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Title: Rapid and Specific Diagnosis of Astrocytomas with Fluorescent Aptamers

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Conflict of Interest Declaration:

None.

Abstract:

Introduction

Neurosurgical tumor resections are routinely assisted by intraoperative histological assessment of biopsies. However, it is difficult to differentiate, via gross appearance or frozen section analysis, high-grade gliomas from non-operative lesions, such as primary central nervous system lymphoma (PCNSL). Aptamers are an emerging class of polynucleotides that function as “chemical antibodies” with high binding affinity to specific cellular targets. They can identify

samples in minutes compared to the typical 24-to-48-hour timeframe via immunohistochemistry (IHC). We have previously developed a PCNSL-specific aptamer and aim to develop a fluorescent aptamer targeting glial fibrillary acid protein (GFAP), a common IHC target for gliomas.

Hypothesis: GFAP aptamers can accurately identify glioma cell lines in twenty minutes, a timeframe that can support intraoperative decision-making for best treatment practices.

Objective: Develop a GFAP aptamer to utilize as an intraoperative assay to diagnose glioma tumors.

Methods

Our data was obtained from human glioma cells (U251) and human PCNSL (Ramos) cell lines. We demonstrate a CD20-specific aptamer labeling protocol to rapidly identify Ramos cells and preliminary data which demonstrates a GFAP aptamer's ability to label U251 cells.

Cell lines were cultured with their appropriate mediums and grown at 37C at 5% CO2. Aptamers had AF488 and Cy5 fluorophores for the CD20 (TD05) and GFAP aptamers, respectively. Annealed aptamers were incubated on ice for 20 min, treated with fixation/permeabilization, and tested at different concentrations. For comparison, we ran positive and negative control validation experiments for each aptamer individually and a mixed aptamer assay with analysis of fluorescence via flowcytometry.

Results

We found a concentration of 400nM or higher of both the TD05 and the GFAP aptamers were highly sensitive and appropriate for labeling Ramos and U251 cells, respectively. When gating baseline fluorescence of non-stained cells, 100% of cells at all aptamer concentrations greater than 400nM showed AF488 fluorescence for Ramos cells stained with TD05 aptamer and Cy5 fluorescence for U251 cells stained with the GFAP aptamer.

We unexpectedly found Cy5 fluorescence with 100% of Ramos stained with GFAP aptamers, AF488 fluorescence with 37.3% of U251 stained with TD05 aptamers.

With a mixed aptamer assay, we found Cy5 fluorescence in 99.8% of U251 stained with GFAP aptamers and AF488 fluorescence in 97.6% of Ramos stained with TD05 aptamers. We found Cy5 fluorescence in 78.7% of Ramos stained with GFAP aptamers and AF488 fluorescence in 96.7% of U251 stained with TD05 aptamers.

Discussion/Conclusion

Our current aptamer protocol to label Ramos and U251 cells are highly sensitive in labeling the appropriate cells. However, we found cells which should not be labeled with an aptamer are displaying fluorescence. Such results should not have occurred as CD20 is a surface receptor

expressed only on lymphoma cells and GFAP is an intracellular protein only expressed in glioma cells. This could be due to non-specific binding. Thus, future refinement of our staining protocol, and testing different concentrations may reduce false positives. Our results could also potentially be explained by a known phenomenon that Cy5 fluorophore can be taken up in mitochondria.

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Introduction

Neurosurgical tumor resections are routinely assisted by intraoperative histological assessment of biopsies. Assessments help determine grade and type of pathology as well as identify tumor margins for proper resection[1]. Some pathologies are challenging to differentiate from frozen section analysis, such as gliomas from lymphomas[2] (Figure 1). It is also difficult to differentiate, via gross appearance or imaging, high-grade gliomas (HGG) from non-operative lesions, such as primary central nervous system lymphoma (PCNSL). In order to develop a final diagnosis, immunohistochemistry (IHC) is the gold standard.

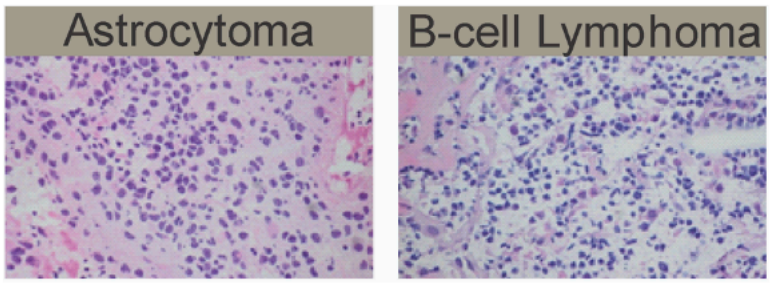

Figure 1. Frozen sections of a high-grade astrocytoma and CNS B-cell lymphoma. These samples appear similar and require IHC for definitive diagnosis.

IHC is too slow to provide surgeons with vital information about the lesion that they are resecting[3]. Thus, surgeons often resect tissue under the assumption of a preliminary, and often vague, diagnosis of the tissue from frozen section. If, for example, the preliminary diagnosis is HGG, the best treatment is aggressive resection in order to reduce the risk of recurrence. If, for example, the preliminary diagnosis is PCNSL, however, the best treatment is non-operative and requires chemotherapy and/or radiation. There have been anecdotal occasions in which the preliminary was HGG and the surgeon performed aggressive resection when days after surgery IHC results in PCNSL. There have also been occasions of the opposite which may require patients to undergo a second operation for full resection of the misdiagnosed tumor. This not only can provide patients at greater risks for morbidity and mortality but also provides patients, families, and healthcare workers with greater morale exhaustion and can increase healthcare costs. Another potential sequence of events might start with a minimally invasive biopsy, performance of IHC, then proper treatment. This pathway also delays treatment by several days.

Aptamers are an emerging class of polynucleotides that function as “chemical antibodies” with high binding affinity to specific cellular targets. They are made of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or peptide strands with unique structures that utilize an induced-fit model to bind to target molecules with high affinity and specificity[4-7]. They are developed via a somewhat “survival of the fittest” selection process by which they are placed with positive and negative molecular targets *in vitro* until an ideal sequence is

developed. This is known as the System Evolution of Ligands by Exponential Enrichment (SELEX)[5, 7]. Once developed, aptamer probes are able to bind to their molecular targets with an equivalent specificity as antibodies but in minutes to hours compared to the typical 24-to-48-hour timeframe of IHC. Researchers have already utilized aptamers to identify tumor cell identification[8-10]. With such capability, surgeons may take a small biopsy, for example, and know in minutes with the confidence of the gold standard of IHC in deciding whether to continue with aggressive resection in the case of a glioma or to avoid further surgical risk and close up for a patient with PCNSL, thus avoiding unnecessary morbidity and/or mortality and providing better standard of care.

We have previously developed a PCNSL-specific aptamer[4, 11]. In this report, we aim to develop a fluorescent aptamer targeting glial fibrillary acid protein (GFAP), a common IHC target for gliomas, and test its utility as an intraoperative diagnostic assay for glioma tumors. We hypothesize that our GFAP aptamers can accurately identify glioma cell lines in twenty minutes, a timeframe that can support intraoperative decision-making for best treatment practices.

Methods

Preparation of Aptamers: All DNA oligonucleotides were purchased from Integrated DNA Technologies, CA with high performance liquid chromatography (HPLC) purification[12, 13]. A TD05 aptamer that targets CD20 and a GFAP aptamer was used.

The name and sequence of fluorophore-labeled aptamers are as follows:

- TD05: 5’ /5Alex488N/AGG AGG ATA GTT AGG TGG CTG TTG AGG GTC TCC TCC TA 3’
- GFAP aptamer: 5’ /5CY5N/AGG AGG AGA TTT TTT TTT TAG GAG GAT AGT TAG GTG GCT GTT GAG GGT CTC CTC CTA /3BHQ_1/ 3’

Our data was obtained from human glioma cells (U251) and human PCNSL (Ramos) cell lines. We demonstrate a CD20-specific aptamer labeling protocol to rapidly identify Ramos cells and preliminary data which demonstrates a GFAP aptamer’s ability to label U251 cells.

Cell lines were cultured with their appropriate mediums and grown at 37C at 5% CO2. Aptamers had AF488 and Cy5 fluorophores for the CD20 (TD05) and GFAP aptamers, respectively. Annealed aptamers were incubated on ice for 20 min, treated with fixation/permeabilization, and tested at different concentrations. For comparison, we ran positive and negative control validation experiments for each aptamer individually and a mixed aptamer assay with analysis of fluorescence via flowcytometry.

Our specific protocol utilized is found below:

1. We developed our aptamers at the proper concentrations. We typically started with a 16uM concentration for each aptamer by including buffer (6 mM MgCl2, 1.2 mM CaCl2, 4.5 g/L glucose, and 0.2% NaN3 in 1× PBS (phosphate-buffered saline buffer)) and PBS, vortexed and annealed at 94C for 5 minutes followed by immediate chilling on ice for 10 min and kept on ice or in 4C fridge as cells are prepared.
2. Yeast tRNA was added at a 0.1mg/mL solution with PBS to reduce nonspecific binding
3. U251 and Ramos Cells were harvested, aliquoting up to $1x10^6$ cells
4. Add proper amount of aptamer solution for each cell vial to planned concentration
5. Cells are then fixed and permeabilized using BD CytoFix/CytoPerm buffer and BD Perm/Wash buffer
6. Add all reagents and aptamers together and incubate for 15 minutes on ice and wash with PBS. Store on ice and cover from light exposure before utilizing flow cytometer

No IRB was required for this research. Our main and secondary outcomes was determining adequate sensitivity and specificity of aptamers binding to the correct corresponding cell types based on percent bound acquired from flow cytometry.

Results

We found a concentration of 400nM or higher of both the TD05 and the GFAP aptamers were highly sensitive and appropriate for labeling Ramos and U251 cells, respectively. (Figure 2A and 2B). When gating baseline fluorescence of non-stained cells, 100% of cells at all aptamer concentrations greater than 400nM showed AF488 fluorescence for Ramos cells stained with

TD05 aptamer and Cy5 fluorescence for U251 cells stained with the GFAP aptamer. We unexpectedly found Cy5 fluorescence with 100% of Ramos stained with GFAP aptamers, AF488 fluorescence with 37.3% of U251 stained with TD05 aptamers. (Figure 1C).

With a mixed aptamer assay, we found Cy5 fluorescence in 99.8% of U251 stained with GFAP aptamers and AF488 fluorescence in 97.6% of Ramos stained with TD05 aptamers. We found Cy5 fluorescence in 78.7% of Ramos stained with GFAP aptamers and AF488 fluorescence in 96.7% of U251 stained with TD05 aptamers. (Figure 3A and 3B).

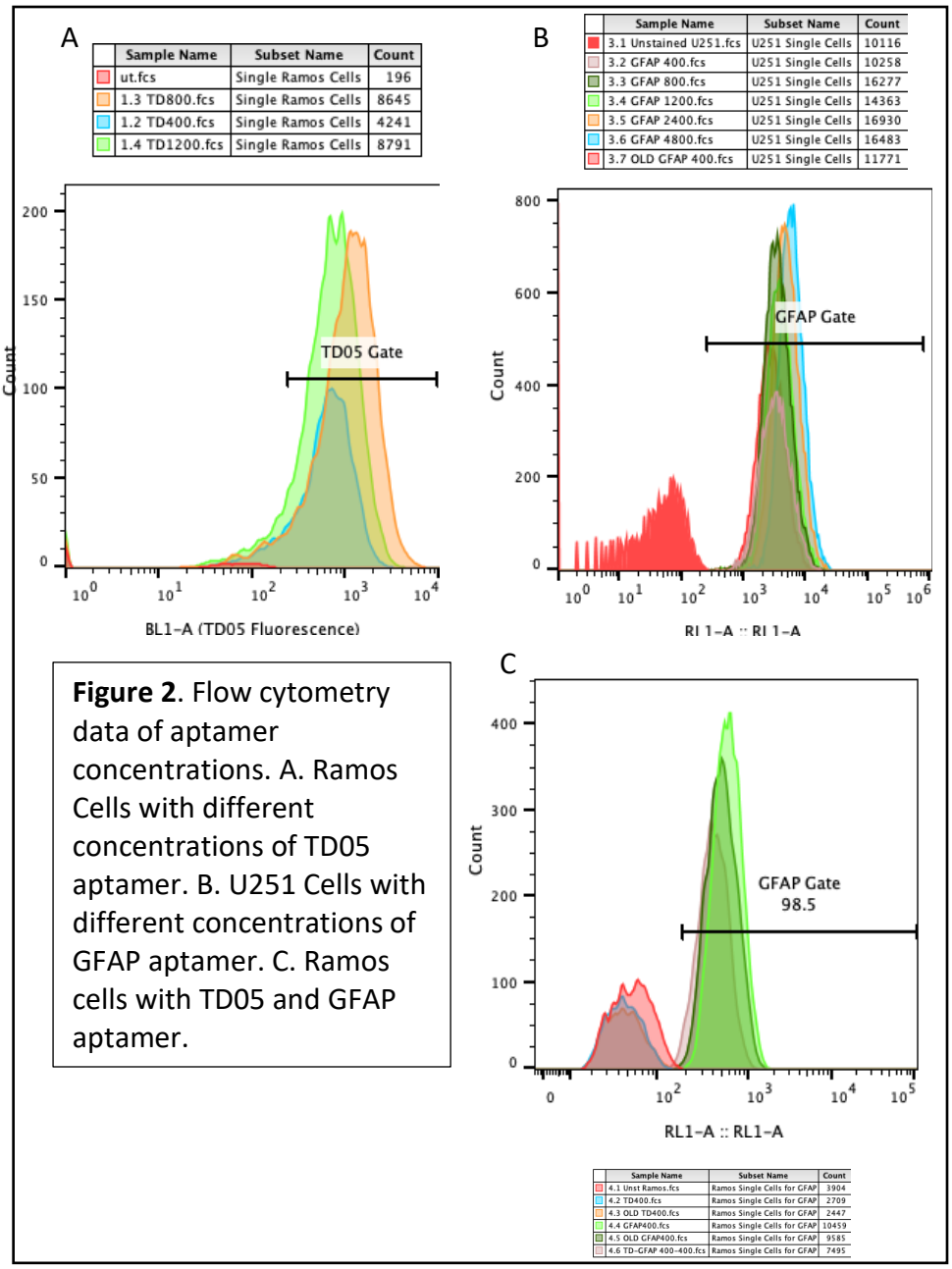

Figure 2. Flow cytometry data of aptamer concentrations. A. Ramos Cells with different concentrations of TD05 aptamer. B. U251 Cells with different concentrations of GFAP aptamer. C. Ramos cells with TD05 and GFAP aptamer.

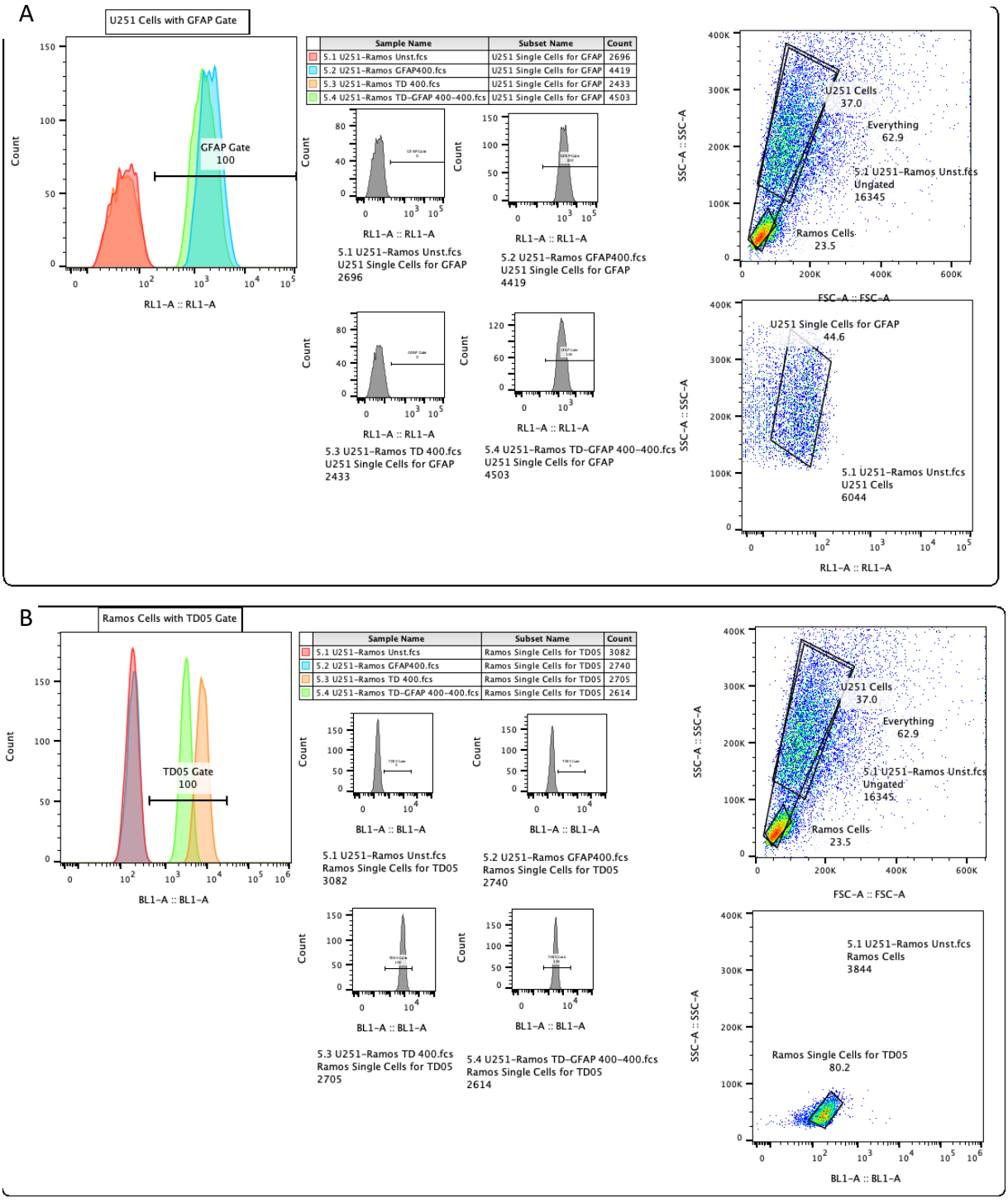

Figure 3. Flow cytometry data of mixed aptamer assay with A. U251 Cells with mixed TD05 and GFAP aptamer and B. Ramos Cells with mixed TD05 and GFAP aptamer assay.

Discussion

Our current aptamer protocol to label Ramos and U251 cells are highly sensitive in labeling the appropriate cells. We found greater than 90% and often near 100% of cells were labeled with the correct aptamer. However, we also found cells which should not be labeled with an aptamer are displaying fluorescence at a high percentage. Such results were not expected as the TD05 aptamer should only be binding to CD20, a cell receptor protein found on lymphoma cells and not glioma cells. We also found the GFAP aptamer caused fluorescence in lymphoma cells which do not express the GFAP protein found intracellularly in glioma cells

These results may be explained by a number of potential confounding factors that are also limitations of this study and data. One hypothesis is that the aptamers have a high amount non-specific binding. The goal of using tRNA found in yeast was to eliminate this confounding outcome but it could explain some fluorescence but seems striking to explain the high false-positive percentage. We also hypothesize that the staining protocol might require further refinement, specifically the annealing step as this seems to be the most vital step. We had utilized the same annealing process as described in our previous studies with the expectation of achieving the same results[4, 11]. However, after further discussions with the 3rd party company that provided the aptamer, it may be useful to conduct a gradual temperature change for annealing. For example, annealing the aptamers at 90C for 5 minutes, then 60C for 5 minutes, and finally 23C for 5 minutes before storing on ice. Furthermore, our cell lines are a number of years old, some as old as 2014 and could have mutated to the point that the cell types express abnormal or atypical proteins that may bind to aptamers. We have also considered the possibility that Cy5 fluorophore has been noted to be taken up by mitochondria in any cell type and could be confounding some of our results [14]. Our limitations certainly do not end there. We would ideally like to test our aptamers against the gold standard of IHC and furthermore, would best test our assay on fresh biopsied tissue as a future experiment. Future directions also include performing “apto-histochemistry” to visualize our results and see the proper cells being labeled under the microscope.

Had our results been as we anticipated, this work aims to provide patients and physicians with the ability to accurately and precisely diagnose brain tumors intraoperatively to provide reassurance that the treatment modality is the best for the patient and avoid unnecessary increased morbidity and mortality. As of now, these aptamers are not ready to provide such capabilities until more data and validation is acquired. There is a difference between clinical and statistical significance here as well. Flow cytometry data provides subjective, near clinical significance whereas true statistical significance requires head-to-head comparisons of aptamer use vs IHC use and developing equivalent sensitivity and specificity results before this technology may be used with confidence. Ultimately, this is the beginning stages of utilizing aptamers for diagnosing intraoperative brain tumors and the potential for

such disruptive diagnostic assays first requires further data and research.

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