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Comparison of the effect of DNA aptamers U31, GR20, GR200 and their combination with radiation therapy on the viability of human glioblastoma cells G22 and Sus\fP2

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ABSTRACT

Aptamers can increase human glioblastoma cell death rate after exposure to radiotherapy. Cell cultures derived from human glioblastomas allow for selection of effective combined therapy.

Objective. To analyze viability of human glioblastoma cell cultures exposed to EGFR-specific aptamers U31, GR20, and GR200 compared to combination of these aptamers and radiotherapy (20 Gy).

Methodology. G22 and Sus\fP2 cell cultures were harvested from tumor tissue. They were exposed to aptamers (10 μM, 72 hours) and irradiated once with 20 Gy. We analyzed viability using flow cytometry.

Results. In G22 cell culture, aptamer U31 reduces apoptosis by 45.8%, but increases necrosis by 67.5%, while GR20 and GR200 increase two types of death (apoptosis and necrosis). In Sus\fP2 culture, all three aptamers significantly increased apoptosis while proportion of necrosis decreased. The most significant decrease in the number of necrotic cells was observed for aptamer U31 (by 77.7%). Radiotherapy combined with aptamers was followed by the following effects for G22 cell culture: U31 and GR200 enhance apoptosis (by 59.4%) and necrosis (by 57.7%), aptamer GR20 increases necrosis by 28.3%. In Sus\fP2 culture, U31 aptamer enhances apoptosis by 132.9% and necrosis by 52.9%, GR20 — 125.4% and 17.7%, GR200 — 27.4% and 30.6%, respectively.

Conclusion. Aptamers have different effects on glioblastoma cell cultures. For Sus\fP2, a combination of radiotherapy with GR20 aptamer is optimal. Either a single application of U31 aptamer or combination of radiotherapy and GR200 aptamer is optimal for G22 cell culture. This result demonstrates the importance of combined therapy and preliminary studies in cell cultures for individualized approach to each CNS tumor.

Keywords: *glioblastoma, DNA aptamer, viability, radiation.*

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Abbreviations

GB — glioblastoma
EGFR — epidermal growth factor receptor
MeV — mega-electron volt
AV — annexin V
PI — propidium iodide
HGG — high-grade glioma

Introduction

Grade IV glioblastoma (GB) is a common primary malignant high-grade brain tumor. It occurs in adults and has a poor prognosis. Despite modern knowledge about genetic characteristics of GB, there is only a little progress in the treatment of patients with this fatal disease [1]. Median survival is 12—18 months [2]. Available treatment options are insufficient, and their side effects significantly reduce the quality of life [3]. Radiotherapy is identified as the most important treatment for GB after resection for better survival. Unfortunately, patients with GB often experience resistance to radiotherapy as a major cause of high mortality. Resistance in GB is often multifactorial and heterogeneous, as well as associated with GB recurrence after surgery [4]. A key challenge in improving the diagnosis and treatment of GB is development of targeted imaging and drug delivery platforms penetrating the blood-brain barrier and specifically targeting tumor cells.

Epidermal growth factor receptor (EGFR) is one of the main markers of GB. It is a receptor tyrosine kinase typically activated in cancer, particularly in GB [3]. Ligand binding to EGFR triggers a cascade of reactions influencing cell growth, survival, adhesion, migration and differentiation. This makes EGFR an important target in antitumor therapy [5].

One of the most effective approaches to malignant tumors is molecular agents capable of selective suppression of key functions in malignant cells. Since overexpression of EGFR receptor is typical for many tumors, targeting EGFR and associated signaling pathways represents a promising therapeutic strategy. To date, several specific aptamers targeting EGFR are identified [5]. Unique properties of aptamers demonstrate their potential to fill the gap in successful diagnostic and therapeutic options where antibodies were previously ineffective. Aptamers possess many characteristics that make them ideal for visualizing and treating GB and other malignant brain tumors. Aptamers are likely to provide patients with better medical care and quality of life. Selectivity, easy modification and low immunogenicity make them ideal agents for drug delivery [6]. All these qualities offer hope for enormous potential of aptamers in the treatment of GB. The first and only clinical trial of NOX-A12 aptamer in patients with GB demonstrated perspective results. There were no dose-limiting toxicities or treatment-related deaths [7].

There is an objective to develop a new aptamer capable of specifically recognizing the EGFR receptor with higher binding affinity. This aptamer is interesting for detection and visualization of EGFR-expressing cells, as well as for targeted delivery of therapeutic compounds. To obtain a new variant with better characteristics, GR20 (a truncated analogue of U31 aptamer) was previously created by removing the 5'- and 3'-terminal sequences (1—10 and 57—76) and replacing 16G with 16C. This modification increases length of duplex region from 5 to 8 nucleotide pairs and stabilizes it [8, 9]. Another aptamer (GR200) was based on GR20 via adding two additional GC pairs and single nucleotide substitution in stem region.

Like original aptamer GR20, GR200 demonstrates secondary structures (one with minimal energy and another containing an unfolded short hairpin with three broken AT pairs) [8].

In our study, we used cell cultures obtained from human GB G22 and Sus\fP2 [10—12] rather than linear cells (cell lines). This allows us to obtain results closer to actual impact on tumor.

The objective of the study was to analyze viability of human GB G22 and Sus\fP2 cell cultures exposed to aptamers U31, GR20 and GR200, as well as combined effects of these aptamers with radiotherapy (20 Gy).

Material and methods

Cell cultures

The experiment utilized human brain GB G22 and Sus\fP2 cell cultures obtained from patients at the Burdenko Neurosurgery Center [10—12].

Obtaining cell cultures

A patient-derived tumor sample was placed in a Petri dish with cold Hank's solution (Gibko — Thermo Fisher Scientific Instruments Co., Ltd., USA) to remove vessels, membranes and necrotic tissues, if present. This sample was then minced and transferred to a 15 ml test tube. Hank's solution was removed, and 0.25% trypsin solution (Gibko — Thermo Fisher Scientific Instruments Co., Ltd., USA) was added. After repeated washing, we placed sample on a shaker at 37 °C for 20 min until tissue softening. Next, DMEM/F12 medium and 10% fetal bovine serum (Gibko — Thermo Fisher Scientific Instruments Co., Ltd., USA) were added at a 1:1 ratio followed by centrifugation and removal of liquid. Tissue dissociation was performed in cold Hank's solution by adding it to the tube and resuspending tissue with glass pipette. Final cell suspension was transferred to clean tube. Hank's solution was added again to residual tissue. We filtered cell suspension through a 40-μm nylon cell strainer (Corning Inc., USA) into a 50-ml tube and centrifuged at 1000 rpm for 10 min.

Liquid was removed, culture medium was added, and tissue was plated into flasks for further cultivation.

Cell cultivation

Cell cultivation was performed in DMEM/F12 medium with sodium pyruvate supplemented with 10% fetal bovine serum (Gibko — Thermo Fisher Scientific Instruments Co., Ltd., USA), 1% HEPES solution (Sigma-Aldrich, USA), 1% GlutaMAX solution and 1% penicillin/streptomycin/amphotericin solution (Gibko — Thermo Fisher Scientific Instruments Co., Ltd., USA). Upon subculture, cells were harvested with 0.25% trypsin solution and washed with phosphate-buffered saline (PBS) (Gibko — Thermo Fisher Scientific Instruments Co., Ltd., USA). Trypsin reaction was stopped by adding 1 ml of growth medium. Cells were centrifuged for 5 min at 1000 rpm. We removed liquid, added fresh medium and reseeded cells into new flasks for further cultivation or used in experiment depending on the objective.

Cell culture irradiation and aptamer addition protocol

For experiment, we seeded 1.5×10^5 cells into flasks with 25 cm^2 culture area. For radiological exposure of cells, we used vertical bremsstrahlung on a TrueBeam STx device (Varian Medical Systems, Inc., USA) (nominal energy 6 MeV, 600 doses/min). Cell cultures were irradiated once with a dose of 20 Gy. Seventy-two hours after irradiation, U31, GR20, and GR200 aptamers were added to the culture medium at a final concentration of 10 μM. After addition, we incubated cell flasks for 72 hours in a CB 150 CO_2 incubator (Binder, Germany) at 5% CO2 and 37 °C. Control flasks were supplemented with aptamers without prior irradiation. Untreated cells comprised control.

Flow cytometry

After incubation, we removed cells with trypsin solution, washed with PBS and resuspended in 500 μl of 1× binding buffer. Cells were stained with 5 μl of anti-annexin V-FITC (AV) and 5 μl of propidium iodide (PI) at room temperature in the dark for 15 min. Samples were analyzed using flow cytometry (CytoFLEX S, Beckman Coulter, Inc., USA).

Results

Patient-derived glioblastoma cell cultures

Experiments were based on two cell cultures harvested from patients with human supratentorial GB: G22 and Sus\fP2 **(Table)** [10—12].

GB cell culture Sus\fP2 **(Fig. 1)** was harvested from a 60-year-old patient. Headache and amnestic disorders developed over a two-week period. MRI revealed extensive lesion in the left temporal lobe, and subtotal resection was performed. Sanger sequencing revealed the wild-type IDH1/2 [12].

GB cell culture G22 [10, 11] **(Fig. 2)** was harvested from a 36-year-old patient with continued tumor growth

Patient-derived glioblastoma cell cultures

Cell culture	Glioma, Grade	IDH1 status	Gender	Age, years	Localization	References
Sus/fP2	IV	Wild type	F	60	Left temporal lobe	[10—12]
G22	IV	Wild type	F	36	Left frontal lobe, corpus callosum, interventricular septum	[10, 11]

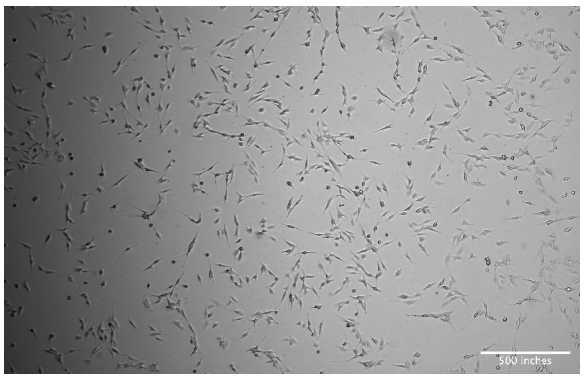


Fig. 1. **Sus/fP2 human glioblastoma cell culture.**

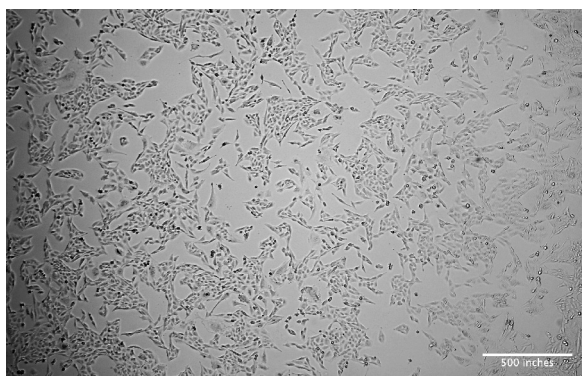


Fig. 2. **G22 human glioblastoma cell culture.**

in the left frontal lobe extending into the corpus callosum and interventricular septum. MRI revealed focal lesion, and subtotal resection was performed. Sanger sequencing revealed the wild-type IDH1/2.

Assessing the effect of U31, GR20 and GR200 aptamers on viability of human glioblastoma cell cultures G22 and Sus\fP2

In G22 human glioblastoma cell culture, proportion of viable cells decreased depending on aptamer added to culture medium (by 31.2% for U31, 41.3% for GR20, and 25.7% for GR200) compared to control cells (G22 without aptamer). Analysis of glioblastoma cell death type using flow cytometry revealed that GR20 and GR200 aptamers in G22 cells increased apoptosis and necrosis. About 88.7% and 55.7% of cells entered necrotic state after use of aptamers GR20 and GR200, respectively. Apoptotic cell death also increased. Aptamer U31 increased necrotic tumor cell death by 67.5%, but significantly reduced apoptotic capacity of glioblastoma cells by 45.8%. The last one is a negative feature **(Fig. 3)**.

In human Sus\fP2 cell culture, all three aptamers significantly increased the number of apoptotic cells and simultaneously decreased proportion of necrotic cells **(Fig. 4)**. The most significant reduction in the number of necrotic cells was observed after addition of aptamer U31 (by 77.7%).

Aptamer GR20 was the most attractive antiproliferative agent. Indeed, its effect on G22 cells increased apoptosis and necrosis, while its effect on Sus\fP2 cells significantly increased apoptosis (650%) and decreased necrosis by 24.7%.

Aptamer GR200 had the least effect on G22 culture compared to other aptamers. This aptamer increased necrosis, in Sus\fP2 culture — increased apoptosis (more than 10-fold) and reduced the number of necrotic cells by 33.1%.

Thus, cultures responded differently to the treatment. Necrosis increased in G22 culture regardless of aptamer, while apoptosis predominated in Sus\fP2 culture. Since aptamers were considered as an additional therapeutic agent enhancing the effectiveness of primary therapy, the next experiment was designed to evaluate the effect of aptamers combined with a single irradiation dose (20 Gy) on viability of human glioblastoma cells.

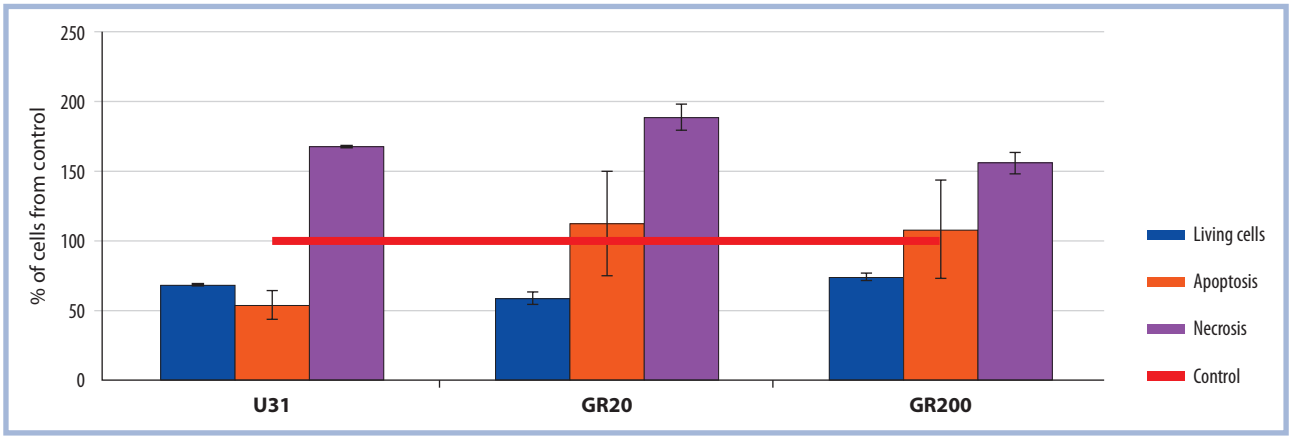


Fig. 3. **Viability of human glioblastoma G22 cell cultures exposed to U31, GR20 and GR200 aptamers.**

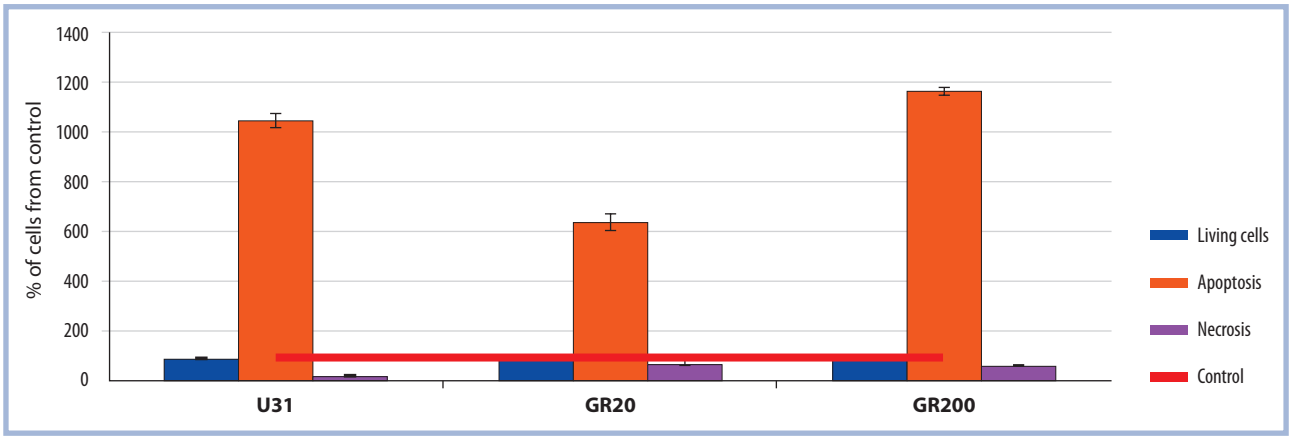


Fig. 4. **Viability of human glioblastoma Sus/fP2 cell cultures exposed to *U*31, GR20 and GR200 aptamers.**

Assessing the effect of U31, GR20 and GR200 aptamers on viability of G22 and Sus\fP2 glioblastoma cell cultures after a single irradiation (20 Gy)

A monolayer of glioblastoma cells was irradiated with a dose of 20 Gy followed by exposure to one of the aptamers (U31, GR20, or GR200). In G22 glioblastoma cell culture, apoptosis and necrosis increased by 59.4% and 57.7% after a single irradiation (20 Gy) and addition of U31 aptamer. Addition of aptamer GR20 did not change apoptotic state, but increased the number of necrotic cells by 28.3%. Aptamer GR200 increased apoptosis by more than 2 times and necrosis by 38.1% **(Fig. 5)**.

A similar effect of aptamer U31 on Sus\fP2 GB cell culture after irradiation resulted in higher proportion of apoptosis by 132.9% and necrosis by 52.9%. Aptamer GR20 increased the number of apoptotic cells by 125.4% and decreased the number of necrotic cells by 17.7%. After addition of aptamer GR200, apoptosis and necrosis increased by 27.4% and 30.6%, respectively **(Fig. 6)**.

Discussion

Radiotherapy is still the most common treatment for many tumors. Approximately 50% of all patients receive radiotherapy that accounts for 40% of all treatments [13]. However, resistant tumor cells remain after radiotherapy and lead to aggressive recurrence. Clearly, there is a need to find molecules that are less toxic to non-pathological cells and higher effectiveness of tumor cell targeting. Combined therapy is becoming popular. Aptamers are an ideal candidate for targeting glioblastoma due to highly structured nature, small size, and ability to penetrate blood-brain barrier and target brain tumors [14]. We used aptamers specific to EGFR receptor (U31, GR20, and GR200) [8, 9].

Flow cytometry revealed differential effects of U31, GR20 and GR200 aptamers on viability of human glioblastoma G22 and Sus\fP2 cells. Similar differences were also observed in case of combined effect of aptamers after a single irradiation of cells. In G22 cell culture, addition of U31 aptamer decreased proportion of viable cells and apoptosis, but increased the number of necrotic cells.

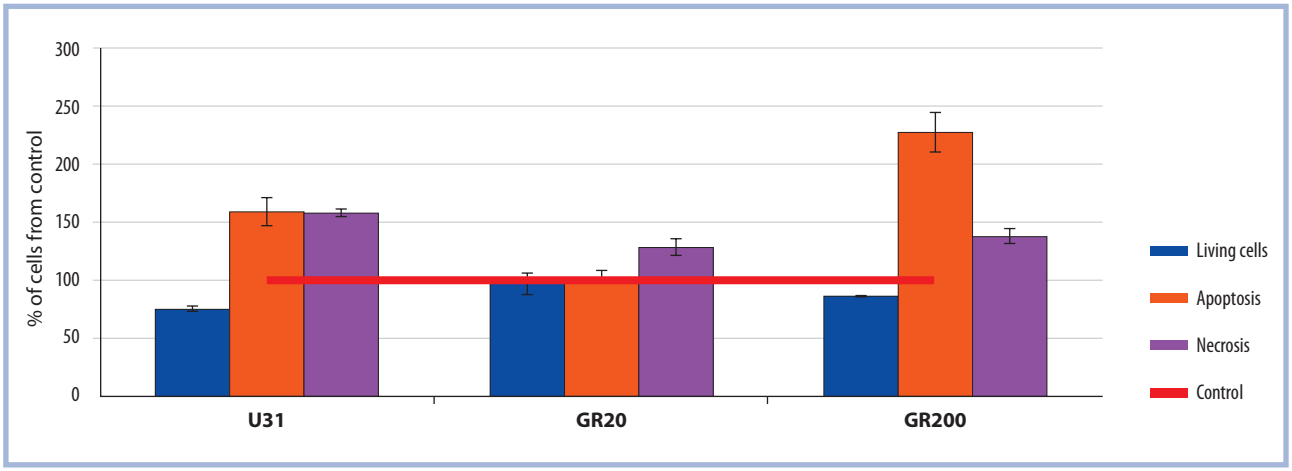


Fig. 5. **Viability of human glioblastoma G22 cell cultures irradiated with a dose of 20 Gy followed by addition of *U*31, GR20 and GR200.**

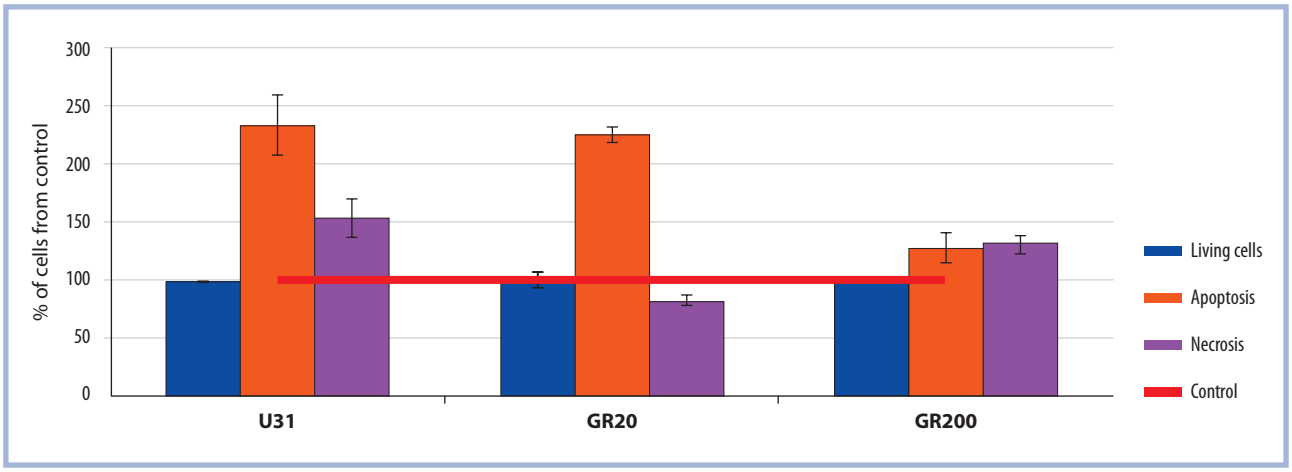


Fig. 6. **Viability of human glioblastoma Sus/fP2 cell cultures irradiated with a dose of 20 Gy followed by addition of *U*31, GR20 and GR200.**

Unlike U31, GR20 and GR200 aptamers enhanced necrotic and apoptotic processes. In Sus\fP2 cell culture, addition of U31, GR20 and GR200 aptamers significantly increased the number of apoptotic cells in all samples, while proportion of necrosis decreased. The most significant reduction in the number of necrotic cells was observed after addition of U31 aptamer. However, GR20 and GR200 aptamers exerted optimal effects in stimulating cell death via favorable apoptotic pathway.

When G22 cell culture was exposed to irradiation and aptamers, the following effects were observed. Combination of radiation with U31 or GR200 increased intensity of apoptosis and necrosis. Meanwhile, combination of irradiation with GR20 had no significant effect on content of apoptotic cells but increased proportion of necrotic cells.

When Sus\fP2 cell culture was exposed to irradiation and aptamers, the greatest effectiveness was demonstrated after addition of GR20 aptamer. It not only increased apoptosis by more than 2 times but also reduced the level of necrosis compared to other aptamers. Different tumor cell responses in GB patients highlight the role of therapeutic agents based on cell cultures of certain tumors. These cell cultures underwent a small number of passages and have heterogeneous profile similar to original tumor. Uniform cell lines, such as U87 or U251, to search for new therapeutic modalities may yield perspective results in vitro and in vivo, but lead to failure in phase II clinical trials due to significant differences between tumors and cell lines.

An equally important result is the need for individual approach to each tumor.

Personalized medicine will significantly improve therapeutic effects on tumors. For example, radiotherapy combined with GR20 aptamer is optimal for Sus\fP2 tumor, while G22 tumor responds optimally to either U31 aptamer alone or combination of radiotherapy and GR200 aptamer.

Conclusion

Aptamers exert different effects on patient-derived glioblastoma G22 and Sus\fP2 cell cultures. Radiotherapy combined with GR20 aptamer is optimal for Sus\fP2 tumors, while G22 tumors require either U31 aptamer alone or combination of radiotherapy and GR200 aptamer. This result demonstrates the importance of combined therapy (aptamers and radiotherapy), as well as the role of preliminary studies on patient tumor cell cultures for individual approach for each central nervous system tumor.

Author contribution:
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Statistical analysis — Sliman Ya.A., Pavlova S.A.
Writing the text — Sliman Ya.A., Pavlova S.A., Pavlova G.V.
Editing — Pavlova G.V.

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The authors declare no conflicts of interest.

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COMMENT

The authors present important data on changes in human glioblastoma cell cultures. Perspective effects of new drugs (DNA aptamers U31, GR20 and GR200) and their combination with radiotherapy on glioblastoma cells are demonstrated. This experimental study is of great importance for development of new treatments for this highly dangerous disease. Patient-derived cell cultures are particularly valuable. Study design is adequate to objective and allows for well-founded conclusions about feasibility of combined therapies for glioblastoma. References reflect current state of the problem and allow for assessment of results. The abstract is clearly structured and adequately reflects the essence of the study. As a recommendation, I would advise the authors to include the approximate number of cells used for flow cytometry.

Revishchin A.V. (Moscow)