotomy open window. Two weeks after cancer-cell implantation, gliomas were resected under fluorescence guidance or under bright light. U87-RFP tumors were clearly visualized with a long-working distance fluorescence microscope. Almost all cancer cells were removed using fluorescence-guided navigation without damage to the brain tissue. In contrast, brain tumors were difficult to visualize under bright light and many residual cancer cells remained in the brain after bright-light surgery. Fluorescence-guided surgery significantly extended the survival of the mice compared to those who underwent bright-light surgery. These results suggest that fluorescence-guided surgery has significant potential for brain cancer treatment.

Fluorescence-guided surgery (FGS) has recently generated much excitement. Kishimoto et al. (1) selectively labeled tumors with green fluorescent protein (GFP) using a telomerase-dependent adenovirus (OBP-401) that expresses the gfp gene only in cancer cells, which, in contrast to normal cells, express the telomerase enzyme (1). It was then possible to resect the labeled tumors under FGS. Tumors that recurred after FGS maintained GFP expression (2), making it possible to carry out follow-up FGS.

In a subsequent report (3), we demonstrated that labeling human pancreatic tumors with fluorescent proteins in orthotopic mouse models, followed by FGS resulted in a significantly improved outcome compared to standard bright-light surgery (BLS). Mice which underwent FGS had a greater extent of resection and improved disease-free survival for one of the most difficult types of cancer to achieve cures.

We have also shown that FGS improved surgical outcomes in fluoroscent orthotopic nude mouse models of human colon cancer expressing a fluorescent protein. Tumors were resected under BLS or FGS. All mice with primary tumors that had undergone FGS had complete resection compared with 58% of mice in the BLS group. FGS resulted in lower recurrence compared with BLS and lengthened disease-free median survival from 9 to >36 weeks. FGS resulted in a cure in 67% of mice compared with only 37% of mice that underwent BLS (4).

5-Aminolevulinic acid (5-ALA), has been used to make gliomas become fluorescent in the clinic. Such labeling resulted in almost a doubling of the frequency of complete resection and doubling of the progression-free survival rates of patients with glioma who underwent FGS, compared to standard surgery in a study of 270 patients (5).

The present report demonstrates the power of labeling gliomas with fluorescent proteins for highly successful FGS in an orthotopic nude mouse model.

Materials and Methods

Human glioma cell line: For RFP gene transduction of cancer cells, 70% confluent human glioma (U87) cells were used. In brief, cells were incubated with a 1:1 precipitated mixture of retroviral
supernatants of PT67-RFP packaging cells and RPMI-1640 (Irvine Scientific, Santa Ana, CA, USA) containing 10% fetal bovine serum (FBS) (Omega Scientific, San Diego, CA, USA) for 72 h. Fresh medium was replenished at this time. Cells were harvested with trypsin/EDTA 72 h post-transduction and subcultured at a ratio 1:15 into selective medium, which contained 200 μg/ml G418 (Invitrogen, Carlsbad, CA, USA). The level of G418 was increased stepwise up to 800 μg/ml (6).

**Cell culture.** U87 cells were maintained in Dulbecco’s Modified Eagle’s Medium (HyClone Laboratories, Logan, UT, USA) supplemented with 10% FBS. The cells were incubated at 37˚C in a humidified atmosphere of 5% CO2 in air. The cells were collected after trypsinization and stained with trypan blue (Sigma-Aldrich, St. Louis, MO, USA). Only viable cells were counted with a hemocytometer (Hausser Scientific, Horsham, PA, USA) (6).

**Mice.** Athymic NCR nude mice (nu/nu) (AntiCancer Inc., San Diego, CA, USA), 6 weeks of age, were used in this study. Mice were bred and maintained in a barrier facility under HEPA filtration. Mice were fed with an autoclaved laboratory rodent diet. All animal studies were conducted in accordance with the principals and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals under assurance number A3873-1.

**Craniotomy open window.** Mice were anesthetized with a ketamine mixture (10 μl ketamine HCL, 7.6 μl xylazine, 2.4 μl acepromazine maleate, and 10 μl H2O) via s.c. injection. After fixing the mice in a prone position, a 1.5 cm incision was made directly down the midline of the scalp. The scalp was retracted and the skull was exposed. Using a skin biopsy punch (Acuderm Inc., Ft. Lauderdale, FL, USA), a 4-mm diameter craniotomy was made over the right parietal bone. The bone fragment was removed carefully in order not to injure the meninges and brain tissue. The craniotomy open window could be covered by the scalp. Scalp retraction enabled multiple imaging sessions of cancer cells in the brain. The incision was then closed with a 6-0 surgical suture (7).

**Stereotactic injection of cancer cells in the brain.** After the craniotomy open window was made, 2×10⁵ U87-RFP cells in 1 μl were injected stereotactically into the mouse brain using a 10-μl Hamilton syringe. Cells were injected in the middle of the craniotomy open window to a depth of 1 mm (7).

**Fluorescence-guided surgery.** Two weeks after inoculation, brain tumors were resected under bright light (BLS group) or under fluorescence guidance (FGS group). An MVX10 Macro View fluorescence microscope (Olympus Corp., Tokyo, Japan) was used for FGS (Figure 1).

**Fluorescence imaging.** An Olympus OV100 Small Animal Imaging System (Olympus Corp.), containing an MT-20 light source (Olympus Biosystems, Planegg, Germany) and a DP70 CCD camera (Olympus) were used for imaging live mice. High-resolution images were captured directly on a personal computer (Fujitsu Siemens Computers, Munich, Germany). Images were analyzed with the use of CellR software (Olympus Biosystems) (8). Seven days after tumor resection, residual brain tumor volume was compared in the BLS group and FGS groups. Tumor volume was calculated by the formula (width² × length × 0.5).

**Survival analysis.** The experimental data are expressed as the mean ± SEM. Statistical analysis was performed using the Student’s t-test. Kaplan-Meier analysis with a log-rank test was used to determine survival and difference between treatment groups.

**Results**

RFP-expressing U87 glioma tumors in the brain were observed through the craniotomy open window using the OV100 imaging system (Figure 2). The craniotomy open window enables imaging of tumor growth in the brain over time while keeping animals alive and without further invasive procedures.
Figure 2. In vivo imaging of U87-RFP glioma growth in the nude mouse brain after bright-light surgery (BLS) or fluorescence-guided surgery (FGS). Representative images of tumor growth in the brain after BLS and FGS. Tumor volume was measured using RFP imaging with the Olympus OV100 at 7 days after tumor resection.

Figure 3. Therapeutic efficacy of fluorescence-guided surgery (FGS) on U87-red fluorescent protein (RFP) brain tumor in nude mice. Two weeks after inoculation, brain tumors were resected under bright light (BLS) (n=5) or under fluorescence guidance (FGS) (n=5). Seven days after tumor resection, residual brain tumor volume was compared in the FGS and BLS groups. Tumor volume was measured using RFP imaging with the OV100 system before and seven days after resection. Tumor volume in the FGS group was significantly smaller than that in control groups (p=0.035).

Figure 4. Survival efficacy after fluorescent-guided surgery (FGS). Survival efficacy after FGS of U87-RFP glioma growing orthotopically in nude mice. FGS significantly prolonged survival. The median survival was increased from 18 days in the bright-light surgery (BLS) group to 61 days in the FGS group (p=0.026). Statistical analysis was performed using the Kaplan-Meier test along with the log-rank test.
Tumor size was measured before surgery and seven days after surgery (Figure 2). The efficacy of tumor resection was evaluated by tumor volume. Before surgery, the average tumor size was 9.20 mm³ in the BLS group and 7.36 mm³ in the FGS group. There was no significant difference between the two groups in tumor volume preoperatively. Tumor volume in the FGS group was 5.07 mm³ on postoperative day 7. This volume was significantly smaller than that in the BLS group (46.4 mm³, p=0.035) (Figure 3).

Survival efficacy after FGS in the orthotopic model is shown in Figure 4. Death from disease was rapid in the BLS group. The median survival time was 18 days. FGS significantly prolonged median survival to 61 days (p=0.026). Two mice treated by FGS survived at day 80.

**Discussion**

We have demonstrated that FGS enables removal of almost all human U87 glioma in the brain of mice in an orthotopic brain tumor model without damage to the brain tissue. FGS prolonged survival of the mice. The craniotomy open window also enables imaging of tumor growth in the brain over time for evaluation of the efficacy of a treatment. These results suggest that FGS surgery has significant potential for brain cancer treatment.

Making tumors glow offers great advantages for cancer surgery. A number of methods to label tumors have been developed. Urano et al. (9) labeled tumors by topically spraying the γ-glutamyl hydroxymethyl rhodamine green probe, use of which is limited to those tumors that overexpress γ-glutamyltranspeptidase. Folate receptor-α (FR-α) has been used for targeting a fluorescence label, but may be limited to a few tumor types such as ovarian cancer, which overexpress FR-α (3). ALA has been used to label glioma for FGS but may be limited to labeling of glioma (10). Fluorescent tumor-specific antibodies (11) have wider applicability for labeling tumors for FGS. However, this method is limited to only those cancer types for which tumor-specific antigens have been characterized. An activatable cell-penetrating peptide (ACPP) labeling method (12) is useful in those tumors that express an appropriate protease which cleaves and thus activates the probe, but the generality of this method is not known.

Because tumors of all types express telomerase, the genetic-labeling method that uses a telomerase-dependent adenovirus to deliver GFP specifically to tumors (1, 2, 13) offers the potential for widespread application and also allows for detection of cancer recurrence (14).

**Conflicts of Interest**

None of the Authors have a conflict of interest in regard to this study.

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**References**


