In Vitro Selection of DNA Aptamers to Glioblastoma Multiforme

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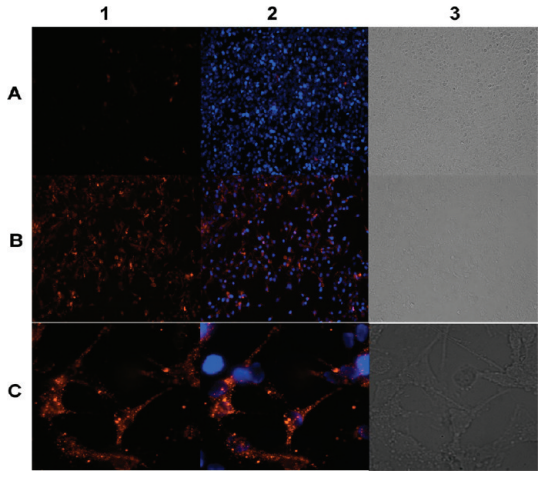
Abstract



Aptamer probes for specific recognition of glioblastoma multiforme were generated using a repetitive and broad cell-SELEX-based procedure without negative selection. The 454 sequencing technology was used to monitor SELEX, and bioinformatics tools were used to identify aptamers from high throughput data. A group of aptamers were generated that can bind to target cells specifically with dissociation constants (K_d) in the nanomolar range. Selected aptamers showed high affinity to different types of glioblastoma cell lines while showing little or no affinity to other cancer cell lines. The aptamers generated in this study have potential use in different applications, such as probes for diagnosis and devices for targeted drug delivery, as well as tools for molecular marker discovery for glioblastomas.

Keywords: DNA aptamer, Cell-SELEX, glioblastoma, cancer

Understanding the molecular characteristics of a disease is fundamental to diagnosis, therapy, and elucidating the nature and mechanism of the disease. Due to the increasing public awareness and statistical data, cancer is an important target for molecular characterization. Although cancer deaths have been predicted to increase from 7.1 million in 2004 to 11.5 million in 2030, the availability of molecular probes for different cancer types is still inadequate (*1*). Successful cancer therapy is based on early diagnosis and accurate staging, both of which require suitable molecular probes and biomarkers (*2*). The gene mutations and alterations associated with cancer often cause changes in proteomic levels, and some of those changes occur on the cell surface. Since cell surface proteins are the first accessible parts of cells, they can be detected relatively easily and can also be used as ports for therapeutics. Thus, cell surface biomarkers are appropriate for early and easy detection, differentiation, and determination of the stage of the cancer. Also they can be used in combination with imaging techniques in vivo both to detect cancer and to follow the response to therapy (*3*).

Glioblastoma multiforme is the most malignant brain tumor in humans. Although it comprises just 1.35% of all primary malignant cancers in the United States (*4*), the median survival time after diagnosis is only 12 months, making glioblastoma multiforme one of the most aggressive types of cancer (*5*). Due to the rapid progress of the disease, chemotherapy, radiotherapy, and surgery are at best palliative measures. Also prognosis of the glioblastoma tumors remains poor because of the high reoccurrence rates after surgical resection (*6*). Glioblastoma multiforme was selected as one of the three initial cancer types to be studied in The Cancer Genome Atlas (TCGA) program, and the genome sequence of the U87MG cell line was recently published (*7*, *8*).

The gene for phosphatase and tension homologue (PTEN) located on chromosome 10 (10q23) is commonly mutated or deleted in glioblastoma. Recent studies indicate that 80% of the human glioblastoma has a deletion or mutation in PTEN (*9*), and a homozygous

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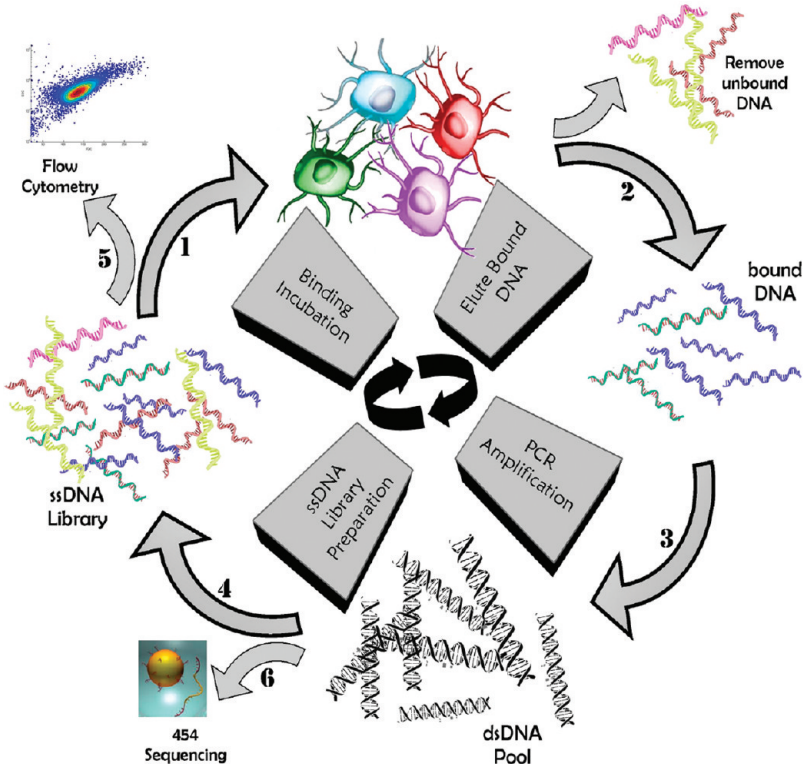

Figure 1. Schematic representation of cell-SELEX. Briefly, the ssDNA pool is incubated with A-172 (target cells) (1). After washing, the bound DNAs are eluted by heating to 95 °C (2). The eluted DNAs are amplified by PCR (3). The double stranded PCR products were separated into ssDNAs for the next round of selection (4) or monitored by flow cytometry for progress (5). When the selected pool was enriched sufficiently, it was sequenced by 454 analysis (6).

mutation in PTEN has been robustly identified in the genomic sequence of U87MG (*8*). Absence of PTEN in glioblastoma promotes angiogenesis and prevents apoptosis, even after treatment with powerful chemotherapeutic drugs (*9*). Due to its high occurrence rate, high vascular endothelial growth factor (VEGF) expression, and poor response to chemotherapeutic drugs, the PTEN-negative glioblastoma cell line A172 was chosen as a model target for aptamer selection in this study.

Aptamers are single-stranded oligonucleotides, which are capable of binding different classes of targets with high affinity and specificity (*10*−*12*), as a result of their unique three-dimensional shapes (*13*). The binding capacity results from the aptamer's three-dimensional conformation and does not involve nucleotide base−base complementarity (*14*). The three-dimensional shape of an aptamer is due to the base composition and intramolecular hybridization that causes folding to a particular molecular shape. This molecular shape causes binding through shape-specific recognition with remarkable structural stability and high affinity.

Aptamers are generated by an in vitro selection and amplification technology called SELEX (Systematic Evolution of Ligands by EXponential enrichment). SELEX is in essence a combinatorial technique, in which an oligonucletide library is searched to determine which components bind to the target molecules of the researcher's choice. Typically, the library used for SELEX consists of 10^{13}−10^{15} random oligonucleotides containing a 30−50 random base region flanked on both sites with predetermined primers for PCR. As shown in Figure 1, SELEX involves cycles of binding to the target, removal of the unbound DNA, and amplification of bound sequences by the polymerase chain reaction (PCR). This cycle is repeated 10−20 times to increase the yield of sequences which bind to the target.

Cell-SELEX is an extension of SELEX using whole cells as a complex target for selection of aptamers. Cell-SELEX has the advantage of needing no prior knowledge of the target site on the cell surface. There are many aptamers developed for cancer variety of diseased cells in the literature such as leukemia (*15*), liver (*16*), lung (*17*), ovarian (*18*), and colon (*20*) cells as well as infectious diseases (*19*) . Thus, a high affinity probe can be obtained for an unknown target, which can later be identified. In addition to their usage as affinity probes in diagnosis, aptamers obtained by cell-SELEX can also be adapted for targeted drug delivery.

In this work, cell-SELEX was used to generate aptamers for glioblastoma multiforme with the aid of

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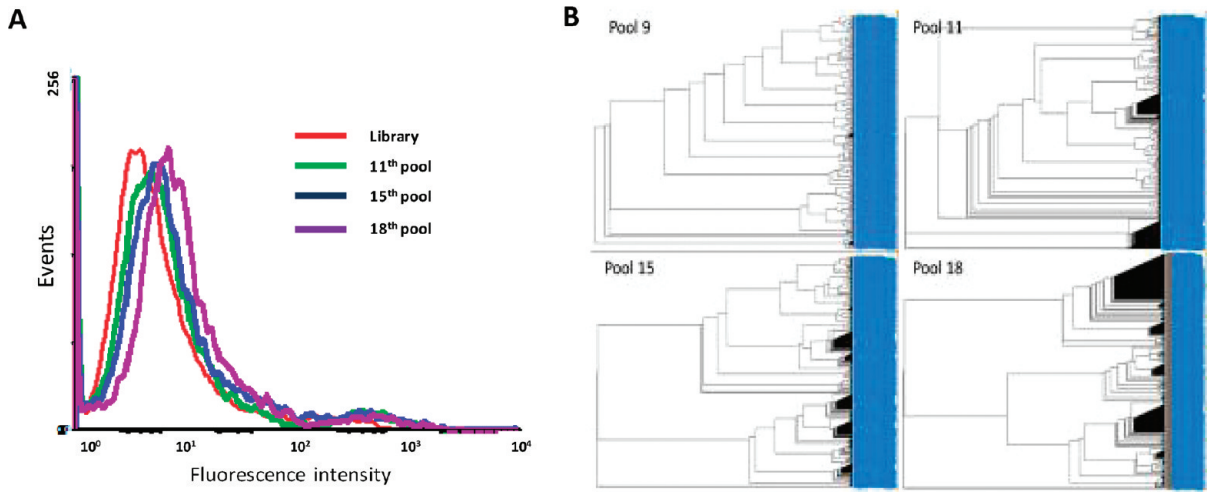

Figure 2. (A) Binding assay of selected pools with A-172 cells. The fluorescence intensity of A-172 cells bound to selected pools increased gradually as the selection progressed. (B) Analysis of individual sequence similarity. Dendogram for visual classification of similarity among sequences of different rounds of selection.

454-pyrosequencing. Incorporation of 454-pyrosequencing in the SELEX procedure increases sample throughput, because screening of sequences from different rounds of SELEX can be done simultaneously. Thus, aptamers can be obtained in earlier rounds with less effort and without a cloning step.

Results and Discussion

Enrichment of Aptamer Candidates

Cell-based SELEX (Figure 1) was successfully used for the selection of DNA aptamers for glioblastoma multiforme using the A172 cell line as the target. Only target cells were used without negative selection to be able to select the aptamers broadly recognizing the cell membrane proteins as implied in our previous study (*21*). Although more specific aptamers can be selected for desired cell lines using negative selection, it is inevitable to miss common cancer biomarkers by this method. SELEX without negative selection has a potential problem of selecting aptamers for common surface proteins, but it can be bypassed by screening more candidate aptamers on different cell lines. Also a smooth and uninterrupted SELEX can gradually and broadly enrich aptamers as it can be seen in following results. As shown in Figure 2A, the fluorescence intensities indicated considerable and consistent enhancement after the ninth round of selection, implying aptamer enrichment. In this study, 454 pyrosequencing (9th, 11th, 15th, and 18th pools) was also used to observe the enrichment process during the selection. Sequencing retrieved 13 094 sequences. After grouping the data for each pool according to their MID sequences, pools were aligned and phylogenetic trees were constructed (Figure 2B). Although pool 9 showed diverse speciation with small numbers and types of families with similar or like sequences, both the numbers and types of enhanced families increased during the selection process.

Characterization of Aptamers

A representative set of nine abundant sequences was chosen for testing their binding abilities to target cells. As shown in Figure 3, five of the sequences showed obvious binding to target cells at both 4 and 37 °C. Sequences GMT 3, GMT 4, GMT 5, GMT 8, and GMT 9 (Table 1) were used for further characterization experiments. As shown in Table 1, these aptamers showed high binding affinities with equilibrium dissociation constants (K_d) in the nanomolar range. Fluorescence microscopy also showed that the aptamers strongly bound to glioblastoma cells (Figure 4).

Determination of Binding Specificity

Aptamers were also tested with other cell lines to determine the specificity for molecular recognition of glioblastoma multiforme (Table 2). Except for GMT 4 and GMT 8, aptamers showed high specificity for glioblastoma. Aptamers GMT 4 and GMT 8 also showed high binding affinity for CCRF-CEM, which is a T lymphoblast cell line. This binding pattern indicates that glioblastoma and acute lymphoblastic leukemia have the same molecular recognition site for these two aptamers. On the other hand, all aptamers showed high affinity for different glioblastoma cell lines (U87MG and GMBJ1). Although SELEX was carried out on the A-172 cell line, GMT 3, GMT 4, and GMT 5 showed higher binding affinities for GMBJ1. This shows that the recognition sites of these aptamers are expressed more by the GMBJ1 cell line. Overall, the data show that cell-SELEX without a negative selection with multiple rounds is a viable strategy for selection of

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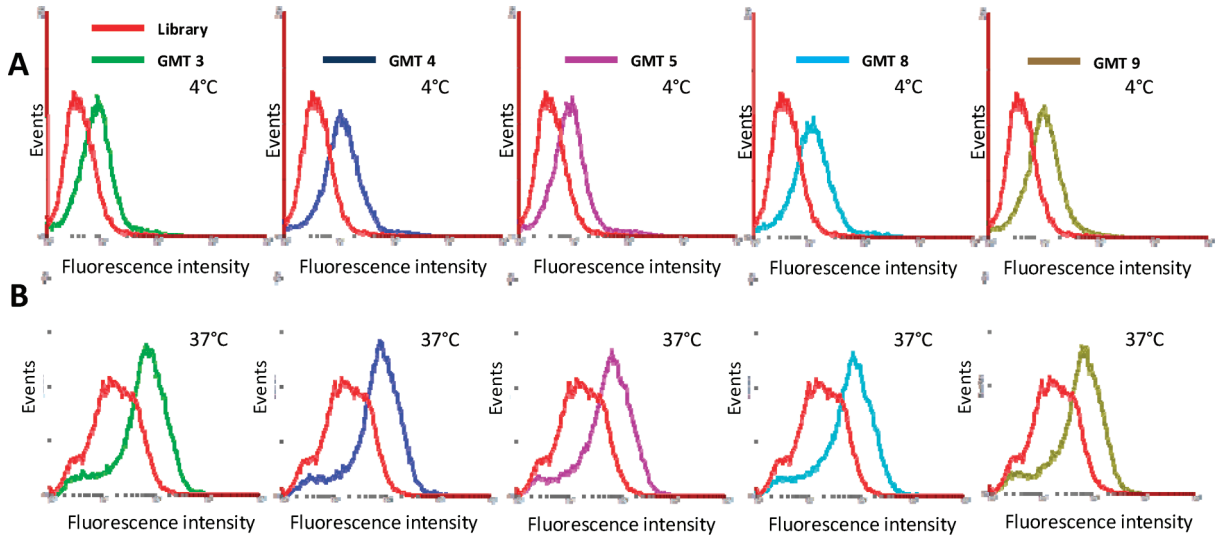

Figure 3. Identification of aptamer candidates. Flow cytometry assay for the binding capacity of the candidate aptamers at 4 °C (A) and 37 °C (B).

Table 1. Sequences and Binding Affinities of Selected Aptamers to A-172 Cells

aptamer	sequence	K_d
GMT 3	TGACGAGCCCAAGTTACCTAAGGGTATACCCCGGGAGGGTGC CAGGTGACCGCACAAGAATCTCCGCTGCCTACA	75.27 ± 7.54
GMT 4	TGACGAGCCCAAGTTACCTTGGTGATGGTTTTTGGTGGTAAC GGGGGCGGGTGAGTAGAATCTCCGCTGCCTACA	61.82 ± 6.37
GMT 5	TGACGAGCCCAAGTTACCTACGGCAGCAGGTGTGTTCGGGTT GGATTGGTGTGGTTAGAATCTCCGCTGCCTACA	168.56 ± 21.57
GMT 8	TGACGAGCCCAAGTTACCTCGATCTTGTGTGTTTAATTGTTTAT TGCTGTACCGTGAGAATCTCCGCTGCCTACA	99.31 ± 17.42
GMT 9	TGACGAGCCCAAGTTACCTCATAACAAAAGTGCCAACACGGACC ACGTCCATTTTGAGAATCTCCGCTGCCTACA	97.26 ± 11.86

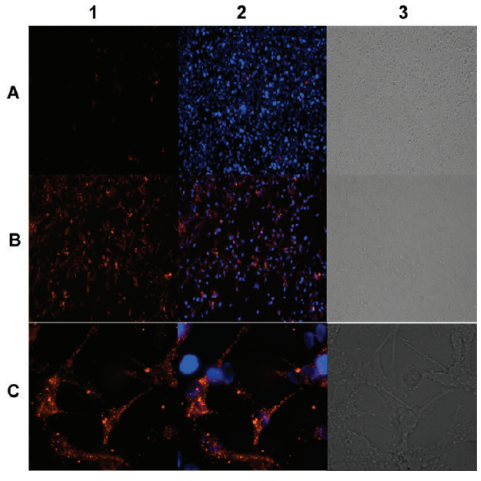

Figure 4. Microscopy images of library and GMT 8-bound A-172 cells. (A) Library 10X, (B) GMT 8 10X, (C) GMT 8 (63X), (1) streptavidin-PE stained florescence image, (2) merge of DAPI and florescence image, and (3) optical image.

aptamers to detect diseases without considering cell lines.

Determination of Target Type

In order to determine if the target of the aptamers is a membrane protein on the cell surface, the cells were treated with two proteinases, trypsin and proteinase K. As shown in Figure 5, after treatment with proteinase K, all aptamers lost their binding abilities. Although proteinase K treatment caused an obvious decrease in binding affinity, trypsin treatment was not as effective. In particular, GMT 3 and GMT 5 showed a very small decrease in binding affinity when treated with trypsin, indicating that the binding entities of these aptamers are resistant to trypsin cleavage. The drastic decrease in binding efficiency after treatment with proteinase K shows that the aptamer targets are most probably proteins or they are closely accompanied by proteins.

In this report, multiple rounds of cell-SELEX without negative selection were applied to obtain molecular probes for glioblastoma multiforme. A new sequencing technology was used to monitor SELEX to select aptamers from the pool sequences. Bioinformatics is used extensively to be able to handle the large amount of data

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Table 2. Binding of Aptamers to Cell Lines from Different Origins[a]

	origin									
	glioblastoma		breast		leukemia		lung	colon	epithelial	ovary
aptamer	A-172	U87MG	GMBJ1	MCF-7	CEM	RAMOS	H23	HT29	HBE-135	CAOV-3
GMT 3	+	+	++	−	−	−	−	−	−	−
GMT 4	++	+	+++	−	+++	−	−	−	−	−
GMT 5	+	+	+++	−	−	−	−	−	−	−
GMT 8	+++	++	+++	−	+++	−	−	−	−	−
GMT 9	++	−	++	−	−	−	−	−	−	−

[a] The fluorescence intensity of the unselected library is used as background binding, and binding efficiencies are expressed as relative to the background binding: high binding (more than 4-fold) is represented as (+++), middle binding (3−4-fold) is represented as (++), low binding (2−3-fold) is represented as (+), and no binding (less than 2-fold) is represented as (−).

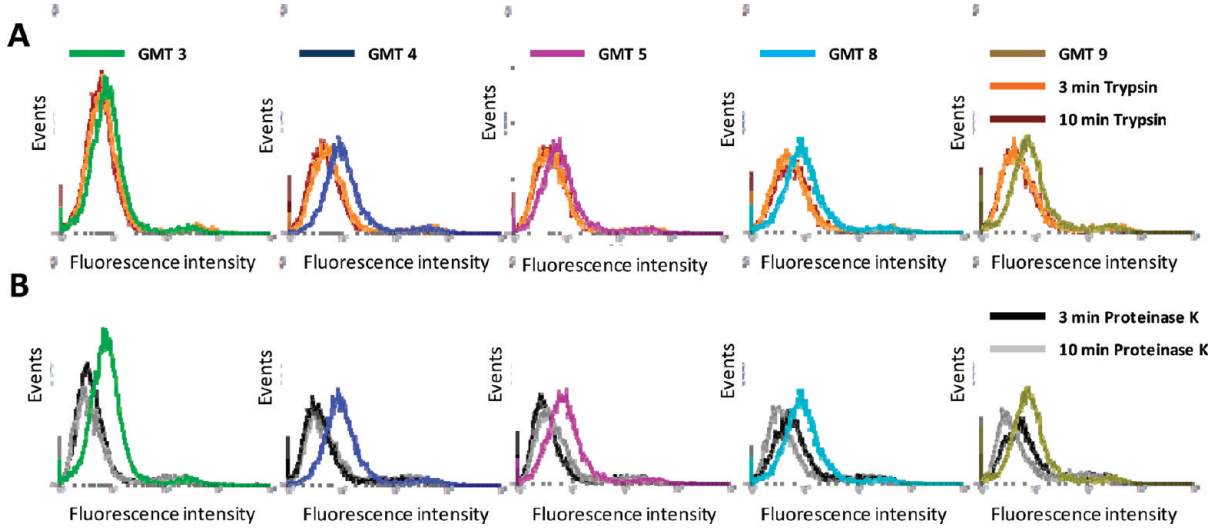

Figure 5. Binding of aptamers to (A) trypsin- and (B) proteinase K-treated A-172 cells for 3 and 10 min.

in the study. By using new sequencing technology and bioinformatics, more representative sequences were easily selected from the pools. Also evolution of sequences throughout the SELEX can easily be observed by bioinformatics tools. Selected aptamers showed high affinity to different types of glioblastoma cell lines and showed little or no affinity to other cancer cell lines. Most of the selected aptamers have K_d values in the nanomolar range and are very specific. It is clear that usage of new sequencing technologies with the help of bioinformatics can provide early and detailed information for progress of SELEX by displaying the intensity of selected sequences in the earlier rounds.

Methods

Cell Lines and Cell Culture

Cell lines A-172 (ATCC.CRL-1620, brain, glioblastoma) and CaOV-3 (ATCC.HTB-75, ovary, adenocarcinoma) were grown in Dulbecco's modified Eagle medium (DMEM, ATCC.30-2002). The MCF-7 (ATCC.HTB-22, mammary gland, adenocarcinoma) and U87MG (ATCC.HTB-14, brain, glioblastoma) cells were grown in Eagle's minimum essential medium (EMEM, ATCC.30-2003). Cell lines CCRF-CEM (ATCC.CCL-119, T lymphoblast, acute lymphoblastic leukemia), Ramos (ATCC.CRL-1596, B lymphocyte, Burkitt's lymphoma), and H23 (ATCC.CRL-5800, lung, non-small cell lung cancer) were grown in Roswell Park Memorial Institute 1640 medium (RPMI-1640, ATCC.30.1001). The HT-29 (ATCC.HTB-38, colon, colorectal adenocarcinoma) cells were grown in McCoy's 5A medium (ATCC.30-2007), and HBE135-E6E7 (ATCC.CRL-2741, lung epithelial, HPV-16 E6/E7 transformed) cells were grown in keratinocyte free medium (Keratinocyte-SFM, GIBCO.-17005-042). All cell lines were supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO.26140-079) and 100 units/mL penicillin−streptomycin (Cellgro.30-002CI).

DNA Primers and Library

The DNA library used for aptamer selection consisted of a central, continuous stretch of 42 randomized sequences flanked by PCR primer sequences (5′-TGACGAGCCCAAGTTACCT-42N-AGAATCTCCGCTGCCTACA-3′). FITC labeled 5′ primer (5′-FITC-TGACGAGCCCAAGTTACCT-3′)

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and biotinylated 3′ primer (5′-biotin-TGTAGGCAGCGGA-GATTCT-3′) were used in the PCR. All sequences were synthesized by standard phosphoramidite chemistry using an ABI 3400 DNA synthesizer (Applied Biosystem Inc., Foster City, CA) and purified by reversed-phase ion pairing HPLC (Varian Analytical Instruments, Walnut Creek, CA)

SELEX Protocol

In this study, human glioblastoma cell line A-172 was used as the target cell line. The initial pool containing 7 nmol of DNA library was dissolved in 500 μL of binding buffer (4.5 g/L glucose, 5 mM $MgCl_2$, 0.1 mg/mL yeast tRNA, and 1 mg/mL BSA in Dulbecco's PBS with $CaCl_2$ and $MgCl_2$ (Sigma. D1283)). The library was denaturated by heating at 95 °C for 5 min and cooled on ice for 10 min before incubation. Then the single-stranded DNA (ssDNA) pool was incubated with A-172 cells on a 5.0 cm diameter cell culture dish (Corning) for 60 min at 4 °C. After incubation, cells were washed twice with 3 mL of washing buffer (4.5 g/L glucose, 5 mM $MgCl_2$ in Dulbecco's PBS with $CaCl_2$ and $MgCl_2$) for 30 s at 120 rpm. Cells were harvested using a cell scraper (Corning) and transferred into 500 μL of water. The bound DNA sequences were eluted by heating at 95 °C for 15 min. The eluted sequences were amplified by PCR with FITC and biotin labeled primers (denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s). For each round of SELEX, the number of PCR cycles was optimized between 10 and 20. The PCR products were separated on 3% agarose gel, and the highest numbered cycle without any nonspecific band was chosen as the cycle number for amplification of the selected sequences. After amplification, selected ssDNA was separated from the biotinylated antisense ssDNA by alkaline denaturation using 200 mM NaOH and affinity purification with streptavidin-coated sepharose beads (GE Healthcare Bio-Sciences Corp.). The separated ssDNA solution was passed through a desalting Nap-5 column (GE Healthcare Bio-Sciences Corp.) to remove the NaOH. The entire selection process was repeated according to the course of enrichment. To acquire more specific and high affinity aptamers, the incubation time was decreased from 60 to 30 min as the number of selection rounds increased, and the washing time was also increased gradually from 30 to 60 s. Additionally, 10% FBS was added to the incubation mixture after the fourth round of selection. Further details of Cell-SELEX can be found in our previous study (*22*, *23*).

Sequencing

After 18 rounds of selection, 4 selected ssDNA pools were amplified using 19-mer 454 fusion and multiplex identifiers (MID) incorporated into the unmodified primers (forward primer 1 with MID1 and 454 fusion, 5′-GCC TCC CTC GCG CCA TCA G−AC GAG TGC GT−T GAC GAG CCC AAG TTA CCT-3′; forward primer 2 with MID7 and 454 fusion, 5′-GCC TCC CTC GCG CCA TCA G−CG TGT CTC TA−G ACG AGC CCA AGT TAC CT-3′; forward primer 3 with MID10 and 454 fusion, 5-GCC TCC CTC GCG CCA TCA G−TC TCT ATG CG−T GAC GAG CCC AAG TTA CCT-3′; forward primer 4 with 454 fusion, 5′-GCC TCC CTC GCG CCA TCA G−TG ACG AGC CCA AGT TAC CT-3′; reverse primer with 454 fusion; 5′-GCC TTG CCA GCC CGC TCA G−TG TAG GCA GCG GAG ATT CT-3′). Pools were sequenced by the Genome Sequencing Services Laboratory at the University of Florida using a Genome Sequencer 20 system with pyrosequencing 454 strategies.

Bioinformatics

Sequences were grouped according to their MID sequences for further analysis. The grouped sequences were aligned using MAFFT-6 software (*24*) and Jalview2.4 (*25*) using the progressive method. ATV 4.0.5 was used to display and manipulate the phylogenetic tree (*26*). After forming the phylogenetic tree, representative sequences from different families were selected as candidate aptamers.

Flow-Cytometric Analysis

Flow cytometry was used to monitor the enrichment of pools during the selection. The FITC-labeled ssDNA pool was incubated with 5×10^5 target cells at a final concentration of 250 nM on ice for 30 min. The cells were washed twice with 500 μL of washing buffer and resuspended in 300 μL of washing buffer. The fluorescence intensity was determined by using a FACScan cytometer (Becton Dickinson Immunochemistry Systems) by counting 30 000 events. The FITC-labeled initial ssDNA library was used as a negative control.

Binding Analyses

The binding affinities of aptamers were determined by incubating various concentrations of biotin-labeled aptamer with 5×10^5 target cells at 4 °C for 30 min. Cells were then washed with 500 μL of washing buffer twice. The cells with bound aptamers were incubated with 200 μL of streptavidin-PE (Invitrogen, SNN1007) at 4 °C for 20 min and washed with 1 mL of washing buffer. The mean florescence intensity of the unselected library was subtracted from that of the aptamer with the target cells to determine the specific binding. The ligand binding analysis function of SigmaPlot (Jandel Scientific) was used to calculate the apparent equilibrium dissociation constant (K_d) of aptamer according to the equation $Y = B_{max}X/(K_d + X)$

Effect of Temperature on Binding

Since the selection was performed at 4 °C, some of the aptamers may not bind well at higher temperatures. Because further studies were planned at physiological conditions, the binding studies of aptamers were also performed at 37 °C with a 30 min incubation period.

Proteinase Treatment

Target cells (1×10^6) were washed with 1 mL of PBS and then incubated with 2 mL of 0.05% trypsin/0.53 mM EDTA in Hank's balanced salt solution (HBSS) or with 2 mL 0.1 mg/mL proteinase K in PBS buffer at 37 °C for 3 and 10 min. DMEM containing 10% FBS was then added to inhibit proteinases. After washing with 2 mL of binding buffer, cells were used in the aptamer binding assay as described in the flow-cytometric analysis.

Imaging of Cells Bound to Aptamers

For imaging, 100 pmol of biotin-labeled aptamer was incubated with the cells in 200 μL of binding buffer for 30 min. After washing with 1 mL of washing buffer, cells were incubated with streptavidin-PE for 20 min. Excess streptavidin-PE was removed using 2 mL of aliquots of washing buffer. Also Vectashield mounting medium (Vector Laboratories) with 4′,6-diamidino-2-phenylindole (DAPI) was used for counterstaining of cellular nuclei. A Leica DM600B microscope was used for imaging.

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Optimization of Aptamers

The secondary structure analysis of aptamers was performed by means of NUPACK analysis algorithms (*27*) using the Internet tool NUPACK nucleic acid package (*28*) (Figure S-1 in the Supporting Information). Using the secondary structures, possible fragments of aptamers having stem and loop structures were synthesized and screened for their binding affinities.

Supporting Information Available

Figure showing the secondary structure analysis of selected aptamers. This material is available free of charge via the Internet at http://pubs.acs.org.

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Abbreviations

X, concentration of ligand; *Y*, specific binding; B_{max}, maximum specific binding; K_d, equilibrium dissociation constant; PTEN, gene for phosphatase and tension homologue.

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