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Identification of Vigilin as a Potential Ischemia Biomarker by Brain Slice-Based Systematic Evolution of Ligands by Exponential Enrichment

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Abstract

Stroke is one of the leading causes of disability and death among adults worldwide and results in numerous biochemical alterations. However, few efficient biomarkers are clinically available to diagnose stroke because of the limitations of biomarkers and their probes. In this work, we utilized frozen brain slices of middle cerebral artery occlusion (MCAO) in a mouse model of ischemia to select a specific binding aptamer, termed LCW17, by tissue-based SELEX (systematic evolution of ligands by exponential enrichment). LCW17 was enhanced in binding in ischemic brain slices compared to sham control. We identified the binding target of LCW17 as vigilin. Vigilin is increased in ischemia brain slices and exhibits enhanced release from cultured hippocampal neurons after oxygen glucose deprivation in vitro. Taken together, ischemic brain slice-based aptamer selection will enable identification of more probes and potential target molecules for diagnosis and therapy of ischemic stroke. Aptamer LCW17 and vigilin may potentially be applied to define the molecular mechanism underlying ischemic stroke, as well as its diagnosis.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b00609.

Monitoring the progress of aptamer selection, analysis of individual sequence similarity, confocal images, expression and purification of vigilin, binding affinity of LCW17 for ischemia slices, DNA sequences employed in this work, and mass spectrometric analysis (PDF)

The authors declare no competing financial interest.

Stroke is one of leading causes of death and permanent disability worldwide, with over 80% of stroke caused by ischemia, i.e., inadequate blood flow to the brain.^{1,2} Previous studies have demonstrated that excitotoxicity and ionic imbalance, oxidative stress, and apoptotic-like cell death were the fundamental mechanisms leading to brain cell death after the onset of ischemia.³ Some biomolecules involved in the pathological mechanisms of stroke have been studied by the researchers.⁴⁻⁷ Cytokines and C-reactive protein (CRP) were the groups of most studied inflammatory blood biomarkers in the stroke field, but still have not been used in stroke clinical practice.⁸ Few effective technologies have been developed to probe the features of cerebral ischemia. It follows that the paucity of biomarkers also limits our understanding of the pathological mechanisms of ischemic brain injury and our ability to identify targets for diagnosis and therapy.

Aptamers have been widely used in biomarker discovery,⁹ cell capture,^{10,11} and in vivo imaging¹² owing to their high specificity, relatively rapid tissue penetration, and facile chemical synthesis.¹³ To obtain specific binding aptamers, cell-SELEX (systematic evolution of ligands by exponential enrichment) was developed by using the differences at the molecular level between any two types of cells, thus identifying the molecular signatures on the surfaces of targeted cells.¹⁴

Cell-SELEX has become a useful tool for targeted therapy and biomarker discovery in cancer and other diseases.^{12,15,16}

However, because neurons are heterogeneous and obtained through primary culture, the enrichment of aptamers that specifically bind biomarkers of neurological diseases remains challenging. Moreover, several types of cells, such as astrocytes, microglia, neutrophils, and macrophages, respond to brain disease, and the extracellular matrix (ECM) generated by these different cells plays a critical role in tissue repair and replacement after an acute insult.¹⁷ Tissue slice-based SELEX was introduced by Shao's group, but only used in selection of aptamers targeting biomarkers of cancer cells.^{18,19} Using a brain slice as the target for SELEX would overcome problems of cell-SELEX and allow the evolution of aptamers for more target molecules, including fragments of ECM and membrane components of various cells.

To investigate the molecular and cellular mechanisms of brain injury after the onset of cerebral ischemia, occlusion of the middle cerebral artery (MCAO) of rodent animals has been acceptably and widely used as a model system.^{2,20,21} In this work, we used frozen brain slices of mouse MCAO to enrich and select specific binding aptamers of proteins expressed after the onset of MCAO ischemia.

MATERIALS AND METHODS

Animals.

Male wild-type mice (C57BL/6J, aged 56 days, Hunan SLAC Laboratory Animal Co., Ltd.) were used for this study. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition). All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Hunan University. Mice were housed separately in a temperature- and humidity-controlled room under a 12 h light–dark cycle with free access to food and water.

Transient Focal Cerebral Ischemia.

Focal cerebral ischemia was induced by intraluminal middle cerebral artery occlusion (MCAO). Mice were anesthetized with 5% isoflurane and maintained with 1% isoflurane in an oxygen/air mixture using a gas anesthesia mask (RWD Life Science). The left common carotid artery and the external carotid artery were exposed by a ventral midline neck incision and clipped. The external carotid artery was ligated with 5–0 silk suture. A 2 cm length of silicone-rubber-coated monofilament (RWD Life Science) was advanced from the common carotid through the internal carotid up to the level of the anterior cerebral artery. The suture was inserted 9 mm from the bifurcation of the common carotid to occlude the middle cerebral artery. The sham operated animals were treated identically, except that the middle cerebral artery was not occluded after the neck incision.

Brain Tissue Slices.

Mice were perfused with saline solution before being perfused with 4% paraformaldehyde, postfixed overnight in 4% paraformaldehyde at 4 °C, cryoprotected in 30% sucrose, and embedded in optimal cutting temperature compound (OCT, 25608–930, Tissue-Tek O.C.T. compound, Sakura). Tissues were cryosectioned at 15 µm and stored in 50% glycerol solution at –40 °C for up to 3 months. The frozen sections were warmed to room temperature, dipped in ice-cold methanol for 10 min, washed three times with washing buffer (DPBS, 5 mM MgCl₂), and incubated in the blocking buffer (2 µg/µL yeast tRNA, 2% BSA (w/v), and 5 mM MgCl₂ in DPBS) for 30 min before experiments.

DNA Primers and Library.

The ssDNA library used for aptamer selection contained a central randomized sequence of 30 nucleotides flanked by two polymerase chain reaction (PCR) primer sequences (5′-AGCGTCGAATACCACTAC-AG-30-nt-CTAATGGAGCTCGTGGTCAG-3′). FAM-labeled forward primer (5′-FAM-AGCGTCGAATACCAC-TACAG-3′) and biotinylated reverse primer (5′-biotin-CTG-ACCACGAGCTCCATTAG-3′) were used in the PCR. All sequences were synthesized by Sangon Biotech, Shanghai, China.

SELEX Procedure.

The process of brain tissue slice-based SELEX is similar to the procedure described in the past.¹⁸ The initial pool containing 5 nmol of ssDNA library was dissolved in 100 μ L of binding buffer (2 μ g/ μ L yeast tRNA, 1% BSA (w/v), and 5 mM MgCl₂ in DPBS), denatured by heating at 95 °C for 5 min, and cooled on ice for 10 min. In the first selection, the ssDNA library pool was incubated with five ischemia slices in a 1.5 mL tube for 2 h at 4 °C. Unbound ssDNA was removed by washing. The binding ssDNA sequences were collected by heating at 100 °C for 10 min and used as a template to prepare the evolved DNA pool by PCR with FAM-labeled forward primer and biotin-labeled reverse primer. The double-stranded DNA was separated from the PCR solution by streptavidin-coated beads (GE Healthcare). Then, the FAM-labeled ssDNA was separated by alkaline denaturation (0.2 M NaOH) and desalted with a NAP-5 column (GE Healthcare). Finally, the FAM-labeled ssDNA pool was concentrated and used for the next selection, temperature melting curve, and confocal imaging. From the second selection, the ssDNA pool was first incubated with the ischemia slices for positive selection; then, the bound ssDNA was collected for incubation with sham slices for negative selection to remove the nonspecific binding sequences. To acquire aptamers with high affinity, incubation time was gradually reduced from 2 to 0.5 h and the number of the ischemia slices from four pieces of brain slice to one piece in the positive selection. After 10 rounds of selection, the ssDNA pool was analyzed by high-throughput sequencing performed by Sangon Biotech, Shanghai, China.

Confocal Imaging of Brain Tissue Slices.

The selected ssDNA pools and aptamers were labeled with FAM. The brain slices were preincubated with blocking buffer, as described above, and then incubated with 10 pmol of ssDNA or aptamers, respectively, in 100 μ L of binding buffer at 4 °C for 40 min. They were then washed three times, stained with 4–6-diamidino-2-phenylindole (DAPI; 75 ng/mL) for 5 min, washed three times, dehydrated, and sealed for imaging. Three different areas of hippocampal CA1 region were imaged in the slice.

All of these tissue slices were imaged on an FV1000-X81 confocal microscope (Olympus, Japan). The analysis size of the imaging area is 105.88 μ m $\times$ 105.88 μ m. The average fluorescence intensity and sum of integrated intensity of the clusters (the integrated intensity of the fluorescent clusters equal to the multiplication of area and intensity) were calculated by Image-Pro Plus, v. 6.0.

Monitoring the Selection Process.

To monitor enrichment, sham slices and ischemia slices were preincubated with blocking buffer (2 μ g/ μ L yeast tRNA, 2% BSA, and 5 mM MgCl₂ in DPBS), and then incubated with 100 nM FAM-labeled evolved ssDNA pools and library in 200 μ L of binding buffer (2 μ g/ μ L yeast tRNA, 1% BSA, and 5 mM MgCl₂ in DPBS) at 4 °C for 30 min. After incubation, nuclei were stained with 1 μ g/mL DAPI in washing buffer (5 mM MgCl₂ in DPBS) at 4 °C for 5 min and subsequently washed three times with 200 μ L washing buffer. Slides were briefly dried and mounted with a water-soluble mounting medium and cover-slips. Finally, the slides were imaged by an FV1000-X81 confocal microscope equipped with a $\times$ 60, 1.35 NA objective (Olympus).

Furthermore, melting curve analysis based on DNA renaturation/reassociation kinetics (c0t) was employed for qualitative estimation of the diversity of aptamer pool, as previously described for monitoring the progress of in vitro aptamer. A real-time PCR assay was established to quantify bound ssDNA pool from each SELEX cycle. The enriched pool was amplified by PCR using unlabeled primers from the KOD SYBR qPCR mix. The Applied Biosystems 7500 Fast Real-Time PCR system was used to detect the amplification curve and melting curve of the PCR products.

Brain Homogenate Preparation and Protein Identification.

For brain homogenate experiments, one mouse ischemia brain was homogenized by glass homogenizer in 3 mL of extraction buffer (0.1 mM EGTA, 1 mM PMSF, 1 µg/mL pepstatin, 1 µg/mL leupeptin and 2 µg/mL aprotinin, 1% Triton X-100 in PBS) and centrifuged at 10 000g for 2 h at 4 °C to collect the supernatant. Using the streptavidin–biotin system, 150 µL of supernatant was incubated with 50 pmol of biotin-labeled aptamer LCW17 (or ssDNA library) and 100 µL of streptavidin-coated Sepharose beads at 4 °C for 40 min. The collected beads were washed four times with washing buffer and mixed with 50 µL of 3× SDS loading buffer, boiled, analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), and stained with Coomassie Brilliant Blue R250. The aptamer-specific protein bands were excised manually from the Coomassie Blue-stained gels and digested automatically using 6 × 5 LC–MS/MS peptide reference mix (Promega, Madison, WI, U.S.A.). Tryptic products were analyzed using an LTQ Orbitrap Velos Pro (Thermo Fisher Scientific). Each spectrum was internally calibrated with mass signals of trypsin autolysis ions to reach a typical mass measurement accuracy of ±10 ppm. Raw data were searched in the Swiss-Prot database. The Proteome Discoverer Software (version 2.0) was employed to do the match score between MS data and Swiss-Prot database.

Vigilin Expression and Purification.

The open reading frame of vigilin was PCR-amplified from mouse cDNA, and the coding sequence of His (HHHHHH)-tagged vigilin was subcloned into the BamHI and XhoI sites of a pcDNA3.2 expression vector with PCR. Human embryonic kidney (HEK) 293T cells were employed to generate recombinant vigilin protein. All of the HEK 293T cell transfections were performed using the polyethylenimine (PEI) method. The PEI (1 mg/mL in ddH₂O)/DNA ratio was 3:1. The PEI/DNA mixture was incubated for 30 min at room temperature before adding to the HEK 293T cell cultures dropwise. For vector, or His-tagged vigilin plasmid, 3 µg of plasmid was transfected into one well of a 6-well plate, together with 1 µg of pFUGW-tdTomato fluorescent protein. His-tagged vigilin protein was purified according to the His-tag protein purification kit (P2226, Beyotime). Briefly, the transfected HEK293T cells were collected and resuspended in lysis buffer for 0.5 h on ice, and insoluble material was removed by centrifugation (10 min at 15 000g). His-tagged vigilin protein was purified by BeyoGold His-tag purification resin with shaking for 0.5 h at 4 °C. His-tagged vigilin protein was collected by centrifugation (10 s at 1000g). After three washes with wash buffer, the His-tagged vigilin protein was eluted by elution buffer, and protein preparations were denatured at 95 °C for 5 min and separated on 10% SDS–PAGE gels for silver staining.

Aptamer-Based Binding Assay.

An ELISA (enzyme-linked immunosorbent assay) plate was coated with 50 μL of purified vigilin protein diluted to 0.1 $\mu\text{g}/\mu\text{L}$ in coating buffer, incubated overnight at 4 $^{\circ}\text{C}$, washed three times with washing solution, and then blocked with assay diluent for 1 h at 37 $^{\circ}\text{C}$. Biotinylated aptamer LCW17 was heated to 95 $^{\circ}\text{C}$ for 5 min and cooled on ice for 10 min. The aptamer was then diluted in aptamer washing buffer (5 mM MgCl_2 , assay diluent) and incubated at room temperature (RT) protected from light for 1 h. The plate was washed four times with aptamer washing buffer and incubated with 100 μL of streptavidin–HRP (horseradish peroxidase) (diluted 1:250, BD Biosciences, 554066) in assay diluent for 1 h at RT. After a final wash, chromogenic reaction was initiated using 100 μL of substrate solution. Thirty minutes later, the reaction was stopped with 50 μL of stop solution. The absorbance was measured at 450 nm. The coating buffer, washing solution, assay diluent, substrate solution, and stop solution were provided in the BD OptEIA reagent set A (cat. no. 550536).

Primary Hippocampal Neuron Culture and Oxygen Glucose Deprivation Treatment.

Cultured neurons were obtained from C57BL/6J mouse hippocampal cells, as described previously.²² Briefly, mouse hippocampal cells were dissected from postnatal day 0 wild-type mice, dissociated with 0.25% trypsin (Gibco, Grand Island, NY, U.S.A.), digested for 12 min at 37 $^{\circ}\text{C}$, plated on poly-D-lysine-coated glass coverslips (8 mm) at a density of 80 000 neurons per coverslip (Scope Cell Counter Basic, C.E.T. Corporation, Beijing, China), and then maintained at 37 $^{\circ}\text{C}$ in a humidified chamber of 95% air and 5% CO_2 for 14 days.

Oxygen glucose deprivation (OGD) experiments were performed using a special humidified chamber kept at 37 $^{\circ}\text{C}$, which contained an anaerobic gas mixture of 90% N_2 , 5% H_2 , and 5% CO_2 . To initiate OGD, the culture medium was replaced with deoxygenated, glucose-free Dulbecco's modified Eagle's medium (Life Technology, 11966–025), and the culture was treated for various durations (1, 2, and 4 h). The conditional medium of both control (maintained at 37 $^{\circ}\text{C}$ in a humidified chamber of 95% air and 5% CO_2 in normal growth medium) and OGD treatment was harvested and subsequently precleaned with a centrifuge at 3000g for 5 min.

Western Blot.

Protein extracts were denatured at 95 $^{\circ}\text{C}$ for 5 min and separated on 10% SDS–PAGE gels at 110 V for about 70 min. Then, the proteins were transferred to nitrocellulose (NC) filters at 80 V for 5 h. The NC membrane was initially blocked with 5% nonfat milk and 2% goat serum (v/v) in Tris-buffered saline with 0.1% Tween 20 (TBS-T) at RT for 1 h. Membranes were then incubated overnight at 4 $^{\circ}\text{C}$ with the following primary antibodies: anti- β -actin (1:1000, Sigma-Aldrich, A5441), anti-vigilin (1:200, CUSABIO, CSB-PA876442), anti-His (1:5000, Bioworld Biotech, AP0032), and anti-GAPDH (1:1000, TransGen Biotech, HC301–01). After three washes of 5 min each with TBS-T, goat antirabbit or antimouse immunoglobulin G (IgG) was added at a dilution of 1:5000 as the secondary antibody. The NC membrane was scanned with an imaging system (MicroChem 4.2, DNR Bio-Imaging, Israel).

In Vivo Injection and ex Vivo Imaging.

Aptamers were injected into the brain of 3 h ischemia mouse. Thirty micromolar Cy5-labeled LCW17 (dissolved in 5 mM MgCl₂ of DPBS) was denatured by heating at 95 °C for 5 min and cooled on ice for 10 min. Glass pipettes were pulled from borosilicate glass capillary tubes (World Precision Instruments) using a P-97 pipet puller (Sutter Instrument). Stereotactic injections (RWD 68526, RWD Life Science, Shen Zhen, China) of 2 µL of aptamers were at coordinates 2.0 mm posterior, 2.0 mm lateral, and 1.8 mm ventral relative to bregma and stopped at the injection site for 5 min, then, respectively, injected 2 µL to the ischemia (left) brain and sham (right) brain, as shown in Figure 6A. The same dose of Cy5-labeled library aptamer was injected into another mouse brain at the same time as a control. After 1 h, the mice were sacrificed. The brains were quickly isolated and sectioned into slices, and then imaged by IVIS Luminar XR living imaging system (PerkinElmer, U.S.A.).

Statistical Analysis.

Every group had three mice used in each experiment. Calculated data are presented as mean $\pm$ SD. The paired Student *t* test for Figure 6B and unpaired Student *t* test for Figure 2, parts B and C, and Figure 5B were used to determine the differences between two groups in confocal images analysis and ex vivo images analysis, respectively. All statistical analyses were done with Origin Pro 8.

RESULTS AND DISCUSSION

Enrichment of Aptamer Candidates to Cerebral Ischemic Slices.

As shown in Figure 1, frozen mouse brain slices at 4 h post-MCAO ischemia were employed for positive selection, and sham slices were used for negative selection to exclude nonspecific binding aptamers. During the selection, both ischemic and sham brain slices were stained with FAM-labeled single-stranded DNA (ssDNA) from the selected aptamer pool. Since neurons in the CA1 region of the brain's hippocampus are highly vulnerable to ischemia,^{20,23} we monitored the fluorescence intensity of this region by confocal imaging to evaluate the enrichment of aptamers. On the basis of quantitative analysis of the fluorescence intensity in the CA1 region, we could detect no significant fluorescence signal from the initial library binding to either sham or ischemic slices. However, the fluorescence intensity was significantly increased in ischemic slices from the 6th to 10th pools, resulting from the enrichment of the specific binding aptamers (Figure S1, parts A and B). Meanwhile, we observed that the melting peak of ssDNA in the 10th pool became narrowed (Figure S1C). These results also suggested that the ssDNA was efficiently enriched during the selection.²⁴

Evaluation of Aptamer LCW17 Accumulation in Cerebral Ischemia.

To identify the aptamer candidates, the ssDNA of the 10th pool was sequenced and grouped based on sequential repeatability, secondary structures, and homogeneity (Figure S2). Three highly abundant sequences, including LCW3, LCW5, and LCW17 (Table S1), were selected to be synthesized and labeled with FAM for further research. The FAM-labeled aptamers of LCW3, LCW5, and LCW17 at 200 nM were applied to bind the frozen sham or ischemic

brain slices, respectively. After aptamer binding, the tissue slices were imaged in the hippocampal CA1 region of the brain by confocal microscopy. We found that the original library did not efficiently bind to sham or ischemic slices (Figure 2). In contrast, the selected aptamer LCW17 had much greater ability to bind to the ischemic slices compared to the sham slice (Figure 2A). The ability of the other aptamer candidates, LCW3 and LCW5, to bind to ischemic slices was lower than that of LCW17 (Figure S3). To assess the specific binding of aptamer LCW17 to the ischemic brain slice, we analyzed the fluorescent clusters of LCW17 in the binding experiment and found that the relative average fluorescence intensity and number of clusters which formed as a result of LCW17 binding to its target in the ischemic slice was significantly higher than that of the sham slice (Figure 2, parts B and C). Furthermore, the frequency distribution of the clusters (Figure 2D) indicated that an increased number of clusters with higher fluorescence intensity in the ischemic brain slices led to a correspondingly increased number of LCW17 aptamers bound to ischemic slices. These data revealed that the selected aptamer LCW17 could, indeed, bind to the cerebral ischemic slices.

Identifying That the Target Molecule of the Aptamer LCW17 Is Vigilin.

To identify the target protein of aptamer LCW17, the homogenate protein of ipsilateral hemisphere 4 h postischemia was extracted and subjected to a pull-down experiment with biotin-labeled aptamer LCW17, respectively. The LCW17 pull-down preparation was subjected to SDS–PAGE, followed by Coomassie Brilliant Blue staining, to separate and analyze the aptamer-bound target proteins. A band at about 140 kDa was more intense in LCW17 than in the library. The band was excised and subjected to mass spectrometric analysis (Figure 3A). The results showed a high level of vigilin in the LCW17 binding band, but only a low level in the corresponding library binding band (Figure 3B, Tables S2 and S3).

To confirm the target molecule of aptamer LCW17 binding, we performed Western blot assays with an antibody of antivigilin and found that the preparations captured from brain homogenate by aptamer LCW17 were specifically recognized by the antibody against vigilin, but that no band was detected in the preparations captured by the library (Figure 3C). To test the dose–effect relationship between vigilin and aptamer LCW17, different amounts of LCW17 were used to capture vigilin from the homogenate of ischemic brain. We found that more vigilin was captured from the same amount of homogenate input when more aptamer LCW17 was applied (Figure 3D). To determine if the LCW17-binding target colocalizes with vigilin in ischemic brain slices, the mouse brain slices were first bound with FAM-labeled LCW17, and then stained with rabbit anti-vigilin primary antibody and Alexa Fluor 546-labeled goat antirabbit secondary antibody. We observed that aptamer LCW17 colocalized with vigilin in ischemic brain slices (Figure S4), indicating that vigilin is a potential target molecule of aptamer LCW17. We also observed the fluorescence signals of LCW17 and MAP2 (microtubule-associated protein 2, enriched in dendrites) were near to each other (Figure S5); the vigilin should be secreted from the dendrites.

To further verify the LCW17-binding target, we tested if aptamer LCW17 could directly bind to purified recombinant His-tagged vigilin. Various amounts of aptamer LCW17 were

used to conduct a pull-down assay. We found that the proteins pulled down by LCW17 were in a dose-dependent manner (detected by immunoblotting with anti-His antibody). When the aptamer library at 200 nM was applied, no vigilin was detected (Figure 3E and Figure S6), suggesting that aptamer LCW17 directly binds to the purified vigilin. Taken together, these data revealed that vigilin is the target molecule of aptamer LCW17.

Evaluating the Ischemic Lesion Growth in the MCAO Model by Aptamer LCW17.

Multiple techniques have been reported to evaluate histopathological damage of stroke, but the sensitivity and reproducibility of quantification methods have not been precisely characterized.^{23,26,27} Because aptamer LCW17 specifically binds to the ischemia slices, we tested if this aptamer could be used to evaluate ischemic lesion growth in a mouse model of MCAO. First, we characterized the binding affinity of LCW17 to vigilin. Various concentrations of LCW17 were applied to the wells of a vigilin-coated ELISA plate (Figure 4), and the equilibrium dissociation constant (K_d) was calculated as 25 ± 3 nM by fitting the dependence of absorbance at 450 nm on the concentration of the aptamer to the equation $Y = B_{\max}X/(K_d + X)$ with Origin software.

Second, we also evaluated the binding affinity based on the fluorescence intensity of specific binding clusters in confocal images, and the K_d was calculated as 17 ± 8 nM (Figure S7).

These results demonstrated that the selected aptamer LCW17 could bind to the target vigilin with high affinity in vitro and within tissue slices. Finally, we incubated FAM-labeled aptamer LCW17 with tissue slices obtained from mice across a time course of 2, 3, and 4 h of ischemia. We found a significant time-dependent increase in the sum of integrated intensity of the fluorescent clusters (Figure 5, parts A and B). These results indicated that aptamer LCW17 may be used to quantify histopathological damage in the ischemic brain.

Vigilin Has Enhanced Release from Cultured Hippocampal Neurons after OGD Treatment.

Previous study revealed that vigilin regulates very low density lipoprotein (VLDL) secretion²⁸ and that it has been detected in extracellular vesicles (EVs).^{29–31} Moreover, since primary cultured neurons subjected to OGD are widely accepted for investigating the pathogenesis and potential treatment strategies for cerebral ischemia in vitro,^{22,32,33} we reasoned that the protein expression of vigilin might change in cultured hippocampal neurons after OGD treatment. To test this hypothesis, the conditional medium of both control and OGD treatment was harvested and subsequently precleaned with a centrifuge followed by immunoblotting. Results showed that the cultured medium with different durations of OGD treatment contained higher protein vigilin levels than those of controls, respectively (Figure 5D), indicating that the release of vigilin is enhanced during OGD in vitro. Previous studies demonstrated that vigilin protects cells from over-accumulation of cholesterol³⁴ and that a lowered cholesterol level is neuroprotective in stroke and traumatic brain injury.^{35,36} Therefore, our findings suggest that vigilin plays a critical role and may be a potential biomarker in brain injury after cerebral ischemia onset.

In Vivo Injection and ex Vivo Imaging.

At last, we injected 60 pmol of Cy5-labeled aptamers into the brain of 3 h ischemia mouse. The biodistribution of the aptamers in the brain was imaged ex vivo at 1 h postinjection (Figure 6B).

Obviously, after stereotactic injection of aptamer Cy5-LCW17, the intensity of the fluorescence in the CA1 area of the ischemia brain is about twice that of the sham (Figure 6C). In the control experiment, after stereotactic injection of 60 pmol of Cy5-Library, the fluorescence signal is very low. These results indicated that the aptamer LCW17 could recognize the target in vivo and have the potential to be a probe of detecting the ischemic stroke.

CONCLUSIONS

In summary, by using ischemic brain slice-based SELEX, we selected aptamer LCW17, which specifically binds to ischemia slices, and we identified vigilin as the target of the aptamer LCW17. Aptamer LCW17 specifically bound recombinant vigilin with K_d value in the nanomolar range, and it was efficiently used to evaluate the ischemic lesion growth in mice. Therefore, this SELEX method could be used in research on other diseases not otherwise amenable to study by cell-SELEX. Aptamer LCW17 will be a potential tool with which to detect incipient ischemic stroke, perform targeted therapy, and understand the molecular mechanism of cerebral ischemia. Additionally, defining the molecular and physiological roles of vigilin in ischemic stroke will be interesting and tantalizing for cardiovascular researchers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic illustration for brain MCAO slice-based SELEX. (A) Illustration of ischemic brain section slice preparation: MCA, middle cerebral artery; ICA, internal carotid artery; ECA, external carotid artery; CCA, common carotid artery. (B) Brain tissue slice-based SELEX method for enriching DNA aptamers toward changed protein levels after ischemia.

Figure 2.

Investigation of the specificity of aptamer LCW17. (A) Confocal microscopy assay of binding of aptamer LCW17 to ischemia slice and sham slices. The unselected initial library was used as control; scale bar is 10 μm . (B) Average fluorescence intensity of clusters in the confocal images. (C) Number of fluorescent clusters per 100 μm^2 . (D) Quantification of the binding affinity of aptamer LCW17 in slices reported as frequency distribution of the fluorescence intensity of the clusters. Defined threshold is 10–255, $n = 4$ slices, one animal of sham, one animal of ischemia 4 h, unpaired Student t test, *** $p < 0.001$).

Figure 3.

Identification of the molecular target of aptamer LCW17. (A) Analysis of LCW17-mediated target separation in Coomassie Blue-stained polyacrylamide gel. Beads binding was used as a control. (B) Specialized protein analysis by mass spectrometry. (C) Western blot assay verification of the target protein with anti-vigilin antibody. (D) Immunoblotting analysis of the target of LCW17 binding. (E) Various concentrations of aptamer LCW17 pulled down the purified protein of mouse vigilin. The unselected initial library (200 nM) was used as control. The structure of recombinant vigilin, as predicted by SWISS-MODEL (ref 25).

Figure 4. Affinity of aptamer LCW17 binding to vigilin. (A) Diagram of aptamer-based ELISA protocol. (B) Equilibrium binding curves for various concentrations of LCW17 with vigilin coated on an ELISA plate; the absorbance at 450 nm was recorded.

Figure 5.

Evaluation of ischemia severity by aptamer LCW17. (A) Confocal images of the brain slices at different time points after MCAO ischemia. Scale bar is 10 μ m. (B) The sum of integrated intensity of the fluorescence in confocal images ($n = 3$ slices, one animal of sham, one animal of ischemia 4, 3, and 2 h, unpaired Student t test, $*p < 0.05$, $***p < 0.001$). (C) Diagram of OGD treatment and medium collection. (D) Immunoblots showing protein levels of vigilin in conditional medium without or with OGD treatment for 1, 2, or 4 h. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control and indicated the cell lysates in medium.

Figure 6.

In vivo injection and ex vivo imaging the aptamers in ischemia mouse. (A) Schematic illustration of the in vivo injection; Cy5-labeled aptamers were injected into the CA1 areas of the brain. (B) The fluorescence imaging of the brain slices; the ipsilateral (left) hemisphere is the ischemia brain, the contralateral (right) hemisphere is the sham brain. (C) Quantification the intensity of the fluorescence in CA1 areas ($n = 3$ mice, three animals of sham, three animals of ischemia 3 h, paired t test, n.s., not significant, $**p < 0.001$).