Detection of brain tumor cells in the peripheral blood by a telomerase promoter-based assay

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Conflict of interest: The authors' institution (University of Pennsylvania) has submitted a patent application based on a component of the technology presented in this manuscript.
ABSTRACT

Blood tests to detect circulating tumor cells (CTC) offer great potential to monitor disease status, gauge prognosis and guide treatment decisions for cancer patients. For patients with brain tumors, such as aggressive glioblastoma multiforme (GBM), CTC assays are needed that do not rely on expression of cancer cell surface biomarkers like EpCAM that brain tumors tend to lack. Here we describe a strategy to detect CTC based on telomerase activity, which is elevated in nearly all tumor cells but not normal cells. This strategy utilizes an adenoviral detection system that is shown to successfully detect CTC in patients with brain tumors. Clinical data suggest that this assay might assist interpretation of treatment response in patients receiving radiation therapy, for example, to differentiate pseudoprogression from true tumor progression. These results support further development of this assay as a generalized method to detect CTC in cancer patients.

PRECIS

The study suggests how the ubiquity of telomerase activation in tumor cells might be exploited as a generalized strategy to detect circulating tumor cells in cancer patients, as a tool to monitor therapeutic response and disease relapse.
INTRODUCTION

High-grade brain tumors are often markedly aggressive and associated with a poor prognosis. For example, despite combination therapy that typically consists of surgical resection, radiation therapy (RT) (1), and systemic chemotherapy (both concurrent and adjuvant) (2), Glioblastoma Multiforme (GBM or Grade IV glioma) remains the most fatal primary malignant CNS neoplasm in adults, with a median overall survival of 14.6 months. The management of patients with brain tumors often includes additional challenges such as accurately differentiating tumor progression from pseudoprogression (3), the latter which consists of treatment-related changes to the normal vasculature and often results in increased signal intensity that is difficult to distinguish from enhancing tumor tissue on Magnetic Resonance (MR) imaging studies.

Given the poor prognoses associated with high-grade brain tumors such as gliomas, and the difficulties monitoring tumor response or progression, there is clearly a need for innovative approaches to improve tumor assessment. Circulating biomarkers are a promising, non-invasive means to evaluate the status of the primary brain tumor, and may potentially help guide patient treatment and management in the future. In this regard, we describe here the novel application of a telomerase-based assay for detecting circulating brain tumor cells, effective in cell culture and animal studies, and in a pilot cohort of patients with high-grade glioma.
MATERIALS AND METHODS

Cell Culture, Western blot and Immunofluorescence

Human glioma cell lines U251 (WHO grade IV), U87 (WHO grade IV), and U373 (WHO grade III) (along with control cell lines SKBR3 (human breast cancer) and PC3 (human prostate cells) were purchased from the American Type Culture Collection (Manassas, VA) and were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and 1.0% penicillin-streptomycin at 37°C in an atmosphere of 5% CO₂.

For Immunoblotting, 3 x 10⁵ cells were seeded into each well of a 6-well culture plate and left undisturbed for 24 hours, prior to lysis with 100 µl of 2X Laemmli sample buffer. The cell lysates were subsequently boiled for 5 min and centrifuged to remove cell debris. An equal volume of each cell lysate sample (15 µl) was loaded into the individual wells of a NuPAGE® Novex ® 4-12% Bis-Tris gel (Invitrogen) and resolved by electrophoresis in 1x NuPAGE® MOPS SDS Running Buffer (Invitrogen) for 1:20 h at 125 V. 20 µl of 2.4 µg/µl of normal brain extract (Santa Cruz Biotechnology, Inc.) served as a negative control. The SeeBlue®Plus2 prestained protein standards (Invitrogen) was included (12 µl per well) for protein size determination. Electrophoretic transfer onto poly-vinylidene fluoride (PVDF) membrane was performed with 1x NuPAGE® Transfer Buffer (Invitrogen) at 30 V for 1 h. Membranes were then blocked with 5% (w/v) dried nonfat milk in PBST buffer (1x Phosphate Buffered Saline buffered containing 0.06% Tween 20) for 1 hour at room temperature and then incubated with primary antibodies. Immunoblotting for Nestin (1:1000,mouse,clone 10C2 , Abcam Inc.), Ran (1:20000,mouse monoclonal, BD Transduction Laboratories), EGFR (Cell Signal)
and β-Actin (1:1000, clone AC-15, Sigma-Aldrich) was performed overnight at 4°C. Alternatively, immunoblotting for EpCAM (1:500, mouse, clone #158210, R&D Systems) and hTERT (1:500, rabbit, clone Y182, Millipore) was performed for 2 h at room temperature. Following incubations with primary antibodies, membranes were washed in PBST buffer and incubated with HRP-conjugated secondary antibodies (Amersham™, GE Healthcare) for 1 h at room temperature. All antibodies were diluted in PBST-5% milk buffer. Immunoreactive bands were visualized via enhanced-chemiluminescence by incubating membranes with the ECL™ Western Blotting Detection Reagents in accordance with the manufacturer’s recommendations (Amersham™, GE Healthcare). Films were exposed to the membranes for the appropriate durations and subsequently scanned with a personal scanner (Epson Perfection 4490 photo). The specificity of the anti-EGFR antibody was tested (via western blotting or immunofluorescence) on cell lines known to express or to be deficient for EGFR (Supplemental Figure 2). Immunofluorescence was performed on formalin- or formaldehyde fixed cells (1% formalin, 10 minute duration at RT), with incubation with primary antibodies for one hour, followed by incubation for 30 minutes with fluorochrome-conjugated secondary antibodies (Molecular Probes).

Orthotopic Brain Tumor Xenografts in Mice

U251 cells constitutively expressing GFP and luciferase, were orthotopically implanted in a nude, athymic mouse per previously published protocol (4,5). Brain tumor growth was confirmed and tracked via serial bioluminescence imaging, prior to removal, sectioning, and formalin fixation. The brain tumor sections were subsequently stained for hematoxylin and eosin (H&E) or for immunofluorescence with the following
antibodies: Nestin (AbCAM), EpCAM (R&D systems), GFAP (Invitrogen), DAPI (Vector Laboratories).

**Adenoviral Probe for Tumor Cell Detection**

The tumor cell detection assay utilizes a telomerase-responsive adenoviral probe that consist of the expression cassette for green fluorescent protein (GFP, inserted within the deleted E3 region), as well as the hTERT promoter driving expression of E1A and E1B for viral replication and amplification of the GFP signal (TelomeScan, Oncolys (6)). The efficacy of the probe in glioma cell lines was first characterized via time course experiments and immunofluorescence staining. For these experiments, cells were seeded onto 8-well Poly-d-lysine-coated chamber slides (typically 5x10^3 cells/well) prior to viral infection. The probe (2X10E8 viral particles) was introduced into each well and time-lapse phase contrast and fluorescent microscopy images were taken every six hours for the duration of each experiment.

**Analysis of Patient Samples**

After receiving written informed consent, peripheral blood collections were obtained from patients or healthy control volunteers under Institutional Review Board-approved protocols. All patients in the study were free of significant comorbid medical conditions or prior cancer, deemed operable, and underwent biopsy, sub-total or gross-total surgical resection, followed by radiation therapy. Peripheral blood was also obtained from healthy, cancer-free adult volunteers, and processed alongside patient samples, to serve as controls. To determine a CTC threshold in patients, we conducted an analysis of thirty blood samples collected from healthy volunteers. This analysis
determined a threshold of 1.3 GFP-positive cells per ml of blood from healthy controls as the “baseline” for determining whether a patient was considered to have detectable CTCs. To minimize the risk of inadvertent collection of epithelial stem cells, the initial tube of collected blood was discarded. For each patient, approximately 10 ml of peripheral blood was collected into green-top vacutainer tubes containing the anticoagulant heparin. The collected blood was then centrifuged in Oncoquick tubes as per a protocol adapted from manufacturer recommendations to remove RBCs and other non-CTC constituents of the blood. The resulting CTC-enriched layer was removed for further washing. The purified CTCs were then placed into media-contained chamber slides, and incubated in the presence of the adenoviral probe for 24 hours at 37 degrees Celsius and then fixed. The CTC-containing chamber wells were then imaged via a computer-driven semi-automated fluorescence microscope, with subsequent analysis performed via Image Pro Plus. Analysis included sorting and filtering based on reproducible parameters such as fluorescent intensity (five standard deviations above background), cell area, cell diameter (between 15 and 70 microns), and absence of clumping or debris. The resultant data can therefore identify tumor cells with specificity, and which are identified as brightly fluorescent due to the probe, while also excluding debris and clumped cells (Supplemental Figure 2).

Statistical Methods

Data were summarized as counts per milliliter (ml). We analyzed data using an ANOVA model estimated in a Generalized Linear Models regression framework, using a negative binomial link for the cell-count outcome. We accounted for repeated measures
from the same subject using the cluster-correlated robust covariance estimate to adjust standard errors. Models treated timepoint/control status as a categorical variable, and treated the before treatment condition as the reference group. A logistic regression-based classifier (applied to all data) was employed to discriminate between control \((n=30)\) and pre-treatment \((n=11)\) levels. To define patients with CTC-detectable disease, we then used the classifier to determine a threshold of \(1.3\) cells/mL (demarcated as “T” in Figure 4; A and B).

**RESULTS and DISCUSSION**

Our initial experiments included those for establishing the specificity of the probe, and testing the usefulness of Nestin as a glioma cell marker for immunofluorescence and subsequent circulating tumor cell (CTC) analysis. Glioma cells do not express epithelial cell adhesion molecule (EpCAM) \((7,8)\), commonly utilized for CTC detection. All glioma cell lines we tested (U251, U373, and U87) showed varying levels of Nestin expression, but virtually no EpCAM. In contrast, EpCAM but not Nestin was detected in breast (SKBR3) and prostate cancer (PC3) cell lines (Figure 1A). Moreover, absence of EpCAM expression in glioma was confirmed via immunofluorescence staining of U251 glioma cells in culture with PC3 prostate cancer cells utilized as positive control (Fig. 1B). The usefulness of Nestin as a glioma marker was also confirmed in an *in vivo* model of glioma. In this system, human glioma cells were implanted into nude mice to generate orthotopic brain tumors as previously described \((4,5)\). As shown in Figure 1C, the resultant tumors continue to express Nestin and show absence of EpCAM *in vivo*. As an additional test of tumor demarcation, we stained the sections for Glial Fibrillary
Acidic Protein (GFAP), and which also readily identified the tumor due to its glial origin. Finally, since our ultimate goal was to track tumor cells based on elevated telomerase expression (9,10), the brain tumor sections were stained for the presence of human Telomerase (hTERT). As expected, hTERT showed specific in vivo tumor staining similar to that of Nestin (Fig. 1C).

The co-localization of Nestin and Telomerase in our brain tumor model in contrast to the lack of either in normal brain was encouraging for utilization of elevated telomerase as a marker of glioma tumor cells. We have confirmed that all brain tumor cell lines (as well as cancer cell lines from other tumor histologies) tested show elevated levels of telomerase, while normal brain (either human or mouse) does not (Figure 1D and data not shown). We next established the effect of the telomerase-detecting probe in glioma cells as a function of time and in the presence of control blood from healthy volunteers. Each of the glioma cell lines tested showed signal at 12 hours after exposure to probe which was robust by 24 hours (Figure 2A and B). The signal was durable and persisted to and beyond 48 hours after initial exposure (albeit the absolute numbers of cells were reduced due to cell death). Finally, we spiked control blood with human glioma cells grown in culture which stably express the fluorescent tag mCherry to allow discernment from non-tumor cells. The entire sample was then exposed to probe and imaged 24 hours later. As shown in the representative image in Figure 2C, only the tumor cells expressed GFP after exposure to probe, resulting in complete concordance with mCherry fluorescence (resulting in only yellow cells when the respective images are merged (“Merge”), with no cells showing only GFP or mCherry alone). In contrast, the nuclei of the white blood cells in the sample also stain with the
nuclear marker DAPI, but showed neither GFP nor mCherry fluorescence. Additional experiments with time-lapse microscopy confirmed the lack of GFP false-positive signal in processed control blood from healthy volunteers exposed to the telomerase-based CTC probe (Supplemental Fig. 1A and B). These experiments together therefore established the specificity of the probe for accurately demarcating glioma cells while leaving normal cells unaffected.

We subsequently demonstrated that Nestin also serves as a useful marker of glioma-derived tumor cells in peripheral blood samples taken from patients. The probe identified tumor cells with elevated telomerase activity (via GFP expression) which was coincident with Nestin expression (Figure 3A). In contrast, the nuclei of surrounding WBCs stained for DAPI but showed neither GFP nor Nestin expression. Furthermore, as an additional verification that the GFP-positive cells that we were identifying in glioma patients were in fact derived from the primary tumor, we conducted immunofluorescence staining for EGFR in the CTCs from patients with tumors that demonstrated amplification of the Epidermal Growth Factor Receptor (EGFR). Figure 3 shows the results of a representative patient (with additional images in Supplemental Figure 3). This was a patient with a WHO grade IV primary tumor who underwent resection, and with the resultant tissue found to have EGFR gene amplification based on solid tumor next generation sequencing panel (Illumina TruSeq Amplicon-Cancer Panel). The amplification was reported as a 10-12 fold increase in the sequencing reads corresponding to the amplicons covering EGFR in the submitted tumor sample. Immunohistochemical analysis of the tumor resection specimen also noted strong EGFR protein expression in the majority of tumor cells. This genetic underpinning
identified in the primary tumor provided us with the opportunity to test the patient’s peripheral blood sample for circulating tumor cells which should also show overexpression of EGFR. We found that the probe identified tumor cells via GFP expression, and which we subsequently confirmed showed strong EGFR overexpression (results with CTCs from additional patients with EGFR-overexpressing tumors are shown in Supplemental Figure 5). These results together indicated that the probe was successful in accurately identifying glioma-derived CTCs in the peripheral circulation of patients with high-grade (and EGFR-overexpressing) glioma. Additionally, EpCAM was not detectable in the CTCs of this patient, as well other patients with gliomas that lack EGFR overexpression (Supplemental Figure 6).

Having established the potential usefulness of the probe, we initiated a pilot study to study CTCs in cohort of glioma patients undergoing radiation therapy (RT) in the Department of Radiation Oncology. Peripheral blood samples were obtained from patients with pathology-confirmed primary high-grade (WHO grade III or IV) glioma, either before initiation of (“Pre-RT”), or following completion of cranial RT (“Post-RT”, total dose of 3000 to 6000 cGy). Healthy volunteer blood samples served as negative controls and were processed alongside patient samples (Figure 4A). We found that CTCs were detectable in eight of eleven (72%) pre-RT patients, compared to one of eight (8%) post-RT patients (Figure 4B). In the pre-RT patients, there was a wide range of CTCs, but the average value was 8.8 CTC per ml of blood.

For a small number of the patients, sequential CTC counts were available and were especially illuminating. One patient (the second patient in Table 1) had been previously treated with adjuvant chemo-radiation for high-grade glioma but then
represented with obstructive hydrocephalus with MRI (Magnetic Resonance Imaging) showing a cystic mass in the left thalamus. A ventriculoperitoneal shunt was placed and salvage radiation was prescribed (3000 cGy). The first CTC analysis performed before the start of the salvage radiation was found to be 5.0 CTC/mL. While the patient appeared stable in the initial post re-irradiation period and with standard MRI appearing unchanged, the CTC count was however found to have increased post-treatment to 15.4 CTC/mL. Subsequent advanced MRI scans then demonstrated increased rCBV in the tumor supporting the early finding of progressive disease, and the patient was later confirmed to have tumor recurrence (Figure 4C, Left panels).

A second patient with high-grade glioma (Table 1, Figure 4C, Right panels) was found to have a pre-RT count of: 12.5 CTC/mL. The patient completed and tolerated chemoradiation without complications. A post-treatment MRI showed persistence of T2 signal (initially interpreted as: “progressive disease versus treatment changes”), while the post-treatment CTC level of 1.0 CTC/mL indicated treatment response. In subsequent post-treatment visits the patient’s MRI scans demonstrated improvement and therefore, the imaging findings were ultimately attributed to “pseudoprogression”, not progressive disease.

Standard monitoring for treatment response and disease progression in high-grade glioma patients can present challenges. Serial MRI obtained before and following completion of therapy is typically involved, but changes resulting from either “pseudoprogression” or radionecrosis may mimic progressive disease. “Pseudoprogression”, commonly associated with Temozolomide therapy, entails new MRI enhancement within three months of completion of RT, which gradually resolves...
and thus non-indicative of true tumor progression. A “pseudoprogession” incidence of 31.1% was recently reported within a cohort of 103 patients with high grade gliomas (11). Pseudoprogession is therefore often mistaken for true tumor progression, potentially resulting in great anxiety for patients and family as well as interfering with the accuracy of MRI in up to half of patients during the first post-RT MRI (12, 13). Radionecrosis, which typically occurs within 3-12 months post-RT (14), can further hamper the accurate diagnosis of tumor progression. Taken together, further research and development of additional tools to monitor tumor status, such as a brain tumor CTC assay, would be a welcome complement to radiographic imaging.

Although detection of CTCs from primary brain tumors presents a unique challenge, a number of technologies have been described for CTC purification and detection of tumors in general. Among the earliest were gradient separation fluid-based methods (eg. Ficoll Paque©) to separate CTCs from red and white blood cells and other constituents based upon buoyant density (15). Isolation by Size of Epithelial Tumor Cells (ISET) method employs an 8-micron pore diameter filter to separate epithelial CTCs from hematopoetic cells, assuming the former to be larger than the latter (16), but overlapping pores created in the filter manufacturing process may result in non-uniform pores. Newer generation systems have been created to attempt to improve and optimize CTC sensitivity (17). Among the most prominent is the CellSearch system (Veridex, LLC) (18) is based on the detection of epithelial cell adhesion molecule (EpCAM). Mikolajczyk et al. attempted to utilize EpCAM and cytokeratin (CK) and other antibody mixtures to identify a greater range of CTCs (19), but these systems may not be effective for tumors that do not express such surface markers, including brain
tumors. Furthermore, epithelial-to-mesenchymal transition (EMT) may occur in tumors of epithelial origin, in which surface cell markers are downregulated and mesenchymal markers are upregulated, and which may negatively affect assay performance (20-22). Of note, tumors that have undergone EMT may represent a more aggressive phenotype, portending worse prognosis (20) and treatment resistance (23).

We utilized a telomerase-based assay to detect CTCs in our brain tumor patients. Telomerase is a protein expressed in most cancer cells that enables cell renewal by maintaining telomeres found at the end of chromosomes (9). Kojima et al. engineered an adenovirus containing the gene for green fluorescent protein that can selectively replicate in cells that express telomerase (6). Because well-differentiated WBCs typically do not express telomerase, the fluorescence detected after replication can help distinguish CTCs from non-cancerous cells found in the blood. The major advantage of a telomerase-based system is that this method is epithelial cell marker-independent. Although a telomerase-based strategy to identify CTCs has been employed in other malignancies (24-26), our report highlights for the first time this approach for the detection of brain tumor-derived CTCs, which to our knowledge has not been reported in the literature previously.

In conclusion, we propose telomerase-based systems may be useful for CTC detection in patients with brain tumors, offering high sensitivity (since greater than 90% of solid tumors, including glioma, express elevated levels of telomerase) (9) and high specificity (as telomerase is not found to be expressed in well-differentiated and normal cells). We recognize the limitations of this study, including the need for more serial measurements throughout the treatment and disease course for each patient, and the
small sample size (which does not permit sub-factor analysis). Furthermore, future investigation is warranted into characterizing the potentially unique genetic signatures expressed within the brain tumor CTCs which would allow for genetic stratification of patients including information on genetic subtypes and characteristics such as IDH1/2, EGFR, and PIK3CA (27). These efforts are currently being pursued in our ongoing studies.

The encouraging pilot data we describe have also prompted us to begin a new protocol to enroll a larger cohort of patients who will be serially followed through their treatment course and follow-up period (up to two years). The results of such prospective studies will be needed to establish the ultimate clinical utility of this telomerase-based brain tumor CTC assay.

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REFERENCES


### TABLE 1. Clinical characteristics of prospectively followed glioma patients

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<th>Post-RT</th>
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<th>Surgery</th>
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<td>33</td>
<td>F</td>
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<td>M</td>
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<td>Proton</td>
<td>5940</td>
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FIGURE LEGENDS

Figure 1. Preclinical validation of telomerase-based assay for glioma tumor cell detection. **A.** Western blot analysis to assess Nestin and EpCAM expression was performed on human glioma cells in culture (U251, U373, U87), alongside Nestin-negative controls: human breast cancer and human prostate cancer cell lines (SKBR3 and PC3, respectively). Nestin, EpCAM, and Ran loading control and their corresponding molecular weight markers are labeled as indicated. **B.** At 24 hours post Probe exposure, U251 human glioma cells (bottom panels) were counter-stained with EpCAM and DAPI as labeled, alongside EpCAM-positive control PC3 human prostate cancer cells (top panels) to confirm lack of EpCAM expression in glioma via immunofluorescence. Merge image is shown in right-most panels. **C.** U251 (constitutively-expressing green fluorescent protein, GFP) human glioma cells were orthotopically implanted in a nude, athymic mouse with post-mortem brain harvesting and sectioning for H&E staining and immunofluorescence staining of markers (hematoxylin and eosin, Nestin antibody, GFAP antibody, EpCAM antibody, hTERT antibody, DAPI antibody), alongside a negative control (normal mouse brain). **D.** Western blot analysis was employed to detect hTERT expression in human glioma cancer cells (U251, U373, U87), human breast cancer cells (SKBR3) and human prostate cancer cell lines (PC3), alongside negative control normal mouse brain extract. hTERT and β-Actin loading control and their corresponding molecular weight markers are labeled as indicated.
Figure 2. Telomerase-based assay probe \textit{in vitro} characterization for glioma tumor cell detection. \textbf{A.} Human glioma cancer cells (U251, U373, U87 as indicated) were infected with the probe at multiplicity of infection (MOI) of 10, and (20X) images were obtained over 72 hours. Representative phase-contrast microscopy images (to depict morphology, bottom panels) and fluorescence microscopy images (to demonstrate GFP expression, bottom panels) are provided. \textbf{B.} At the respective indicated time points of the \textit{in vitro} time course experiment, automated computer software was employed to determine quantity of cells transduced by the probe (demarcated by GFP expression) and total quantity of cells present on phase microscopy. Ratio of cells infected by the probe to maximal glioma cells present was calculated to establish the ideal time-point for optimal specificity and sensitivity of tumor cell detection by the probe. \textbf{C.} U251 human glioma cells (constitutively-expressing the red fluorescent mCherry protein) were spiked into control human blood followed by standard isolation and processing. Cancer cell specific identification by the probe was demonstrated by the co-localization of mCherry (red) and GFP (green) fluorescence, against a background of non-transduced WBCs (DAPI-positive, GFP-negative cells).
Figure 3. Clinical results: glioma CTC detection and verification. A. A peripheral blood clinical sample was obtained from a representative glioma patient, and subjected to standard processing and enrichment for circulating tumor cells with fluorescent microscopy images acquired 24 hours following addition of the probe. Secondary immunofluorescence staining was conducted for Nestin (red). Panels show representative imaging of a CTC identified with white arrows indicating the colocalization of a CTC after probe (green) identification and Nestin (red) staining. DAPI was used to delineate the nuclei of all cells. B. A peripheral blood clinical sample was obtained from a glioma patient (whose primary tumor was known to have amplification of the EGFR gene and overexpression of EGFR protein) and subjected to standard processing and enrichment for circulating tumor cells with fluorescent microscopy images acquired 24 hours following addition of probe. Secondary immunofluorescence staining was conducted for EGFR (red) as indicated. White arrows indicate the colocalization of two separate CTCs (upper and lower panels) identified after probe (green) identification and EGFR (red) staining. DAPI was used to identify the nuclei of cells.
Figure 4. Clinical results: serial enumeration to monitor treatment response in pilot study. A. Peripheral blood clinical sample was obtained from pre-radiation therapy GBM patient, and subjected to standard processing and enrichment for circulating tumor cells with fluorescent microscopy images acquired following antibody (Nestin, DAPI) staining at 24 hours after exposure to the assay Probe. “T” indicates classifier threshold, defined as the level observed in controls (healthy volunteers). B. CTC counts are elevated in most glioma patients prior to the start of radiation therapy (“Pre-RT”) with marked overall decrease after treatment regimen completion (“Post-RT”). “T” indicates classifier threshold. C. Comparison of CTC trends and brain axial MRI of “progressive disease” versus “pseudoprogression”. Magnetic resonance imaging (MRI) was performed within two weeks prior to initiation of radiation therapy (“Pre-RT”) and approximately one month following completion of treatment (“Post-RT”). CTC results (in “CTC/mL”) are included below axial MR images at the respective time points. Red arrows indicate a left thalamic lesion prior to and following RT (left panels). Inset box delineated by the dotted red line (left panels: Post-RT) demonstrates the tumor area of interest and the associated advanced MR imaging rCBV map conducted in the post-RT setting. Advanced MR imaging confirmed active tumor progression after analysis of rCBV fraction. Blue arrows indicate MR signal abnormality in midbrain lesion and surrounding area on axial view prior to and following RT (right panels).
FIGURE 1.

A. 

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<th>U373</th>
<th>U87</th>
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~176 kDa
~42 kDa
~27 kDa

B. 

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C. 

H&E  Nestin  DAPI  EpCAM  DAPI

D. 

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FIGURE 4.

A. Cells/mL

Healthy Controls

B. CTC/mL

Pre-RT  Post-RT

C. PROGRESSIVE DISEASE

Pre-RT  Post-RT

CTC/mL: 5.0  15.4

PSEUDOPROGRESSION

Pre-RT  Post-RT

CTC/mL: 12.5  1.0
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