

Is it possible to detect surface antigen CD133 on patient-derived glioblastoma continuous cell cultures using fluorescent aptamers?

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ABSTRACT

Theranostics combines diagnostics and therapeutic exposure. Regarding glioblastomas, theranostics solves the problem of detecting and destroying tumor stem cells resistant to irradiation and chemotherapy and causing tumor recurrence. Transmembrane surface antigen CD133 is considered as a potential marker of tumor stem cells.

Objective. To detect CD133 in patient-derived glioblastoma continuous cell cultures using fluorescence microscopy and modified aptamers (molecular recognition elements) anti-CD133.

Material and methods. To detect CD133, we used mouse fluorescence monoclonal antibodies anti-CD133 MA1-219, FAM-modified DNA aptamers anti-CD133 AP-1-M and Cs5. Non-aptamer DNA oligonucleotide NADO was used as a negative control. Detection was performed for three samples of patient-derived glioblastoma continuous cell cultures coded as 1548, 1721 and 1793.

Results. MA1-219 antibodies brightly stained cell culture 1548, to a lesser extent — 1721. There was diffuse staining of cell culture 1793. Cs5-FAM aptamer stained cells in a similar way, but much weaker. AP-1-M-FAM aptamer interacted with cells even weaker and diffusely stained only cell culture 1793. Non-aptamer NADO did not stain cell culture 1548 and very weakly diffusely stained cell culture 1793.

Conclusion. For both molecular recognition elements (MA1-219 antibody and Cs5 aptamer), 3 cell culture samples can be arranged in the following order possibly reflecting CD133 status decrease: strong signal for cell culture 1548, much weaker for 1721, even weaker for 1793. Only cell culture 1548 can be considered CD133 positive with combination of Cs5+ and NADO signals. Cell culture 1793 is CD133 false positive with combination of Cs5+ and NADO+ signals.

Keywords: glioblastoma, CD133, tumor stem cells, fluorescence aptamers, antibodies.

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TO CITE THIS ARTICLE:

Moiseenko VL, Antipova OM, Pavlova SA, Pronin IN, Pavlova GV, Kopylov AM. Is it possible to detect surface antigen CD133 on patient-derived glioblastoma continuous cell cultures using fluorescent aptamers? *Burdenko's Journal of Neurosurgery = Zhurnal voprosy neirokhirurgii imeni N.N. Burdenko*. 2024; 88(1):56–62. (In Russ.). <https://doi.org/10.17116/neiro20248801156>

Abbreviations

CD133 — transmembrane protein, surface antigen

TSC — tumor stem cells

GB — glioblastoma

CHO-K1 — Chinese hamster ovary cell

Cy2 — cyanine-2, fluorescent dye

FAM — fluorescein amidites

FBS — fetal bovine serum

DMEM/F12 — Dulbecco's modified eagle medium with addition of F12 medium in a 1:1 ratio

PBS — phosphate buffer solution, pH=7.4

DAPI — 4',6-diamidino-2-phenylindole, fluorescent dye in blue spectrum staining nuclear DNA

Introduction

The CD133 protein was first identified a quarter of a century ago in two independent studies of mouse neuroepithelial cells and human hematopoietic stem cells [1, 2]. CD133 is found in the neuroepithelium and localized

in microvilli on the apical surface of various epithelial cells, including ependymal layer of the brain [1]. The second study verified CD133 as a marker of hematopoietic stem and progenitor cells in bone marrow and blood [2]. Functions of CD133 are still undefined. Nevertheless, this membrane protein on plasma membrane protrusions and human CD133 gene mutation preventing its exposure on cellular surface led to proposal that CD133 is an organizer of plasma membrane protrusions [3].

The human CD133 gene is located on chromosome 4 and contains at least 37 exons [4]. Gene transcription is determined by five tissue-specific alternative promoters resulting different splicing isoforms of mRNA [5]. There are several splice variants of CD133 with alternative cytoplasmic C-termini [6]. The CD133 protein contains five transmembrane domains, two large glycosylated extracellular domains of ~250 amino acid residues in size and two intracellular loops of ~30 amino acid residues in size [7].

According to one assumption, CD133 may be a marker of normal stem cells [8]. CD133 is expressed in various stem cells, but its role is unknown. Perhaps, CD133 may be involved in key functions of stem cells (self-renewal and multipotent differentiation). Although the role of this protein is unclear, association of CD133 with the Wnt and Notch signaling pathways suggests its ability to induce cell proliferation [9, 10]. Tumors contain stem-like cells (the so-called tumor stem cells). Perhaps, CD133 can be used as a candidate biomarker for TSCs [11]. TSCs are of particular interest because they are capable of initiating tumor growth. Over the past two decades, the authors found TSCs in many solid tumors including GB [12]. Chemotherapy and radiotherapy are not thought to be effective in killing TSCs. Therefore, detection and targeted suppression of TSCs may be considered as a future therapeutic strategy. CD133 appears to be a good candidate for such targeted eradication of TSCs.

Currently, antibodies are used as molecular recognition elements for the CD133 marker. The first derived antibodies to CD133 (AC133 and AC141, commercially available as CD133/1 and CD133/2, respectively) recognize different epitopes. Both antibodies recognize glycosylated epitopes [13]. In addition to AC133 and AC141, antibodies recognizing unmodified extracellular epitopes

of CD133 are commercially available. There were attempts to obtain antibodies to CD133 by immunizing mice with transfected CD133-expressing human cells [14].

According to available data on the properties of CD133-positive and CD133-negative cells derived from human GB, we can assume at least two types of TSCs differing in expression of CD133 and/or presence of CD133 epitopes (epitope-positive and epitope-negative for CD133) [15].

Antibodies are the classic option for clinical cytological analysis [16]. Recognition of the CD133 protein by antibodies, especially the AC133 epitope, depends on functional state of the cell (for example, alternative glycosylation masks this epitope preventing recognition) [17]. Therefore, detection of the CD133 protein by aptamers may be an alternative to antibodies.

Aptamers (chemical antibodies) are short oligonucleotides binding to their molecular targets with high affinity and specificity. Aptamers including DNA aptamers Ap1M [18] and Cs5 [19] were selected for cells expressing CD133. Aptamers are superior to antibodies in some parameters (available chemical synthesis, higher thermal stability, simpler quality control and injection of fluorescent labels into aptamers).

The purpose of the study was to detect CD133 in patient-derived glioblastoma continuous cell cultures using fluorescence microscopy and modified aptamers (molecular recognition elements) anti-CD133.

This approach will significantly simplify detection of CD133 for cytological and subsequent histological examination of human GB samples. Specialists can use this method as an alternative/supplement to standard immunocytochemistry with fluorescently labeled antibodies.

Material and methods

Reagents

Nucleotide sequences of aptamers are presented in **table**. Lyophilized FAM-modified oligonucleotides were synthesized and purified by the GenTerra JSC (Russia). Solutions of oligonucleotides were prepared in phosphate-buffered saline with addition of 5 mM MgCl₂.

Nucleotide sequences of oligonucleotides.

No.	Cipher	Nucleotide sequence 5'-3'	Length, units	Target for selection	Reference
1	Ap1M	TACCAGTGCCGTTTCCCC GGAGGGTCACCCCTGAC GCATTCCGGTTGAC	48	CD133 transfected HEK293T	[18]
2	Cs5	TTACATCGAGTGGCTT ATAAAGTAGGCGTAGGG CTAGGCGGAGAGATGTAA	51	CD133 transfected CHO-K1	[19]
3	NADO	CATTTAGGACCAACACAA	18		

Note. NADO — non-aptamer oligonucleotide.

We used mouse monoclonal antibodies to CD133 (MA1-219, Invitrogen, USA). Secondary antibodies to mouse immunoglobulin contained a Cy2 tag (Jackson ImmunoResearch, UK).

Cultivation of continuous GB cell cultures

We used continuous GB cell cultures derived from patients after resection. Culture samples were obtained in laboratory of molecular cellular neurogenetics of the Burdenko Neurosurgery Center and coded by numbers 1548, 1721 and 1793. Cells were cultured in a humid atmosphere containing 5% CO₂ at 37°C in DMEM/F12 medium (PanEco, Russia). The last one contained 1% L-glutamine, 10% FBS and 0.1% antibiotic-antimycotic (Biowest, France).

Fluorescence microscopy

We seeded cell cultures onto 6-well plates (10,000 cells per a well) and incubated in growth medium and 5% CO₂ for 24 hours at 37 °C. Aptamer solution at a concentration of 1 μM in 50% phosphate buffer with addition of 5 mM MgCl₂ and 50% growth medium was added to the cells. After that, we incubated cells for 30 min at 4 °C, washed and fixed by 4% formaldehyde. Cellular nuclei were stained by DAPI solution. Final cells were stored in PBS buffer at +4 °C.

To test human GB cell cultures with antibodies, we preliminarily fixed cells by 4% formaldehyde and permeabilized cell membrane by 0.1% Triton X-100 solution. After that, we added 3% BSA solution to the cells and incubated for 2 hours at +4 °C. Cells were kept in solution of antibodies to CD133 MA1-219 (dilution 1:50) overnight at +4 °C, washed and then incubated with secondary antibodies and Cy2 fluorescent label at a dilution of 1:100. Cellular nuclei were stained by DAPI solution.

We visualized the samples using NIB 920 fluorescence microscope (NexCope, China), as well as 485- and 365-nm filters to detect green fluorescence of FAM tag or Cy2 tag and DAPI fluorescence. Signals were processed using ImageJ (NIH, USA).

Results

We incubated tumor cells for 30 min at 4°C to avoid internalization of aptamer-CD133 complexes into the cell. Non-aptamer DNA oligonucleotide NADO with a length of 18 nucleotides was used as a control for CD133-independent interaction with cells. The figure demonstrates microphotographs after incubation of continuous GB cell cultures with fluorescently labeled antibodies and oligonucleotides.

Incubation of GB cell cultures from patients 1548, 1721 and 1793 with antibodies to CD133 (fragment of re-

combinant protein, amino acids 20–208) and subsequent incubation with secondary antibodies with fluorescent Cy2 label leads to intense staining of all GB cell cultures (**figure a–c**). The most intense luminescence of label is observed on GB cell cultures from patient 1548.

The DNA aptamer FAM-Cs5 demonstrated results similar to the data for antibodies when interacting with continuous GB cell cultures. After staining the GB cell culture 1548 by FAM-Cs5 aptamer, overlay of micrographs obtained in fluorescence channels of aptamer label and DAPI allows us to note that fluorescence signal of FAM-Cs5 aptamer coincides with signal for cell nuclei (**figure d**). Green signal is weaker in case of FAM-Cs5 aptamer and 1721 cell culture (**figure e**). Signal is even weaker for cell culture 1793 (**figure f**), and staining with Cs5 aptamer has no clear boundaries.

The DNA aptamer FAM-Ap-1-M does not interact with cells of cultures 1548 and 1721 as evidenced by the absence of green staining of cells in figures g and h. When superimposing the signals obtained in fluorescence channels of FAM-AP-1-M aptamer and DAPI for 1793 cell culture, we found no clear boundaries of cell staining. This may be due to low presence of CD133 on cell surface or internalization of FAM-Ap1M aptamer into the cell (**figure i**).

The non-aptamer DNA oligonucleotide FAM-NADO did not stain continuous GB cell culture 1548 (**figure j**). There was a green fluorescence signal of FAM-NADO for cell culture 1721 (**figure k**). The brightest fluorescence was observed after staining the primary GB cell culture 1793 by FAM-NADO (**figure l**). At the same time, there are no clear boundaries of cell staining in the microphotographs.

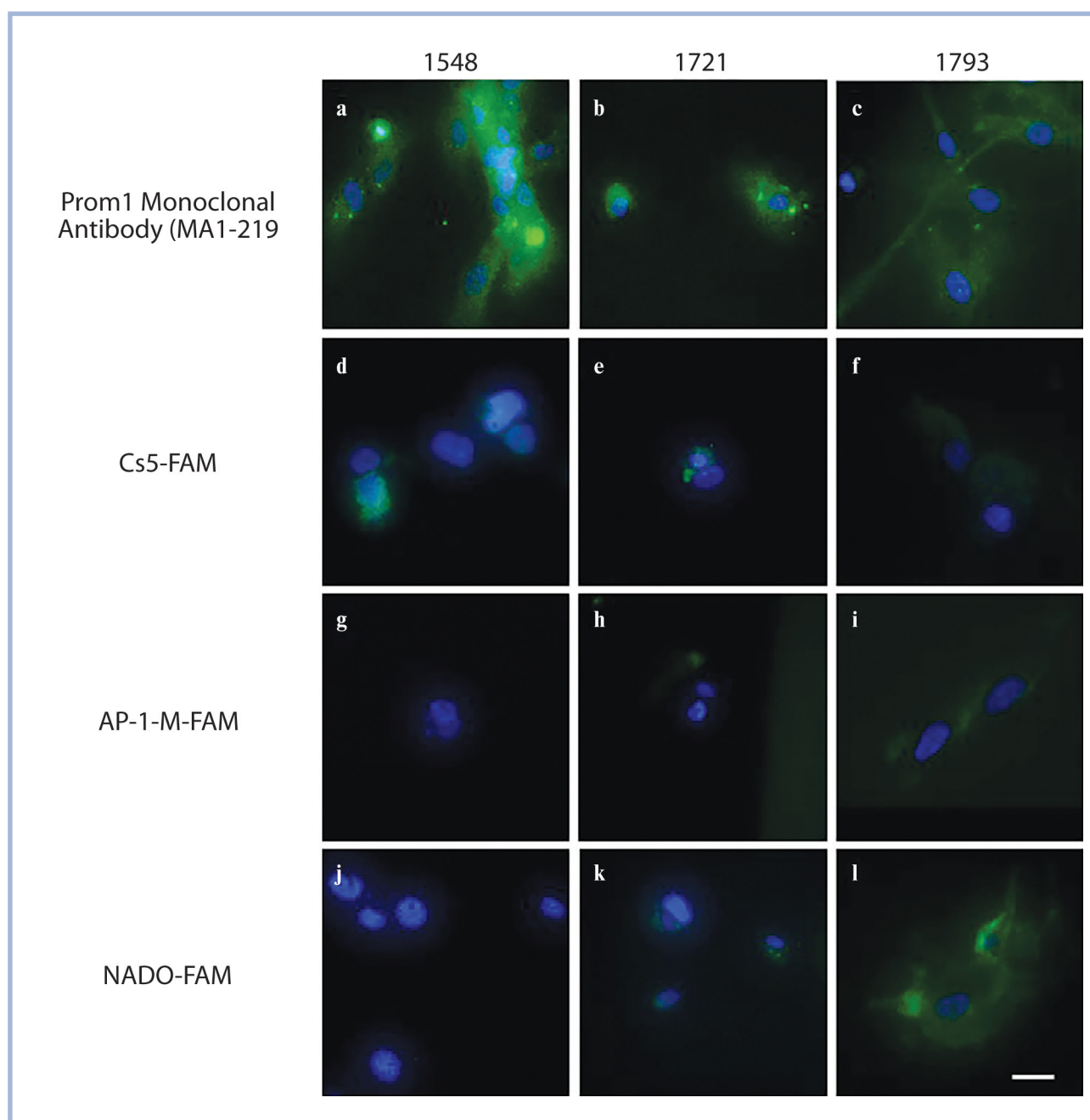
Discussion

Analysis of intraoperative tumor samples is an important stage for postoperative diagnosis of GB. Accurate diagnosis of tumor is impossible without cytological and histological examination. Biopsy regarding various markers determines certain therapy. It is extremely difficult to detect poorly represented markers and/or rare cells. TSCs and their possible marker CD133 are this example [20]. The first step for aptamers in diagnosis of markers in specimens is testing of transplantable tumor cultures with different CD133 expression.

We analyzed the value of fluorescence aptamers against CD133 for detection of tumor marker on continuous GB cell cultures.

MA1-219 antibodies are a clone of mouse antibodies 2F8C5 with a fragment of CD133 protein expressed in bacteria without glycosylation as an antigen. Therefore, these antibodies can lead to inadequate results on human cells. Antibody staining is performed on fixed and permeabilized cells to make all CD133 protein molecules accessible.

In this study, the aptamers used were selected for CD133-expressing eukaryotic cells. The Ap1M ap-



Staining the GB cell cultures of patients 1548 (a, d, g, j), 1721 (b, e, h, k) and 1793 (c, f, i, l) by fluorescently labeled antibodies and aptamers. Green — fluorescence of Cy2 or FAM label, blue — cell nuclei stained by DAPI dye. a, b, c — interaction of anti-CD133 antibodies; d, e, f — interaction of FAM-Ap1M aptamer; g, h, i — interaction of FAM-Cs5 aptamer; j, k, l — interaction of non-aptamer oligonucleotide FAM-NADO. Scale bar = 20 μ m.

tamer is a truncated version of the AP-1 aptamer selected for CD133-expressing transfected HEK293T cells [19]. Interestingly, the AP-1-M aptamer demonstrated higher affinity to CD133-expressing FRO anaplastic thyroid cancer cells than its precursor AP-1 aptamer. Dissociation constant decreases by approximately 3 times when the Ap1-Ap1M nucleotide sequence is shortened (288 vs. 101 nM). Importantly, it is more correct to use the term “half-saturation concentration” for cells instead of “dissociation constant”.

Other authors selected DNA aptamers for transfected Chinese hamster CHO-K1 cells expressing the human

CD133 protein [20]. In this study, Cs5 aptamer was obtained via C5 aptamer truncation. Interaction of Cs5 with transfected CHO-K1-CD133+ cells was characterized by dissociation constant of 16.3 ± 6.8 nM. Flow cytometry revealed interaction of Cs5 aptamer with various cells expressing CD133 (human colorectal carcinoma HCT116 cells, HT29 colorectal carcinoma, HCT8 colorectal adenocarcinoma, A549 lung carcinoma) and no binding with control CD133-minus CHO-K1 cells [20].

In our study, MA1-219 antibodies stained all cells of continuous GB cell cultures.

Aptamers stain samples differently. The FAM-Cs5 aptamer stains cells similarly to antibodies, but noticeably weaker. The FAM-Cs5 aptamer is internalized into cells of culture 1548, and label signal coincides with localization of cell nuclei. The Ap-1-M aptamer does not stain cells. This is consistent with its high half-saturation concentrations. The non-aptamer NADO oligonucleotide stained 1793 cells diffusely similar to antibodies and Cs5 aptamer. Perhaps, staining may be due to interaction of NADO with cell membrane in the absence of a target for NADO. We have previously observed this phenomenon for other GB cell cultures [21].

Conclusion

Fluorescence FAM-DNA aptamers to CD133 are capable of staining continuous GB cell cultures that may be detected by fluorescence microscopy. Continuous GB cell cultures are capable of internalizing aptamers, apparently through endocytosis. Specific properties of continuous cell culture 1793 is a special case of apparently active endocytosis, since 1793 cells are capable of capturing both antibodies and any oligonucleotides, including non-target ones.

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Thus, positive results of aptamer-assisted diagnosis of GB cell culture imply fluorescence signal only with aptamer oligonucleotide. There should be no signal with non-aptamer oligonucleotide.

Fluorescence DNA aptamer Cs5 meets these criteria. Therefore, we can apply this oligonucleotide for aptamer-assisted diagnostics of GB using cytochemistry for CD133 antigen.

Author contribution:

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The study was supported by a grant from the Ministry of Science and Higher Education of the Russian Federation (agreement No. 075-15-2021-1343 dated October 4, 2021).

No conflict of interests to declare.

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Received 02.10.2023

Accepted 17.11.2023