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NANOBIOLOGY, GENETICS, AND OMICS TECHNOLOGIES

New Gliobastoma Cell Lines: Analysis of Genetic Changes, and Assessment of Sensitivity to Radiotherapy and Immunotherapy

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Abstract—The characteristics of six new glioblastoma cell lines obtained from tumor material from patients are presented. The studied glioblastomas do not have mutations in the genes *IDH1* and *IDH2*, which indicated a poor prognosis for their therapy. Two glioblastomas carry the pathogenic mutation p.Arg110Pro in the gene *TP53*. All cell lines studied express RNA of tumor suppressor and oncogenic isoforms of the p73 protein. The glioblastomas responded differently to radiotherapy, with five of them being more resistant to γ irradiation than the standard A172 glioma line. All six cell lines express RNA genes for vascular endothelial growth factor and its receptor (VEGFR-1) in different ratios. Testing of an immunotherapeutic regimen with monoclonal antibodies to VEGFR-1 on one of the cell lines confirms that the studied glioblastomas are sensitive to blocking vascular growth factor and its receptor. Thus, these glioblastomas can become a promising model for studying the formation of tumor-cell resistance to radiotherapy and the effectiveness of immunotherapy that blocks growth factors and their receptors.

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INTRODUCTION

Glioblastomas are the most common and aggressive grade IV (highest) primary brain tumors that are difficult to treat [1, 2]. According to the 2021 WHO (World Health Organization) classification, they belong to the adult type of diffuse gliomas with non-mutated genes *IDH* [3]. Glioblastomas do not have clear boundaries and penetrate deeply into neighboring tissues. It is almost impossible to completely remove them surgically; therefore, complex approaches are used for treatment, combining surgery, radiotherapy, and chemotherapy with alkylating agents [4]. However, despite aggressive treatment, tumor recurrences occur in 80% of cases; moreover, resistance to therapy often develops, which makes these tumors de facto incurable [5].

Glioblastomas have a high degree of heterogeneity. They can vary significantly between patients; moreover, the cell populations that make up the tumor themselves are not homogeneous and can have significant differences in morphology, phenotype, and genetic characteristics [6]. Obtaining cell lines from tumor biopsies makes it possible to study in detail the phenotypic, biochemical, and genetic characteristics of various glioblastomas, to identify molecular markers, on the basis of which in the future it will be possible to select more specific, perhaps even individual, treatment regimens for brain tumors.

One of the most important molecular markers for gliomas, which allow determination of the prognosis of the disease and a treatment strategy to be chosen, is the status of isocitrate dehydrogenases *IDH1* and *IDH2* [7, 8]. Isocitrate dehydrogenase is an enzyme that catalyzes the conversion of isocitric acid to α-ketoglutaric acid within the tricarboxylic-acid cycle. *IDH* is involved in many metabolic processes, such as signal transduction, lipid synthesis, oxidative stress, and oxidative respiration. Mutations in genes *IDH1* and *IDH2* are found in 70–80% of diffuse astrocytomas of II–III degree of malignancy, oligodendrogliomas, and sec-

ondary glioblastomas, but are almost never found in primary glioblastomas [9].

Another marker that determines the nature of the development of gliomas and their response to therapy is the status of the p53 transcription factor [8]. The p53 protein is the main "guardian of the genome", which is activated in response to hypoxia, oxidative stress, hypothermia and hyperthermia, DNA damage (including that which occurs during radiotherapy), a decrease in the nucleotide pool, activation of oncogenes and causes apoptosis, cell-cycle arrest, and the suppression of neoangiogenesis. The largest number of mutations leading to dysfunction of the p53 protein is located in the "zinc finger": a DNA-binding domain encoded by exons four to eight. The majority of mutations are concentrated between amino acids 125–300, the main part of which leads to the replacement of one amino acid with another, which in turn causes a change in the structure of the protein and limits its functionality [10, 11]. As a tumor suppressor, p53 has also been well studied in gliomas [9]. It has been shown that in glioblastomas *TP53* mutations are observed much less frequently than in other types of grade-IV gliomas [12]. However, in 78% of cases of glioblastomas, there are violations, if not in the p53 protein itself, then in the pathways of its regulation [13].

The p73 protein is a homolog of p53 and regulates the expression of a number of common target genes, but mutations in the *p73* gene are rare (1% of cases) [14]. In many tumors, overexpression of the p73 protein is even observed [15]. From the *p73* gene both full-length (tumor suppressor) and truncated (oncogenic) isoforms can be expressed [16]. It was shown that truncated isoforms can suppress the transcriptional activity of both TAp73 with complete transactivating domain and p53, forming heterotetramers with them and competing for DNA binding sites. Thus, the fate of a cell depends on the ratio of the amounts of these isoforms. [17]. The p73 protein may play an important role in the differentiation of glioblastomas [18]. It has been shown that there is a correlation between the RNA levels of the TAp73 and ΔNp73 isoforms, tumor malignancy, and patient survival [19]. Thus, p73 can be considered as a potential biomarker of glioblastoma malignancy.

A modern approach to the treatment of glioblastomas is immunotherapy, which is aimed at blocking growth factors and their receptors with synthetic antibodies. Gliomas are among the most vascularized and edematous tumors because they express high levels of vascular endothelial growth factors (**VEGF**) [20]. Antiangiogenic therapy has long been used in the treatment of gliomas. For example, bevacizumab (trade name Avastin), a humanized monoclonal antibody against VEGF, has demonstrated therapeutic benefits in many patients with glioblastomas when used alone or in combination with other therapies [21]. Vascular endothelial growth factor receptor-1 (**VEGFR-1**) is a tyrosine-kinase receptor that binds to ligands of the VEGF family [22]. In addition to expression in the tumor endothelium, VEGFR-1 is found on the surface of tumor cells themselves and cells of the tumor microenvironment. Blocking VEGFR-1 leads to the suppression of proliferation and increased apoptosis of tumor cells, the reduction of vascularization of the tumor node, and the suppression of tumor invasion and metastasis [23]. Moreover, in nontumor cells, VEGFR-1 is indirectly involved in angiogenesis and its blocking with antibodies is not toxic to the patient's body [24].

In this study, we genetically characterize new glioblastoma cell lines, as well as assess their sensitivity to radiotherapy and immunotherapy with monoclonal antibodies that bind the growth factor VEGF-A and its receptor VEGFR-1.

EXPERIMENTAL

Cell cultures. The cell line A172 (glioblastoma) was obtained from the collection of the Institute of Cytology, Russian Academy of Sciences (Russia); the glioblastoma cell lines Gl–R, Gl–Tr, Gl–L, Gl–Sh, Gl–C, and Gl–F were obtained in the Laboratory of Cell Biology (Konstantinov St. Petersburg Institute of Nuclear Physics, National Research Center "Kurchatov Institute", Russia) from tumor tissue of patients. The cells were cultured in complete DMEM-F12 medium (Biolot, Russia) containing 10% fetal bovine serum (HiMedia, India) and 0.5% gentamicin at 37°C in an atmosphere of 5% CO_2. The cell numbers were counted using an automatic cell counter LUNA-II™ (Logos Biosystems, Korea).

DNA extraction, PCR, and sequencing. DNA extraction from a sediment of 1 million cells was carried out using a KR-012 kit (Omnix, Russia) according to the manufacturer's instructions. The DNA concentration in the samples was measured using a NanoDrop One spectrophotometer (Thermo FS, USA). The amplification of DNA gene fragments *IDH-1*, *IDH-2*, *KRAS*, and *TP53* was carried out using PCR. The reaction mixture (60 μL) contained a single Turbo buffer for Taq-HS polymerase, 2.5 units of Taq-HS polymerase (Evrogen, Russia), 250 μM of each dNTP, 300 nM of forward and reverse primers (3'–5' IDH-1: f-gag aat cgt gat gcc acc aa, r-ttg gaa att tct ggg cca tga; IDH-2: f-tct ggc tgt gtt gtt gct tg, r-aga gac aag agg atg gct ag; KRAS: f-ggt cct gca cca gta ata tgc, r-tac gat aca cgt ctg cag tca ac; p53, third–fourth exons: f-cag ccc cct agc aga gac ct, r-ggt gaa gag gaa tcc caa agt tcc; fifth–sixth exons: f-tct cct tcc tct tcc tac agt act cc r-gga ggg cca ctg aca acc ctt; seventh–eighth exons: f-gcc tca tct tgg gcc tgt gtt atc tc, r-tgg tct ctc cac cgc ttc ttg t), and 0.2 μg DNA. The reaction was carried out on a T100 Thermal Cycler (BioRad, USA): 5 min, 95°C (polymerase activation); 35 cycles: DNA denaturation (95°C, 30 s), primer annealing (60°C, 25 s),

and elongation (72°C, 45 s). Visualization of the resulting fragments was carried out using electrophoresis in a 6% polyacrylamide gel, followed by staining with ethidium bromide. The resulting DNA fragment was purified from low-molecular-weight fragments and enzyme residues using the Cleanup Standard kit (BC022S, Evrogen, Russia) according to the manufacturer's instructions. Sanger sequencing was performed on a Nanofor 05 genetic analyzer (Institute for Analytical Instrumentation, Russian Academy of Sciences, Russia). The results were analyzed using the SnapGene Viewer program.

Total RNA extraction and real-time RT-PCR. Total RNA was isolated using LIRA reagent (LRU-100-50, Biolabmix, Russia) according to the manufacturer's instructions. The concentration of total RNA in the samples was measured using NanoDrop One spectrophotometer (Thermo FS, USA). The total RNA was treated with DNase I (Thermo FS, USA) at 37°C and 40 min for the degradation of genomic DNA. The expression of genes *TP73, VEGF-A*, and *VEGFR-1* was assessed by RT-PCR. A Genta Single-tube RT-PCR master mix kit (RT-M-003, GenTerra, Russia) was used for the reaction. The reaction mixture (25 μL) contained a one-shot master mix, 375 nM forward primer, reverse primer and probe ([25], 3'–5' VEGF-A f-gag gca gct tga gtt aaa c, r-ttc tgt cga tgg tga tgg tg, p-FAM-tgc aga tgt gac aag ccg agg c-BHQ1, VEGFR-1 f-tca gca cat tcc cta gtg ag, r-cac agg tgg ttt gcg tat gt, p-FAM-tac tgg ctc ctg gca gcg gct-BHQ1, actin f-atg cag aag gag atc act gc, r-ata ctc ctg ctt gct gat cc, p-FAM-atc att gct cct cct gag cgc aa-BHQ1), and 0.5-μg RNA. The reaction was carried out using a CFX96 Touch™ thermal cycler (BioRad, USA): 30 min, 50°C (reverse transcription), 15 min, 95°C (polymerase activation), and 45 cycles: denaturation of a DNA fragment (95°C, 15 s), primer and probe annealing (58°S, 30 s), elongation (72°S, 60 s). The relative expression of target genes was determined from threshold-cycle values and normalized to actin gene expression.

Cell irradiation and viability assessment. The cells of all glioma lines were irradiated with graded doses of 1–8 Gy of X-ray radiation with an energy of 35 KeV or γ radiation with an energy of 1.2 MeV using the ^{60}Co-source of γ rays "Issledovatel" (Konstantinov St. Petersburg Institute of Nuclear Physics, National Research Center "Kurchatov Institute", Russia), and their viability was assessed using the alamarBlue (resazurin) test [1].

Flow cytometry. Cells of the A172 and Gl–Tr lines were fixed with 4% formaldehyde. Some cells were additionally treated with a 0.5% Triton solution to permeabilize the membrane. They were incubated with primary antibodies of clones 4C1 and 3B12 to VEGFR-1 (monoclonal mouse, 0.1 μg/mL) for 12 h, 4°C; stained with secondary antibodies antimouse-Alexa488 (17c01220, Hansa BioMedLife Science, Estonia, 1 ng/mL) for 1 hour at 4°C, and visualized on a CytoFlex flow cytometer (Beckman Coulter) at a laser wavelength of 480 nm, accumulating a minimum of 20 000 events.

Real-time cell-viability assessment. The cultures of cells A172 and Gl–Tr were transplanted to E-plates at a concentration of 2×10^4 cells/well and placed into the xCELLigence Real-Time Cell Analysis instrument (37°C, CO_2). After 24 hours, the medium was replaced with a new one with monoclonal antibodies to VEGFR-1 receptors (clones 4C1, 3B12), Avastin at a concentration of 1 mg/mL or their combination at a concentration of 0.5 mg/mL each. Cell growth was monitored for 10 days.

Statistical analysis. Experiments to analyze expression and cell proliferation were carried out in at least triplicate. Data visualization and analysis were performed using GraphPad Prism software. The processing of flow cytometry data and their visualization were carried out using the freely available Floreada.io plugin. The results in the histograms are presented as mean ± standard deviation (SD). The data were compared using one-way ANOVA with Tukey's test to correct for multiple comparisons. The differences were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

The heterogeneity of glioblastomas is one of their features. The populations of cells that make up a tumor can differ both in different patients and within one tumor, therefore, to search for prognostic molecular markers and develop therapeutic regimens, it is advisable to use several cell lines that differ from each other. Work on the production and characterization of new glioblastoma cell lines has been carried out since the middle of the 20th century and still remains relevant due to the ineffectiveness of antitumor therapy and low patient survival [6]. The most promising research strategy is to use cultured cell lines derived from patient biopsies and compare them with existing standard glioma lines. Figure 1 shows photographs of glioblastomas Gl–R, Gl–Tr, Gl–L, Gl–Sh, Gl–C, and Gl–F obtained from the tumor material of patients. Cell cultures vary in cell size, shape, and growth patterns, making them potentially interesting models for testing anticancer therapy regimens.

Mutations in isocitrate dehydrogenase genes. The presence of mutations in isocitrate dehydrogenase genes is an important prognostic marker. Gliomas with mutated genes *IDH1* and *IDH2* respond better to therapy and have a more favorable treatment prognosis [26]. In glioblastomas, the genes *IDH1* and *IDH2* are changed extremely rarely. The most common mutations in IDH are replacements of conservative arginines at positions R100, R109, R119, and R132 for type-1 IDH and R140, R149, R159, and R172 for type 2 with histidines, which leads to the appearance of an

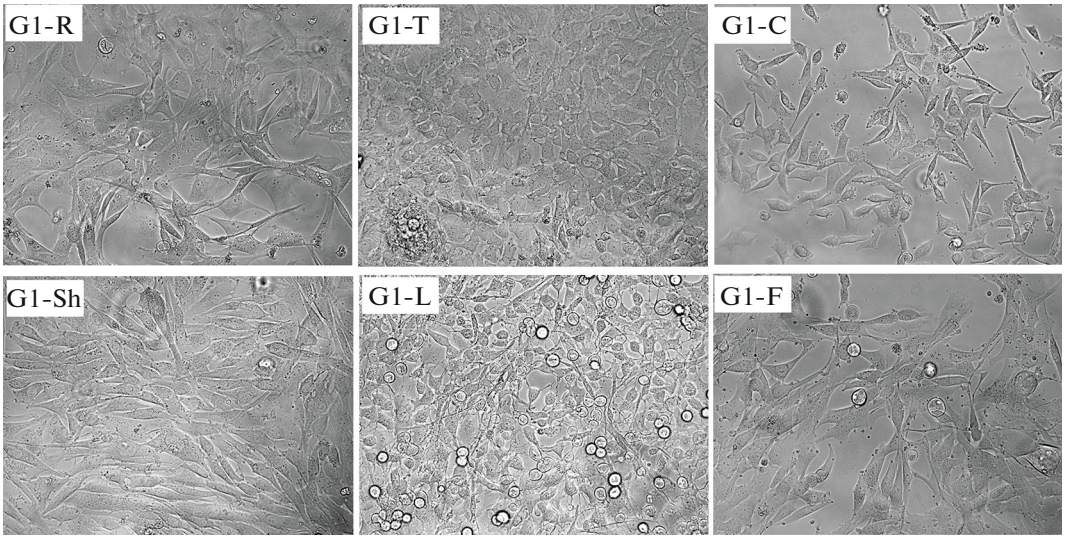

Fig. 1. Microphotographs of glioblastomas. Microphotographs of cells of the Gl–R, Gl–Tr, Gl–L, Gl–Sh, Gl–C, and Gl–F lines were taken using a Life Technologies Evos microscope (Thermo FS, UK) at a magnification of ×20.

additional function of the enzyme, through which it converts α-ketoglutarate (α-KG), the normal product, to D-2-hydroxyglutarate (D-2HG). How this contributes to carcinogenesis is currently unclear, but is likely due to the effect of D-2HG on DNA demethylases, which promotes DNA and histone methylation [8, 27]. The selected primers completely covered the region of genes *IDH1* and *IDH2* containing "hot spots", but in none of the studied glioblastomas were substitutions in conservative arginines identified. Therefore, Gl–R, Gl–Tr, Gl–L, Gl–Sh, Gl–C, and Gl–F are wild-type glioblastomas according to IDH; they belong to gliomas with a poor prognosis for treatment and are a good model for selecting and testing regimens of antitumor therapy.

Mutations in the TP53 gene. Mutations in IDH are often associated with mutations in p53 or codeletion in chromosomes 1p/19q. In glioblastomas, mutations in the gene *TP53* occur in only 20–30% of cases, in grade-IV gliomas with a mutated *IDH* gene, mutations occur in 60–70% of cases. The overwhelming number of described mutations in the gene *TP53* is concentrated between codons 125 and 300. The selected primers were located in introns and covered all "hot regions."

In Gl–Tr and Gl–L, the pathogenic mutation rs11540654 was detected at the 110th amino-acid position c.329G > C (p.Arg110Pro). This mutation is poorly described in publications. It occurs in Li–Fraumeni syndrome [28]. The replacement of arginine with proline can lead to conformational changes in the active center of the protein. Arginine carries two main centers: an amino group in the α position, capable of forming multiple hydrogen and ionic bonds, and a guanidine group in the δ position. In a proline molecule, the nitrogen atom is not bonded to a hydrogen atom and cannot be a hydrogen donor when forming a hydrogen bond. In addition, proline greatly bends the peptide chain. Thus, the replacement of Arg with Pro in the DNA-binding domain of the p53 protein can greatly affect its functions.

The rs1042522 polymorphism in the fourth exon at the 72nd amino-acid position c.215C > G (p.Pro72Arg) is a benign change widely described in publications [29, 30]. It is believed that the choice of amino acid at the given position occurs under the influence of "natural selection" depending on the latitude and amount of UV radiation. In Europeans, the composition of this polymorphism is 60, 30, and 10% for Arg/Arg, Arg/Pro and Pro/Pro, respectively. In the studied glioblastomas, the Pro/Pro polymorphism was observed in Gl–R, Gl–Tr, and Gl–L, the Arg/Arg polymorphism in Gl–Sh, and the Arg/Pro polymorphism in Gl–C and Gl–F.

A 16-bp deletion of rs59758982 in the third intron, which is classified as a polymorphism and refers to benign changes that do not affect the functions of the p53 protein, was found in all six glioblastomas studied.

Polymorphisms rs12951053 and rs12947788 (14181C > T, 14201T > G) are linked, located in the seventh intron, and can contribute to the formation of defective p53 protein [31]. In the studied glioblastomas, polymorphisms were observed in Gl–R, Gl–Tr, and Gl–L in the homozygous variant, and in Gl–C and Gl–F in the heterozygous variant.

Thus, the studied glioblastomas differ in polymorphisms in the gene *TP53*. In addition, two of them have a pathogenic mutation, which makes their combined use promising for the study of new strategies in radiotherapy and chemotherapy.

Expression of p73 protein isoforms. In many tumors, including glioblastomas, overexpression of the full-length (tumor suppressor) isoform TA of the protein p73 and the appearance of its truncated (oncogenic) isoforms are observed, which promotes tumor progression, mediating angiogenesis, and increasing the survival of tumor cells, and is an unfavorable prognosis for therapy. Isoforms of the p73 protein can differ in the N- and C-terminus [32] (Table 1). Among the isoforms truncated at the N-terminus, the most significant role in tumor development is played by Δex2p73, Δex2/3p73, ΔN'p73, and ΔNp73 [25]. Analysis of N-terminal isoforms revealed (Fig. 2a) the expression of the full-length TAp73 isoform in glioblastomas Gl–R, Gl–Tr, Gl–Sh, Gl–C, and Gl–F, and the appearance of truncated isoforms in all six cell lines, and in Gl– L, Gl–Tr, Gl–Sh, and Gl–C the expression of all four analyzed isoforms was observed, while in Gl–R and Gl–F there was no expression of the Δex2/3p73 isoform.

In Gl–R, Gl–Tr, and Gl–C, p73 expression was significantly higher than in the A172 culture. In the Gl–Sh cultures, the total RNA expression of truncated isoforms is higher than the expression of the full-length TAp73 isoform (the ratio of TAp73 expression to the total expression of truncated isoforms is 0.5). In Gl–L, expression of the full-length TAp73 isoform was completely absent. In Gl–F, the ratio of expression of the full-length isoform to the truncated ones is equal to one. For Gl–R, Gl–Tr, Gl–C, and A172, the expression of RNA of the full-length isoform was higher than the expression of the truncated isoforms by 3.6, 1.5, 2, and 1.3 times, respectively.

Table 1. Exonic composition of p73 protein isoforms

N-terminus		C-terminus	
Isoform	Exon composition	Isoform	Exon composition
TA	1–2–3–4	α	10–11–12–13–14
Δex2	1–3–4	β	10–11–12–14
Δex2/3	1–4	γ	10–12–13–14
ΔN'	1–2–3–3'–4	ε	10–12–14
ΔN	3'–4	ζ	10–13–14
		δ	10–14

Analysis of the C-terminal isoforms of the p73 protein showed that full-length α and truncated β, γ, ε, and δ were found in all glioblastomas studied (Fig. 2b). In the Gl–Tr line, the ζ isoform was additionally observed.

Currently, radiotherapy is one of the main methods of treating brain tumors, but many glioblastomas have quite high radioresistance, and increasing the dose of radiation is also detrimental to healthy brain cells. One of the reasons for radiation resistance may be increased expression of the p73 protein and the

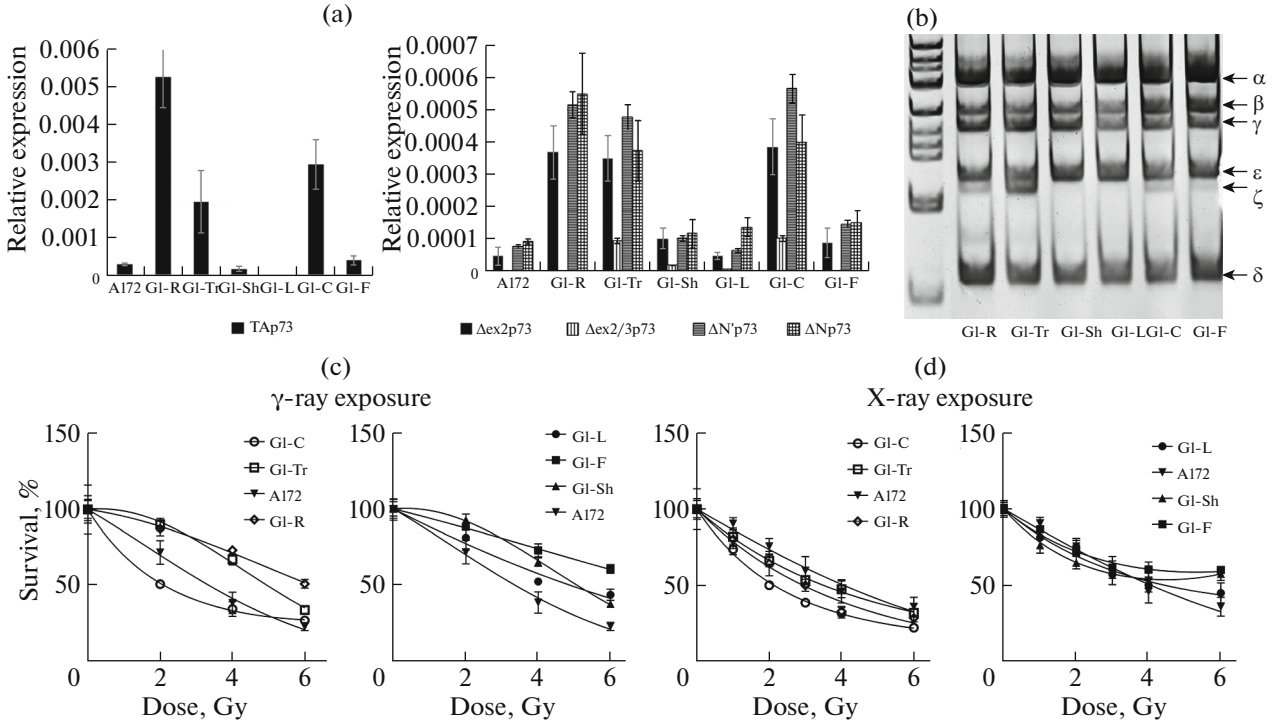

Fig. 2. Expression level of gene isoforms *TP73* in glioblastoma cell lines and their sensitivity to radiotherapy: (a) relative level of RNA expression of N-terminal isoforms of the gene *TP73*, (b) presence of C-terminal isoforms of the gene *TP73*; survival rate of glioblastoma lines (resazurin test data) after irradiation with γ radiation (c) or X-rays (d) in the dose range of 0–6 Gy.

appearance of its oncogenic isoforms in glioblastomas [33]. The accumulation of DNA breaks in response to irradiation should lead to the activation of p53-family proteins and the initiation of apoptosis. The transcription factors p53 and p73 function as tetramers. N-terminally truncated isoforms of the p73 protein can integrate into tetramers of both p73 and p53 and interfere with their normal functioning [34]. In addition, defective proteins compete for binding sites with full transcription factors and prevent the initiation of the RNA expression of target genes responsible for the development of apoptosis. Moreover, the isoform ΔNp73 is capable of attaching directly to the site of DNA damage, interacting with the sensor protein 53BP1, which detects the presence of a break, and inhibiting ATM activation, phosphorylation of the p53 protein, and the initiation of apoptosis [26]. Different ratios of oncogenic and tumor suppressor isoforms of p73 may lead to glioblastomas responding differently to different doses and types of radiation. In the cell lines studied, the gene *p73* is highly expressed, and the N-terminally truncated isoforms are present in different amounts, which makes the combined use of Gl–R, Gl–Tr, Gl–Sh, Gl–L, Gl–C, and Gl–F a promising model for studying radioresistance.

Sensitivity of glioblastoma lines to γ irradiation. Of the glioblastomas studied, Gl–C turned out to be the most sensitive to γ radiation (Fig. 2c). The most stable was Gl–F. It is interesting that these glioblastomas have the same p-53 status (there are no pathogenic mutations, and all detected polymorphisms occur in a heterozygous state), while the ratio of RNA expression of full-length and truncated p73 isoforms for Gl–C is 2.0, and for Gl–F, it is 1.0. Gl–R, Gl–Tr, Gl–Sh, and Gl–L were more resistant to γ radiation than the A172 culture. In this case, Gl–Sh with nonmutated p53, but with a p73 protein isoform ratio of 0.5, behaved in the same way as Gl–Tr with a p53 protein carrying a pathogenic mutation and a p73 isoform ratio of 1.5. Gl–L should have been the most resistant to radiotherapy, but at doses of 2 and 4 Gy it responded to γ radiation more strongly than Gl–Tr, and Gl–Sh, but at 6 Gy it retained more viable cells. In Gl–R, the ratio of p73 isoforms was noticeably shifted towards TAp73, there were no mutations in the p53 protein, but the cell line turned out to be resistant to γ radiation. It is likely that the mechanism of resistance here is formed due to damage to some other molecular pathways.

Sensitivity to X-ray irradiation. Of the glioblastomas studied, Gl–C was also the most sensitive to X-rays (Fig. 2d). The remaining glioblastomas showed approximately the same sensitivity to doses of 1–3 Gy. At a dose of 4 Gy, Gl–F was less sensitive to radiation than the others. At a dose of 6 Gy, Gl–Sh, Gl–F (TAp73/truncated isoforms ≤1), and Gl–L (pathogenic mutation in the p53 protein and absence of TAp73) were more resistant to X-ray irradiation than A172, Gl–R, and Gl–Tr.

Thus, the studied glioblastomas have different p53 and p73 statuses, respond differently to radiotherapy with γ- and X-ray irradiation and can be used as models for selecting radiation doses and testing sensitizing agents.

Mutations in the KRAS gene. Immunotherapy is a promising direction for the treatment of glioblastomas. In 57% of glioblastomas, amplification of the epidermal-growth-factor (**EGF**) receptor gene is observed [35, 36] and an increase in its expression levels on the cell membrane. The use of antibodies both against the receptor itself and against EGF could become a promising therapeutic regimen for the treatment of glioblastomas. The KRAS protein (a member of the RAS protein family) is activated when EGF binds to the receptor and is deactivated after cleavage of the associated GTP molecule, which interrupts the signal. Replacement in *KRAS*-gene codons 12 and 13, encoding glycine, the only amino acid without side radicals with any amino acid, leads to a violation of the spatial confirmation of the protein and prevents its inactivation. As a result, the EGF cascade remains active regardless of receptor status and this makes it impossible to carry out immunotherapies associated with EGF and EGFR. The selected primers covered the regions of codons 12–13. In none of the studied glioblastomas were substitutions in the 12th and 13th glycines identified. Thus, Gl–R, Gl–Tr, Gl–L, Gl–Sh, Gl–C, and Gl–F can be used as models for the study and optimization of immunotherapy associated with EGF and its receptor.

RNA expression of the VEGF-A and VEGFR-1 genes and VEGFR-1 protein. Another target for the immunotherapy of glioblastomas is vascular endothelial growth factor and its type-1 receptor. In [6], it was noted that in glioblastomas Gl–R and Gl–Tr there is high activity of the growth factors TGFβ1, VEGF, and FGF2(b). In this work, we assessed the expression of RNA genes *VEGF-A* and *VEGFR-1* in all glioblastomas studied and found that its level was higher than in the A172 cell line (Fig. 3a). For Gl–Tr and Gl–C, the expressions of the RNA factor and receptor were 2–5 times higher than the corresponding values for A172. In Gl–Sh and Gl–F, receptor RNA expression was 2.6 and 13.2 times higher, while *VEGF-A* RNA expression also increased significantly by 11.1 and 44.2 times, respectively. In Gl–L, the levels of RNA expression of both the receptor (20.8 times) and the factor (352.4 times) significantly increased. In Gl–R, on the contrary, the expression of the *VEGFR-1* RNA gene increased significantly (178.5 times) with an increase in the expression of the RNA factor by 4.3 times relative to A172.

Interestingly, in nontumor cells such as HEK-293 and DF-2, *VEGFR-1*-gene RNA expression is higher than the RNA expression of the factor itself. In the studied glioblastomas only in Gl–R was the same ratio observed; in the remaining five tumor cultures, the

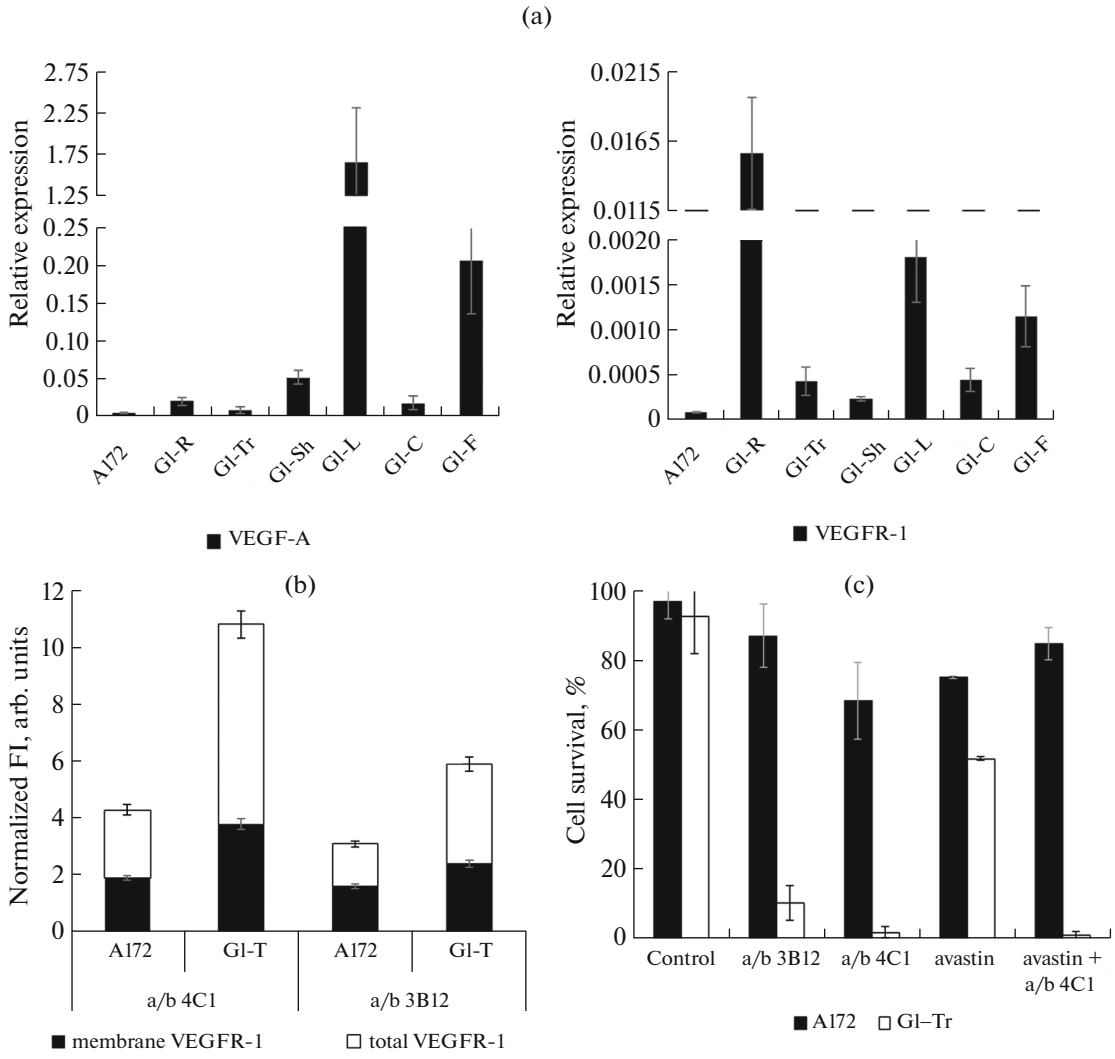

Fig. 3. Immunotherapy for glioblastomas. Expression level of VEGF-A and VEGFR-1 mRNA in cells of the A172, Gl–R, Gl–Tr, Gl–L, Gl–Sh, Gl–C, and Gl–F lines according to real-time RT-PCR data (a). Immunofluorescence analysis of the expression of total VEGFR-1 protein and its receptor form located on the membrane of the A172 and Gl–Tr cell lines. Flow-cytometry data on the visualization of VEGFR-1 with monoclonal antibodies 4C1 and 3B12 (b). Survival of cells of the A172 and Gl–Tr lines when incubated in the presence of monoclonal antibodies to human VEGFR-1 4C1 or 3B12, the drug Avastin, which binds VEGF-A, and their combination (c).

expression of the VEGF-A factor significantly exceeded the expression of its receptor. Thus, the studied glioblastomas differ in the levels of RNA expression of genes *VEGF-A* and *VEGFR-1*, and their relationships with each other.

Using cytometric measurements for A172 and Gl–Tr glioblastomas, we assessed the expression levels of the VEGFR-1 protein and the ratio of its receptor and soluble forms (Fig. 3b). In Gl–Tr, the expression of the total protein and the receptor form of VEGFR-1 was higher than in A172. At the same time, in Gl–Tr, 42 and 54% of the VEGFR-1 protein was located in the receptor form on the cell membrane (when stained with antibodies to VEGFR-1 clones 3B12 and 4C1, respectively), and in A172, 66 and 71%, respectively.

Immunotherapy. Testing of the immunotherapeutic treatment regimen using antibodies of clones 4C1 and 3B12 to the VEGFR-1 receptor was carried out on A172 and Gl-Tr cultures using real-time cell-growth assessment on the xCELLigence device (Fig. 3c). The A172-cell line was not sensitive to immunotherapy with Avastin and monoclonal antibodies to VEGFR-1. In contrast, Gl–Tr survival decreased by 50% when cells were treated with Avastin. When using antibodies to VEGFR-1 clone 3B12, cell survival decreased to 10%, and when using antibodies from clone 4C1 it dropped below 5%. The combined use of Avastin and antibodies to VEGFR-1 clone 4C1 led to complete suppression of the growth of Gl–Tr glioblastoma cells. Thus, Gl–Tr can be used as a model for testing immu-

nological therapy regimens aimed at blocking vascular endothelial growth factor and its type-1 receptor.

CONCLUSIONS

The described cultures of glioblastomas Gl–R, Gl–Tr, Gl–L, Gl–Sh, Gl–C, and Gl–F are stably cultured cell lines that differ from each other in cell size and shape, p53 and p73 statuses, and levels of RNA expression of the genes for vascular endothelial growth factor and its type-1 receptor. All this makes the described cell lines promising models for studying the formation of resistance of glioblastomas to radiotherapy and for testing new sensitizing agents. In addition, the described glioblastoma lines can be used as models for testing synthetic antibodies against VEGFR-1 and developing immunotherapy regimens aimed at blocking VEGF and its receptor.

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CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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