Aptamer Identification of Brain Tumor–Initiating Cells

Youngmi Kim1, Qiuilian Wu1, Petra Hamerlik1,8, Masahiro Hitomi1, Andrew E. Sloan3,4,5,7, Gene H. Barnett2, Robert J. Weil2, Patrick Leahy6, Anita B. Hjelmeland1, and Jeremy N. Rich1

Abstract

Glioblastomas display cellular hierarchies with self-renewing tumor-initiating cells (TIC), also known as cancer stem cells, at the apex. Although the TIC hypothesis remains controversial and the functional assays to define the TIC phenotype are evolving, we and others have shown that TICs may contribute to therapeutic resistance, tumor spread, and angiogenesis. The identification of TICs has been informed by the use of markers characterized in normal stem cells, but this approach has an inherent limitation to selectively identify TICs. To develop reagents that enrich TICs but not matched non-TICs or tissue-specific stem cells, we adopted Cell-Systematic Evolution of Ligands by Exponential Enrichment (Cell-SELEX) to identify glioblastoma TIC-specific nucleic acid probes—aptamers—that specifically bind TICs. In this study, using Cell-SELEX with positive selection for TICs and negative selection for non-TICs and human neural progenitor cells, we identified TIC aptamers that specifically bind to TICs with excellent dissociation constants (Kd). These aptamers select and internalize into glioblastoma cells that self-renew, proliferate, and initiate tumors. As aptamers can be modified to deliver payloads, aptamers may represent novel agents that could selectively target or facilitate imaging of TICs.

Introduction

Cancers invoke molecular programs expressed during development and wound responses to promote the initiation and maintenance of complex neoplastic tissue systems that include not only transformed cells but also supportive vasculature, immune components, stroma, and extracellular matrix (1). Standard human cancer models based on established cell lines are subjected to passage under conditions that select for rapid proliferation and survival through mechanisms often distinct from the original tumor. Although proliferation and resistance to apoptosis are hallmarks of cancer, other aspects of cancer—involution of normal tissues, metastasis, resistance to cytotoxic insults, and vascular recruitment—critically contribute to the lethality of cancer (1). An increasing number of cancers have been shown to display cellular hierarchies with a subset of the neoplastic compartment activating molecular mechanisms and cellular phenotypes similar—but not identical—to embryonic or tissue-specific stem cells (2, 3). These tumor-initiating cells (TIC), also called cancer stem cells or tumor-propagating cells, are functionally defined through assays of self-renewal and tumor propagation (4). We and others have shown that TICs are relatively resistant to conventional cancer therapies (radio- and chemotherapy) and promote tumor growth through angiogenesis (5–8). The TIC hypothesis has been questioned because of potential plasticity of the cellular hierarchy and difficulties with TIC identification but these challenges are products of our attempts to simplify complex systems with limited technical resources. On the basis of this background, creating agents that prospectively identify TICs may not only permit the interrogation of the cellular hierarchy in cancers but also serve as a platform for the development of novel targeted therapies and imaging reagents.

Glioblastoma (GBM) is the most prevalent and lethal primary brain tumor and ranks among the most lethal of all cancers (8). Through the work of many groups the presence of a cellular hierarchy has been supported not only in glioblastoma but also other central nervous system cancers (9, 10). The characterization of TICs is based on paradigms developed from embryonic and tissue-specific stem cells, but TICs are distinct from these normal cells so the immunophenotypes may not fully overlap. Several surface TIC markers have shown promise in glioblastoma, including CD133 (Prominin-1; refs. 5, 11, 12), CD15/Lewis X-antigen/stage-specific embryonic antigen-1 (SSEA-1; ref. 13), CD44 (14), L1CAM (15), integrin α6 (16), EGFR receptor (EGFR; refs. 17, 18), platelet-derived growth factor receptor β (PDGFRβ; ref. 19), and the EphA2 receptor (20). Functional assays, including ALDEFLUOR and side population, have been less reliable in glioblastoma but useful in other cancer types (21). Neurosphere formation has been used to enrich TICs but this method prevents the prospective...
separation of tumorigenic and nontumorigenic cells that define a cellular hierarchy. Although these markers have been useful in some studies to prospectively enrich or deplete TICs, many of these have been limited by a shared antigen with normal neural progenitors. Several groups have taken other approaches to identify TIC targets. RNA interference screens have identified key transcription factors, kinases, phosphatases, or ubiquitin-modifying enzymes (22, 23). One group reported a TIC screen in prostate cancer stem cells (24) and another identified CD133-binding aptamers (25). However, we are not aware of a large screen to identify novel TIC-enrichment reagents in glioblastoma. On the basis of this background, we hypothesized that the application of a large, unbiased screening method could develop highly specific reagents to identify functional glioblastoma TICs.

Aptamers are short nucleic acids capable of specific and tight binding to target structures that can include proteins, lipids, other nucleic acids, or any three-dimensional structures. Aptamers are named from the Latin aptus (fitting) and Greek meros (part). Aptamers can be selected by the in vitro iterative process SELEX (Systematic Evolution of Ligands by Exponential enrichment) that enriches RNA or DNA aptamers capable of discriminating between molecular targets with even subtle differences (26–29). Cell-SELEX is a modified procedure that uses cells as an evolutionary selection source and does not require foreknowledge of a molecular target (28, 30). In general, Cell-SELEX is a combination of in vitro evolution and combinatorial chemistry involving a series of steps including incubation, partitioning, and amplification.

In Cell-SELEX, the initial pool of library is chemically created by solid-phase technology. This library must contain at least one, but preferably several, sequences(s) having the unique folded conformations required to facilitate selective binding with the target. Cell-SELEX typically involves positive selection to isolate target cell-interacting sequences and counter selection to eliminate nonspecific sequences recognizing nontarget cells (28). Counter selection results in the depletion of aptamers that bind common cell surface receptors in the resulting pools. Thus, the probability of recognizing unique molecules exclusively expressed in the target population is greatly enhanced. Aptamers bind to their targets with high affinity with typical binding dissociation constants \( K_d \) in the pmol/L to nmol/L range. Furthermore, aptamers are structurally stable under a wide range of temperature and storage conditions while instantly reconstituting their active tertiary structures in some conditions. Aptamers can be synthesized quickly and cost-effectively with minimal interbatch variability, in contrast to monoclonal antibodies. Aptamers can also be easily modified to increase stability and affinity for therapeutic and diagnostic purposes (26). Together, these data suggest that there may be strong benefits of identifying TIC-binding aptamers.

### Materials and Methods

#### Isolation and culture of cells including TICs

We designed a two-stage process for Cell-SELEX with an initial discovery phase to enrich TIC-binding aptamers using functionally validated TICs followed by a validation phase–interrogating aptamer specificity with minimally cultured bulk cancer cells derived directly from in vivo environments. To obtain the large numbers of cells necessary for the initial phase, functionally validated TICs were derived from pooled tumors of the same parental glioblastoma xenograft. Tumors were dissociated using the Worthington Biochemical Papain Dissociation System according to the manufacturer’s instructions. To facilitate recovery from enzymatic digestion, cells were then cultured in Neurobasal Media supplemented with B27 without vitamin A (Invitrogen), basic fibroblast growth factor (bFGF; 10 ng/mL), and EGF (10 ng/mL) for at least 6 hours before magnetic sorting. In selecting an enrichment method for TICs, we considered several cell surface markers that we and others have used. Although CD133 has been controversial in some reports as some glioblastomas may not express CD133 and others have tumorigenic CD133-negative cells, CD133 can identify TIC phenotypes in specific glioblastoma models in our studies (5, 31, 32). After minimal culture, we separated CD133-positive and -negative cells using microbead-conjugated CD133 antibodies (Miltenyi Biotech). To prevent the effects of culture, we conducted Cell-SELEX in parallel with confirmatory TIC assays. CD133-enriched neoplastic cells were functionally validated in assays of self-renewal and tumorigenesis (data not shown) then designated as TICs and used as targets to enrich TIC-specific aptamers and were maintained in the above stem cell media. CD133-negative cells depleted for tumorigenic or self-renewing capacity (data not shown) were used as non-stem glioma cells (non-TICs) and cultured in the presence of 10% FBS. Human glioblastoma xenografts maintained exclusively in vivo (GBM10, GBM12, and GBM14) were kindly provided by Jann Sarkeria (Mayo Clinic, Rochester, MN). These xenografts were maintained as previously described (33). Human neural progenitors were commercially obtained (Lonza).

#### DNA primers and library

The DNA library used for TIC-specific aptamer Cell-SELEX was a pool of DNA sequences consisting of a combination of common and unique nucleotides. Nineteen common nucleotides were present on the 5′-end, 50 randomized base sequences in the middle, and an additional 18 common nucleotides on the 3′-end. The 5′-end was labeled by fluorescein isothiocyanate (FITC; ref. 5′-FITC-sequence-N45-sequence-3′) to monitor enrichment of selection using fluorescence-activated cell sorting (FACS). The forward primer was labeled at the 5′-end with FITC (5′-FITC-sequence-3′) and the reverse primer with biotin at the 5′-end (5′-biotin-sequence-3′). To amplify each eluted pool, PCR was used, and ssDNA was isolated by capturing the biotinylated complementary strand by streptavidin–biotin interactions and denaturing double-stranded DNA with 200 mmol/L NaOH. PCR mixtures were prepared and PCR reactions were carried out according to the manufacturer’s instructions.

#### Cell-SELEX

Minimally cultured TICs isolated from pooled 08–387 xenografts were used as target (positive cell) and matching non-TICs and neural progenitors were used for counter selection.

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(negative cell). Of note, 5 nmol of DNA library was dissolved in 1,000 µL of binding buffer containing 4.5 g/L glucose, 5 mmol/L MgCl₂, 0.1 mg/mL TRNA, and 1 mg/mL bovine serum albumin in Dulbecco’s PBS. The DNA library or enriched pool was denatured at 95°C for 5 minutes, cooled on ice for 10 minutes, and incubated with TICs on ice in an orbital shaker for 1 hour. After TICs were washed three times to remove unbound DNA sequences, the bound DNA sequences were eluted using 500 µL binding buffer at 95°C for 10 minutes with centrifugation. To carry out a counter selection, each aptamer pool was incubated with non-TICs for 1 hour, and then the supernatant was collected to carry out the positive selection. Pool enrichment was monitored using FACS and subjected to cloning into Escherichia coli using TOPO TA Cloning Kit for sequencing (Invitrogen) to identify the aptamer candidates.

Flow cytometry to monitor aptamer binding

Each FITC-labeled aptamer candidate was incubated with TICs or non-TICs in binding buffer on ice for 30 minutes. The cells were washed three times with binding buffer, and the pellets with the bound sequences were resuspended in 200 µL binding buffer. The fluorescence intensity was determined with a LSR II (BD Immunocytometry Systems) by counting 10,000 events. The FITC-labeled unselected ssDNA library was used as a negative control. To determine the specificity of the selected aptamers, aptamer binding to additional glioblastoma cells (08–322, 4121, U87/MG) or neural progenitors (RenCell c-myc immortalized human neural progenitors, Millipore; human fetal neural progenitors, Lonza) was determined. For determination of aptamer binding to differentiated TICs, TICs were plated on Geltrex-coated plates in the presence of 10% human fetal neural progenitors, Lonza) was determined. For determination of aptamer binding to differentiated TICs, TICs were plated on Geltrex-coated plates in the presence of 10% serum and harvested after 5 days for FACS analysis.

Determination of aptamer affinity for TICs

To determine the binding affinity of the aptamers, 1 × 10⁶ target cells were incubated with varying concentrations of FITC-labeled aptamer or library and analyzed by flow cytometry. The mean fluorescence intensity of the unselected library was subtracted from that of the aptamer with the target cells to determine the specific binding of the labeled aptamer. The apparent equilibrium K_d of the aptamer–cell interaction was obtained by fitting the dependence of intensity of specific binding on the concentration of the aptamers to the equation

\[ Y = B_{max} \frac{X}{(K_d + X)} \]

Isolation and in vitro characterization of aptamer high and aptamer low glioblastoma cells

To validate the specificity of the selected aptamers, we interrogated key TIC functional phenotypes in minimally cultured and unselected bulk tumors from either xenografts or patient surgical resected tumors. After overnight recovery of glioblastoma cells from tissue dissociation, each Cy5-labeled aptamer was incubated with each specimen for 30 minutes. After washing three times, labeled glioblastoma cells were subjected to cell sorting. For the sphere formation assay, 10 cells of either aptamer high and aptamer low glioblastoma cells sorted by FACS were directly plated into each well of a 96-well plate containing 200 µL of TIC-culturing medium similar to our prior descriptions. After 10 to 14 days, the number of tumor-spheres in each well was counted and plotted. For the cell proliferation assay, aptamer high and aptamer low glioblastoma cells were sorted into each 15 mL tube, and equal numbers of cells were plated into 96 wells. Differences in cell growth were measured using CellTiter-Glo (Promega) according to the manufacturer’s instructions and our prior reports (16, 19, 31, 34). GraphPad Prism software was used to complete t test comparisons of aptamer high and aptamer low values for individual aptamers.

In vivo tumorigenic potential

To determine tumorigenic potential, 300 or 3,000 viable aptamer high or aptamer low glioblastoma cells were intracranially implanted into 4- to 6-week-old athymic/nude immunocompromised mice obtained from Charles River Laboratories, as we have previously described. Animals were maintained until the development of neurologic signs including lethargy, seizure, ataxia, or paralysis or for a maximum of 60 days, when they were sacrificed. All animal procedures were carried out in accordance with a Cleveland Clinic (Cleveland, OH) Institutional Animal Care and Use Committee–approved protocol. Kaplan–Meier curves and log-rank statistical analysis were completed with GraphPad Prism software.

Results

SELEX screening against TICs enriched for a pool of aptamers

SELEX is a method of repeated rounds of in vitro selection used to identify DNA or RNA aptamers, which bind specific targets, including cells (29, 30). As the identification and targeting of TICs has become a focus of some therapeutic-targeting strategies (35), we hypothesized that Cell-SELEX could selectively distinguish TICs from normal neural progenitors and differentiated tumor cells (non-TICs). We developed a two-stage approach with an initial discovery phase that used minimally cultured functionally validated TICs as a positive selector and functionally validated non-TICs and normal neural progenitors in negative selection. A validation phase described later involved testing the use of aptamers on minimally cultured and unselected bulk tumors.

To identify TIC-specific aptamers, a library of randomized DNA sequences was exposed to glioblastoma cells that fulfilled functional criteria of TICs or non-TICs as a positive selection (Fig. 1A). Aaptamers were first exposed to non-TICs or non-neoplastic human neural progenitor cells (NPC) as a counter selection to eliminate sequences not specific for TICs. DNA sequences that did not bind NPCs or non-TICs were incubated with TICs to select for binding aptamers as a positive selection (Fig. 1A). To select highly specific aptamers to TICs with high affinity, the number of NPCs and non-TICs was increased, whereas the number of TICs was decreased in the subsequent rounds of enrichment (Fig. 1B). In addition, concentration of competitors was increased, whereas the reaction time decreased to further enrich high-affinity TIC-specific aptamers (Fig. 1B). Eight rounds of selection enriched a pool...
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Figure 1. TIC-specific aptamer candidates were identified from Cell-SELEX. A, to develop TIC-specific aptamers, glioblastoma cells were obtained from subcutaneous xenografts initiated from a glioblastoma patient specimen. To obtain enough cells for experiments, cells dissociated from multiple xenografts of GBM1 (08–387) were pooled. TICs and non-TICs prospectively sorted from these xenografts using CD133 were used for positive or counter selection, respectively. To enhance the cancer-specific activity of selected aptamers, human NPCs were also used for counter selection. B, as the number of rounds of enrichment increased, more stringent selection conditions were applied to increase the specificity and affinity of the TIC-specific aptamers identified. These conditions included increasing the number of non-TICs, decreasing the number of TICs, increasing the concentration of competitor, and decreasing the incubation period. C, FACS analysis confirmed that a pool of aptamers present after 8 rounds of selection was enriched with DNA sequences, which bind to TICs but have minimal binding to non-TICs or normal neural progenitors. D, DNA sequencing of the DNA sequences present in the pool of aptamers enriched after selection revealed nine different sequences with more than two copy numbers from 96 clones.
Figure 2. Aptamer candidates bind with high affinity to TICs. FACS analysis was conducted using fluorescent aptamers with TICs, non-TICs, and differentiated TICs isolated from GBM1 (08–387) [A], GBM2 (4121) [B], or GBM3 (08–322) [C] xenografts. Fluorescence shift was calculated using the equation \((F_{\text{aptamer}} - F_{\text{cell}})/(F_{\text{library}} - F_{\text{cell}})\). \(F_{\text{aptamer}}, F_{\text{library}},\) and \(F_{\text{cell}}\) refer to the fluorescence value with the aptamer, library, and cell itself. D, the equilibrium \(K_d\) of the aptamer–cell interaction was obtained by fitting the dependence of intensity of specific binding on the concentration of the aptamers to the equation: \(Y = B_{\text{max}} X/(K_d + X)\).
of DNA sequences preferentially bound to TICs in comparison with non-TICs and NPCs as determined via flow cytometry with fluorescently labeled aptamers (Fig. 1C). Having confirmed the ability of the enriched DNA pool to recognize TICs, we then cloned and sequenced the enriched DNA pool to identify individual TIC-specific aptamer candidates. We obtained nine DNA sequences that were repeatedly detected in multiple colonies of bacteria out of 96 colonies analyzed for sequencing. The most frequent sequence domains repeated 8 to 27 times (Fig. 1D; Supplementary Table S1).

**Individual aptamers bind TICs**

To confirm the ability of individual aptamer candidates to specifically bind TICs, flow cytometry was conducted with FITC-labeled individual aptamer candidates (Fig. 1D; sequences A1–A6) and compared with signal obtained from FITC-labeled library containing randomized DNA sequences as a negative control. A positive shift along the FITC axis was observed with each TIC-enriched aptamer compared with the library when incubated with TICs isolated from GBM1 (Supplementary Fig. S1A). In contrast, negligible shifts in the histogram occurred with non-TICs isolated from the same tumor (Supplementary Fig. S1B) or TICs differentiated with FBS (Supplementary Fig. S1C). Similar preferences for aptamer binding to TICs in comparison with matched non-TICs was observed for cells derived from two additional glioblastoma xenografts for the majority of the aptamers tested (Supplementary Fig. S2A–S2D). TIC-enriched aptamers also failed to bind to the well-characterized U87MG glioblastoma cell line (Supplementary Fig. S2E), which was passaged under differentiating conditions in serum. To further characterize the ability of the aptamers to bind non-neoplastic cells in the brain, flow cytometry was also conducted with ReNCell CX immortalized human neural progenitors (Supplementary Fig. S1D) or primary human fetal neural progenitors (Supplementary Fig. S1E). The majority of the TIC-enriched aptamers showed minimal binding to non-neoplastic neural progenitors, although A4 bound both sets of non-neoplastic brain cells, indicating some of the aptamers could more broadly recognize neural progenitors (Supplementary Fig. S2D and S2E).

To further evaluate the specificity of TIC-enriched aptamers, the fold change in fluorescence was calculated. Fluorescence shift was calculated using the equation \( \frac{F_{\text{aptamer}} - F_{\text{cell}}}{F_{\text{library}} - F_{\text{cell}}} \), where \( F_{\text{aptamer}} \) and \( F_{\text{library}} \) refer to the fluorescence value of the aptamer, library, and cell itself. Using this calculation, specificity of aptamers A2–A6 for TICs in comparison with non-TICs was shown for all three glioblastomas tested (Fig. 2A–C). A1 also showed greater binding to TICs in GBM1 and GBM2, but not GBM3 (Fig. 2A–C). The affinity of A1–A6 binding to TICs was also very high with \( K_d \) in the nmol/L range (Fig. 2D). Together, these data strongly suggest that we have identified several aptamers that specifically bind to TICs with high affinity. From antibody and minibody studies, it is apparent that optimal targeting of tumor cells with cell killing payloads (toxin or radioisotope) is achieved when specificity of binding to the target cells is also...
associated with internalization. Using matched TICs and non-TICs, we found that aptamers bind with specificity to the cell surface and also internalized specifically in TICs (Fig. 3A and Supplementary Fig. S3), suggesting their potential to target TICs.

**TIC-specific aptamers enrich glioblastoma cells with increased growth and tumorsphere formation capacity**

Having identified aptamers that bound functional TICs preferentially, we conducted validation studies to determine the ability of the aptamers to select TICs from unselected bulk cells. FACS analysis was used to segregate aptamer\(^\text{High}\) and aptamer\(^\text{Low}\) cells from GBM1 (08-387) [A], GBM2 (4121) [B], and GBM4 (3691) [C] xenografts. Cells were plated at equivalent density and growth measured over time using the CellTiter-Glo assay. *P < 0.05. Error bars represent SD.

Figure 4. Aptamer\(^\text{High}\) cells are enriched for cell growth in vitro. FACS analysis was used to segregate aptamer\(^\text{High}\) and aptamer\(^\text{Low}\) cells from GBM1 (08-387) [A], GBM2 (4121) [B], and GBM4 (3691) [C] xenografts. Cells were plated at equivalent density and growth measured over time using the CellTiter-Glo assay. *P < 0.05. Error bars represent SD.
xenografts and patient specimens. We found that cells that bound the aptamer with high affinity (aptamer\textsuperscript{high}) expressed high levels of stem cell markers such as Sox2, whereas cells with low aptamer (aptamer\textsuperscript{low}) binding expressed differentiation markers including glial fibrillary acidic protein (GFAP; Fig. 3B).

As TICs are functionally defined, we examined the ability of the TIC-enriched aptamers to identify cells that could proliferate and self-renew. In cell titer assays, each of the five aptamer\textsuperscript{high} fractions (A1–A5) showed significantly enhanced cell growth over time in the three glioblastomas tested (Fig. 4).

As neurosphere formation assay has been used as a surrogate measure of self-renewal, we used this assay to determine if TIC-binding aptamers could enrich glioblastoma cells with self-renewing capacity. We found that A2, A3, and A5 significantly segregated cells with neurosphere formation capacity in all glioblastomas tested (Fig. 5). A1- and A4-enriched cells also showed greater neurosphere formation capacity in the majority of glioblastomas tested (Fig. 5). We confirmed these studies in three xenografts propagated without cell culture. When we dissociated these xenografts and segregated cells based on aptamer binding without culture, aptamer\textsuperscript{high} cells were enriched for neurosphere formation capacity (Fig. 5E–G).

These data show that TIC-enriched aptamers can be used to segregate fractions of glioblastoma cells with TIC properties as measured in vitro.

Aptamer\textsuperscript{high} cells are enriched for tumorigenic potential

As TICs by definition must be able to propagate the parental tumor in vivo, we determined whether the TIC-specific aptamers could segregate for differences in tumorigenic potential. Using Cy5-labeled A3 (Fig. 6A and B) and A4 (Fig. 6A and B) as probes, aptamer\textsuperscript{high} and aptamer\textsuperscript{low} fractions were sorted from bulk cells isolated from GBM1 xenografts. Three thousand (Fig. 6A and C) or 300 (Fig. 6B and D) live aptamer\textsuperscript{high} and aptamer\textsuperscript{low} cells were implanted into the brains of immunocompromised mice, which were then monitored daily for
neurologic signs. We sacrificed a small cohort of the group as well as animals implanted with cells from an additional xenograft (GBM43) upon the initial development of neurologic signs and compared tumor size. The aptamer\textsuperscript{high} cells displayed substantially greater tumor volumes than the aptamer\textsuperscript{low} cells (Fig. 6E). We further noted that aptamer\textsuperscript{high} cells displayed greater propensity to invade into normal brain (Supplementary Fig. S4). These differences translated into survival differences as the survival of mice implanted with either A3 or A4 aptamer\textsuperscript{high} cells was significantly reduced with decreased mean survival (Fig. 6F). All mice implanted with the lower number of aptamer\textsuperscript{high} cells (300) developed tumors, whereas 3 of 4 tumors developed with A3 aptamer\textsuperscript{low} cells and only 2 of 4 tumors developed with A4 aptamer\textsuperscript{low} cells (Fig. 6F). These data suggest that TIC-enriched aptamers can segregate for glioblastoma cells with tumorigenic potential.

**Principal component analysis shows aptamer\textsuperscript{high} and aptamer\textsuperscript{low} cells are distinct**

Having shown that the TIC-enriched aptamers could segregate for functional measures of TIC biology, we sought to determine if there were similarities in the molecular profile of...
Commonly differentially expressed genes for aptamer A3 high vs. low cells

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<td>–7.18</td>
<td>–5.46</td>
</tr>
</tbody>
</table>

Figure 7. Microarray analysis reveals clustering of aptamerA3high and aptamerA3low cells. A, PCA revealed clustering of aptamerA3high and aptamerA3low cells. FACS analysis was used to segregate aptamerA3high and aptamerA3low cells from GBM1 (08–387), GBM2 (4121), and GBM4 (3691) xenografts using fluorescently labeled aptamers A1, A2, A3, A4, or A5. The data for the aptamerA3high fractions from all xenografts were pooled and compared with data for the corresponding group of
aptamer<sup>high</sup> or aptamer<sup>low</sup> cells as determined via gene expression and principal component analysis (PCA). PCA is a multivariate analysis resulting in a three-dimensional visual representation of the data useful for identifying similarities within large datasets. We used FACs analysis with fluorescently labeled aptamers (A1–A5) in GBM1, GBM2, and GBM4 xenografts to segregate aptamer<sup>high</sup> and aptamer<sup>low</sup> cells. Microarray data from the aptamer<sup>high</sup> and aptamer<sup>low</sup> populations for each of the individual aptamers in each of the cell types (total of 15 datasets with matched aptamer<sup>high</sup> and aptamer<sup>low</sup> cells) was then processed via PCA. The resulting analysis showed two distinct groups (shown as ellipsoids) corresponding aptamer<sup>high</sup> and aptamer<sup>low</sup> datasets (Fig. 7A). The PCA analysis therefore indicates the data from the aptamer<sup>high</sup> and aptamer<sup>low</sup> cells is distinct showing different molecular profiles between the groups.

**Aptamer<sup>high</sup> cells are enriched for molecular signatures associated with the regulation of cellular development**

To identify specific molecular differences between aptamer<sup>high</sup> and aptamer<sup>low</sup> cells, microarray data from GBM1, GBM2, and GBM4 cells segregated with A3 (Fig. 7B and C) or A4 (Supplementary Fig. S5) fluorescently labeled aptamers was further analyzed. Comparison of genes differentially elevated or repressed in A3<sup>high</sup> cells in comparison with A3<sup>low</sup> cells, revealed a set of 27 molecules that were commonly altered across all three lines (Fig. 7B). Only one gene transcript, chemokine (C–C motif) ligand 2 (CCL2) was consistently elevated, whereas the expression of the remaining 26 targets were repressed in A3<sup>high</sup> cells (Fig. 7B). A similar analysis of A4<sup>high</sup> cells in comparison with A4<sup>low</sup> cells, identified a set of 25 targets whose expression levels were commonly altered across all three glioblastoma cultures tested (Supplementary Fig. S5A). DEAD box polypeptide 60 (DDX60) was commonly elevated, whereas expression in the remaining targets was repressed in A4<sup>high</sup> cells (Supplementary Fig. S5A). When the A3 or A4 gene expression profiles were analyzed using Ingenuity software, the top biologic networks represented by these profiles were both found to regulate cellular development (Fig. 7C, Supplementary Fig. S5B). In fact, several genes were commonly downregulated in the A3<sup>high</sup>, and A4<sup>high</sup>-enriched cells (Fig. 7B and Supplementary Fig. S5A), suggesting that independent TIC-specific aptamers can select a subfraction of glioblastoma cells with common phenotypes and some similarities in their expression profiles.

**Discussion**

Glioblastomas rank among the most lethal of human cancers with median survival of only 12 to 15 months despite surgical resection followed by radio- and chemotherapy with the DNA-alkylating agent temozolomide. The highly infiltrative and invasive feature of glioblastomas hinders complete resection of most tumors, and recurrence is nearly universal (8). TICs are thought to be the portion of glioblastoma cells that are responsible for both cellular invasion and therapeutic resistance (9–12), indicating the need for therapies that kill these cells and for imaging reagents that can identify them in vivo. To identify TIC-specific aptamers, we used a two-stage approach with initial selection using functionally validated TICs followed by independent validation in bulk tumor models with minimal culture to avoid drift. We believe that the TIC-specific aptamers identified in this study may help to fill these needs by providing methods to better target TICs for detection and treatment.

One method through which the TIC-enriched aptamers identified here may be developed for clinical use is through conjugation to increase the affinity and specificity of therapeutic nanoparticles for subgroups of cancer cells that drive tumor maintenance. Nanoparticle technology has already been approved by the U.S. Food and Drug Administration to treat patients with breast cancer in the form of Nab-paclitaxel, and paclitaxel-loaded nanoparticles showed efficacy against glioblastoma cell lines (36). Paclitaxel-loaded nanoparticles cross the blood–brain barrier (37, 38) and reduce glioblastoma growth in animal models (36, 38), suggesting the potential of enhancing the efficacy of these reagents through conjugation to TIC-specific aptamers. Aptamer–drug conjugates for delivery of doxorubicin (39) have recently been shown to have efficacy with liposomal-mediated delivery in glioblastoma animal models (40). Postoperative/chemoradiotherapy with doxorubicin liposomes was well tolerated in phase II clinical trials with liposomal delivery, but did not have significant improvement in outcome over historical averages (41). Directed delivery to TICs through conjugating to TIC-specific aptamers may generate more promising results. These data suggest that TIC-targeting aptamers conjugated to nanoparticles may provide additional benefit for glioblastoma therapies.

In addition to therapeutic use, labeled TIC-specific aptamers may be useful as imaging reagents. In glioblastoma, tumor recurrence most often occurs within 2 cm of the primary resection site, indicating the difficulty of complete resection of the tumor cells due to their infiltrating behavior. The ability to visualize invasive cells during surgery could therefore provide significant benefit. Fluorescent dyes encapsulated in nanoparticles have shown some success for crossing the blood–brain barrier to serve as imaging reagents in glioma, and quantum dot-labeled nanoparticles with aptamers targeting tenascin-C on human glioma cells have shown the ability to image glioma cells in vitro (42). Recently Hwang do and...
colleagues showed the ability to use MRI, fluorescent imaging, and radionucleotide imaging together to visualize tumors after treatment of mice bearing C6 glioma xenografts with radiolabeled nanoparticles containing rhodamine and a cancer-targeting aptamer (against nucleolin; ref. 43). Although intracranial tumors were not imaged, the data suggest the use of aptamer-conjugated nanoparticles as promising for imaging reagents.

In addition to the ability to either identify or target TICs, aptamers could have many potential applications for brain tumor biology, which have not yet been fully explored. For example, aptamers could be identified that specifically recognize proneural, mesenchymal, or classical glioblastoma subtypes, allowing for rapid profiling of patient biopsies. Aptamers that recognize glioblastoma cells in specific regions of the tumor associated with therapeutic resistance, such as the hypoxic niche, could also be identified and be useful for directing therapies to these difficult to target areas. Even aptamers showing specificity for non-neoplastic neural stem cells could be useful as these cells are being considered for delivery of antiangiogenic therapies.

TIC-specific aptamers also serve as a powerful tool to delineate critical molecular mechanisms in glioblastoma. We have used microarray analysis to determine a set of genes that are differentially regulated in aptamer-high cells. These studies identified common targets that were involved in pathways associated with the regulation of cellular development and have recognized roles in cancer. For example, CCL2 that was elevated in the aptamer-high cells is implicated in an interleukin-6–dependent paracrine loop to promote cellular invasion in vivo (44) and has been linked to the promotion of a cancer stem cell phenotype through IFN-regulatory factor 7 (45). Neutralizing antibodies against CCL2 have also shown efficacy in xenograft models as an antiangioma therapy (46). Hexamethylene bisacetamide-inducible protein 1 (HEXIM1) promotes the stability of p53 (47) and inhibits metastasis (48), suggesting that the reduced expression levels present in aptamer-high cells would promote tumorigenesis. Growth arrest and DNA damage-inducible 45b (GADD45b) is a cellular stress sensor, which is suggested to be a tumor suppressor (49), confirming it would be beneficial for cancer cells to have decreased GADD45b expression as shown in our study. Furthermore, GADD45b has been shown to be overexpressed in non-side population cells depleted for cancer stem cell characteristics in embryonic carcinoma cells (50) consistent with the notion it is enriched in TICs. Thus, our molecular analysis suggests TICs identified with specific aptamers have gene expression profiles, which could promote tumorigenesis.

These studies represent an initial development of tools that may inform analytic techniques for molecular pathways involved in the tumor hierarchy and growth as well as therapeutic targeting. We are currently investigating the expression levels and possible functional contributions of the genetic targets identified in the gene expression studies through comparison of TICs and non-TICs in different glioblastoma subgroups with targeting of expression in these models. It is notable that five different aptamers with completely different sequences identified tumor cell populations with very tightly clustered gene expression programs, suggesting the identification of a convergent tumor population (Fig. 7A). This is an important biologic finding as the widely diverse set of putative TIC markers in brain cancer, such as other cancers, has not clearly defined a common population. In addition, preliminary studies suggest that our TICs can be bound to nanoparticles to act as delivery vehicles into the brain (J. Heddleston, Q. Wu, A. Hjelmeland and J. Rich; unpublished data). Collectively, these results suggest that aptamer technology reinforces the presence of a phenotypically similar stem-like tumor population that may be selectively identified and—in future studies—targeted. Aptamer-based therapies may be designed for combinations with either conventional therapeutics or targeted therapies to disrupt tumor subpopulations that contribute to therapeutic resistance, invasion, and angiogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: Y. Kim, A.B. Hjelmeland, J.N. Rich
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Kim, Q. Wu, P. Hamerlik, A.E. Sloan, G.H. Barnett, R.J. Weil, J.N. Rich
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Kim, P. Hamerlik, M. Hitomi, P. Leahy, A.B. Hjelmeland, J.N. Rich
Writing, review, and/or revision of the manuscript: Y. Kim, P. Hamerlik, M. Hitomi, A.E. Sloan, G.H. Barnett, R.J. Weil, A.B. Hjelmeland, J.N. Rich
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Kim, A.B. Hjelmeland, J.N. Rich
Study supervision: A.B. Hjelmeland, J.N. Rich

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References


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Youngmi Kim, Qiulian Wu, Petra Hamerlik, et al.


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