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Stromal and Tumor Glioma-derived Cells with Similar Characteristics Have Differences in α -Smooth Muscle Actin Expression and Localization

I. Gin¹ I. Chistyakova¹ V. Zenin¹ S. Koshkin¹ A. Musorina¹ Y. Lahina² G. Timin³

V. Pospelov¹ S. Prikhodko² A. Petukhov^{1,2,4} E. Tolkunova^{1*}

1. Institute of Cytology, Russian Academy of Sciences, St. Petersburg, 194064, Russia

2. Almazov National Medical Research Centre, St. Petersburg, 197341, Russia

3. Peter the Great Saint-Petersburg Polytechnic University, St. Petersburg, 195251, Russia

4. Scientific Technological University «Sirius», Sochi, 354340, Russia

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ABSTRACT

Gliomas are solid brain tumors composed of tumor cells and recruited heterogenic stromal components. The study of the interactions between the perivascular niche and its surrounding cells is of great value in unraveling mechanisms of drug resistance in malignant gliomas.

In this study, we isolated the stromal diploid cell population from oligodendroglioma and a mixed population of tumor aneuploid and stromal diploid cells from astrocytoma specimens. The stromal cells expressed neural stem/progenitor and mesenchymal markers showing the same discordant phenotype that is typical for glioma cells. Moreover, some of the stromal cells expressed CD133. For the first time, we demonstrated that this type of stromal cells had the typical myofibroblastic phenotype as the α -SMA⁺ cells formed α -SMA fibers and exhibited the specific function to deposit extracellular matrix (ECM) proteins at least in vitro. Immunofluorescent analysis showed diffuse or focal α -SMA staining in the cytoplasm of the astrocytoma-derived, A172, T98G, and U251MG glioma cells. We could suggest that α -SMA may be one of the main molecules, bearing protective functions. Possible mechanisms and consequences of α -SMA disruptions in gliomas are discussed.

1. Introduction

Gliomas represent a heterogeneous group of brain tumors characterized by a highly aggressive nature. Malignant glioblastoma exhibits a high self-renewal and proliferation capacity of glioma stem cells and perivascular niche cells. The study of the interactions between the perivascular niche and its surrounding cells is of great value in unraveling mecha-

nisms of drug resistance in malignant gliomas^[1].

A neoplastic component of glioma consists of the transformed cells of astrocytic or oligodendrocytic origin. Like all solid tumors in addition to the neoplastic component, glioma also contains a heterogeneous stroma which is comprised of prominent vessels and recruiting cells such as antigen-presented cells, reactive astrocytes, mesenchymal stem cells (MSC), and glioma-associated stromal

**Corresponding Author:*

Elena Tolkunova,

Institute of Cytology, Russian Academy of Sciences, St. Petersburg, 194064, Russia;

Email: entolk62@mail.ru

cells (GASC), termed also cancer-associated fibroblasts (CAFs)^[2-4]. Neural stem or progenitor cells from the adult brain which are considered as the most putative cell of origin for glioma^[5-8] may also be a part of the glioma tumor stroma. Some studies in xenograft models have shown that neural stem cells (NSC) can display extensive tropism toward the tumors^[9-11]. In PDGF-induced gliomas stem or progenitor-like cells expressing neural markers can occupy large areas and even predominate in a tumor bulk^[12-15]. Gene expression profiles of these cells resemble ones of tumor cells. Moreover, the ability of the stromal cells to acquire genetic alterations typical for gliomas has been shown in some experimental models^[13]. Thus, identification of this type of stromal cells and obtaining its pure culture is rather difficult because it is poorly distinguishable from the tumor cells. The culture of neural stem/progenitor-like stromal cells has been obtained by using an artificial xenograft model^[16]. The existence and proportion of this stromal cell component in gliomas are not clearly defined.

The role of the tumor microenvironment in cancer progression deserves significant attention. Cancer cells recruit and transform the stromal cells, which acquire myofibroblastic phenotype determined by the expression of alpha-smooth muscle actin (α -SMA)^[17-22]. Thus, Öhlund et al.^[19] have identified a stromal cell subpopulation with elevated expression of α -SMA located immediately adjacent to neoplastic cells in mouse and human pancreatic ductal adenocarcinoma tissue. Holm Nielsen^[20] has found that the proportion of the α -SMA- positive myofibroblasts are upregulated in lung fibrosis and cancer. Vacz et al.^[21] have demonstrated that the proportion of α -SMA(+)/CK(+) cells is significantly higher in colorectal cancer samples compared to healthy or adenoma samples. The myofibroblast function is associated with extracellular matrix (ECM) deposition in both normal physiological and pathological conditions^[4,23]. Glia progenitor cells provide these functions in the CNS. During embryonic and postnatal development these purely differentiated cells exhibit a myofibroblastic phenotype and provide homing of neuron and glia precursors through the emerging brain areas^[24-26]. In the adult nervous system reactive glia demonstrates myofibroblastic features in response to injury^[27]. These cells originate from the pool of progenitor cells disseminated through the brain. Reactivated with inflammatory cues they take part in the formation of a glial scar in the central and peripheral nervous system of adults. The levels of the same inflammatory cytokines (TGF β 1, IL6, etc.) were found to be elevated in neoplastic areas of the brain^[28] that can result in the appearance of myofibroblasts accumulating in the tumor microenvironment. Recruiting

CAFs, MSC and reactive astrocytes could be responsible for the production and modulation of ECM in gliomas. The ECM composition and stiffness are altered in malignant gliomas and the correlation between these alterations and malignancy is discussed^[29]. Whether neural stem/progenitor-like stromal cells could demonstrate myofibroblastic features and contribute to ECM deposition during gliomagenesis remains unknown.

Glioma cells according to their probable origin should have a basic opportunity to exhibit the myofibroblastic phenotype and α -SMA shift. However, it has been shown that α -SMA expression is disrupted in transformed cells^[30,31]. In oncogenic transformed fibroblasts these disruptions were mediated by repression of α -SMA promoter activity with serum response elements^[32]. In a transformed rat cell line α -SMA was involved in the regulation of cell growth and motility and led to the suppression of the malignant phenotype of this cell line^[33]. In tumor cells from primary and secondary glioblastomas α -SMA expression is absent in the cases when the loss of heterozygosity on chromosome 10q occurs^[34]. In secondary glioblastomas, the repression of the α -SMA gene may be also associated with p53 mutations as the α -SMA gene has been shown to be a transcriptional target of this tumor suppressor protein^[35]. It is not yet well explored if all glioma cells are unable to express α -SMA and to acquire myofibroblastic phenotype.

In this report, we isolated the stromal cell population from oligodendroglioma as well as the stromal and tumor cells mixed population from anaplastic and fibrillar astrocytoma specimens. Both stromal and tumor glioma-derived cells showed the same molecular profile indicating that the cells belonged to the same lineage. We first demonstrated that the stromal cells expressing neural stem and progenitor markers had the typical myofibroblastic phenotype as they express α -SMA and exhibit their specific function to deposit ECM proteins at least in culture conditions. We also attempted to determine whether glioma cells retain the expression of α -SMA.

2. Materials and methods

2.1 Tumor Specimens and Glioma-derived Cell Lines

Tumor samples were obtained from three patients undergoing surgical treatment in the Polenov Neurosurgical Institute at Federal Almazov Medical Research Centre (St. Petersburg, Russia). These tumors were histologically characterized according to the WHO classification as oligodendroglioma grade II (Olig II), fibrillar astrocytoma grade II (FAII) and polymorphic anaplastic astrocytoma grade III (AAIII). After surgical removal tumor samples

were kept in PBS at room temperature up to use and then they were washed, minced up to 1 mm³ pieces, enzymatically treated, and passed through a syringe with a needle 20G. The obtained suspension contained single cells and undissociated tissue pieces were plated on a culture plate. These cultures were further designated as “mix” cultures. Overnight unattached tumor pieces were transferred on a culture plate. Cell lines were obtained from these cultures by outgrowth of the cells from tumor pieces. When the cells reached confluence they were trypsinized, diluted and transferred on a plate. This standard procedure was repeated for all cell lines derived from primary cultures. The cell cultures were maintained in DMEM/F12 (Lonza, Belgium) supplemented with 10% fetal calf serum (HyClone Laboratories, Utah, USA), 2.5 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin (Biolot, Russia) under standard conditions (37°C, atmosphere of 5% CO₂ in air). Permanent human glioma cell lines A172, U251MG and T98G were obtained from the Vertebrate Cell Culture Collection at the Institute of Cytology RAS (St. Petersburg, Russia) and were maintained under the same culture conditions.

2.2 Magnetic Cell Separation of CD133+ Cells

The cells were harvested and resuspended in PBS containing 0,5% BSA and 2 mM EDTA. Positive magnetic cell separation (MACS) was performed using the CD133 MicroBead Kit (Miltenyi Biotec, Germany). The ratio of CD133+ cells was estimated by cell counting.

Immunocytochemical staining cells were grown on coverslips at high or low density for the indicated periods of time, then fixed with 4% formalin (Sigma, USA), permeabilized with 0.1% Triton X-100 or 0.5% Triton X-100 for nucleolin staining, and incubated with PBS containing 1% BSA (Sigma, USA) and 2% FCS for 30 min to prevent unspecific binding of antibodies. Then, the samples were incubated with the following primary antibodies: mouse anti- α -SMA (1:300 dilution, Sigma), rabbit anti-GFAP (1:50, Sigma), mouse anti-CNPase (1:50, Chemicon), rabbit anti-nestin (1:50, Millipore), mouse anti-vimentin (1:150, Sigma), rabbit anti-collagen type I (1:40, Chemicon), mouse anti-fibronectin (1:200, Sigma), rabbit anti-CD133 (1:150, Abcam) or rabbit anti-nucleolin (1:100, Proteintech Europe) in 1% BSA overnight at 4°C. After washing with PBS samples were incubated with secondary antibodies Alexa-Fluor-488-conjugated goat anti-mouse-IgG (1:400, Invitrogen) or Atto-550 conjugated goat anti-rabbit-IgG (1:300, Sigma), followed by staining with rhodamine-phalloidin (1:40, Invitrogen) and DAPI (2 µg/ml, Sigma). Then, the coverslips were mounted in Vectashield medium (Vector Laboratories). Samples were

analyzed with the confocal fluorescence microscope Leica TCS SL (Leica Microsystems, Germany).

2.3 RNA Isolation and RT-PCR

Total RNA was isolated from cells with the GenJET RNA purification kit (Thermo Scientific, Lithuania) according to the manufacturer's instructions. RNA concentration was determined with an ND1000 spectrophotometer (NanoDrop, USA). RNA solutions were stored at -80°C. Sample volumes from 0.5 to 11 µL of total RNA solution (depending on RNA concentration) were used in a volume of 20 µL for each reaction of the reverse transcription. The reaction was performed with a RevertAid reverse transcriptase kit (Thermo Scientific, Lithuania) according to the manufacturer's instructions using random hexamer primers. cDNA was stored at -20°C. PCR was performed at a volume of 25 µL with DreamTaq DNA polymerase (Thermo Scientific, Lithuania) according to the manufacturer's instructions. DNA was amplified for 35 cycles. PCR products were analyzed by electrophoresis in 2% agarose gel. Gel image acquisitions were made with the ChemiDoc system (Bio-Rad, USA). Primers were designed using primer-BLAST resources of the National Center for Biotechnology Information (NCBI) and an IDT OligoAnalyzer (<http://eu.idtdna.com/calc/analyzer>). Primers for vimentin gene identification corresponded to the reported sequences (Velpula et al., 2011). The following primers sequences were used:

Vimentin, 5'-GAACGCCAGATGCGTGAAATG-3' (forward),
5' CCAGAGGGAGTGAATCCAGAITA-3' (reverse);
CD44, 5' AAGGTGGAGCAAACACAACC-3' (forward),
5'-ACTGCAATGCAAACACTGCAAG-3' (reverse);
GFAP 5'-GCACGCAGTATGAGGCAATG-3' (forward),
5'-TAGTCGTTGGCTTCGTGCTT-3' (reverse);
NG2, 5'-GGTGGTTTCAGATCGGGAGG-3' (forward),
5' CAGTGACGTTCACTACGGCT-3' (reverse);
CD133, 5'-GGTGCTGTTCATGTTCTCCA-3' (forward),
5' ACCGACTGAGACCCAACATC-3' (reverse);
 β -actin, 5'-TTCCTGGGCATGGAGTCCT-3' (forward),
5'-AGGAGGAGCAATGATCTTGATC-3' (reverse).

2.4 Fluorescence-activated Cell Sorting Analysis

The cells were grown to 80-90% confluence, trypsinized with 0.25% Trypsin-EDTA (Gibco), washed with PBS, centrifuged at 1,000 rpm for 5 min, and the pellet was resuspended at a concentration of 1 x 10⁶/mL. Glioma cells were stained with fluorochrome-conjugated antibodies for at least 30 minutes at 4 °C and then the samples were diluted by FACS buffer (PBS with 1% BSA and 0.05% sodium azide). The analyses were made on a flow

cytometer CyFlowSpace (Partec, Germany) and $\geq 10,000$ events were collected in each analysis with forward scatter and side scatter. Data were analyzed by FloMax 2.82 software (Partec, Germany). The following antibodies were employed: CD44-FITC, CD73-PE, CD105-PE, CD90-FITC, CD29-PE, CD13-PE, CD9-FITC, CD130-FITC, CD146-PE, CD10-FITC, CD11b-PE, CD45-FITC, and HLA-DR-PE (Beckman Coulter, BD Pharmingen, Chemicon, Caltag Laboratories, Becton Dickinson, and eBioscience). Mouse IgG-FITC and IgG-PE isotype controls (DAKO) were used for assessing the background staining of cells.

For DNA content analysis, cells were suspended in 300 μ l PBS containing 200 μ g/ml of saponin (Fluka, NY, USA), 250 μ g/ml RNase A (Sigma, St. Louis, MO, USA, number R4642), and 50 μ g/ml PI, incubated for 60 min at room temperature and subjected to FACS analysis. At least 10,000 cells were measured per sample. Normal human lymphocytes were used as internal diploid control. Samples were analyzed by flow cytometer CytoFLEX. Histograms were prepared using CytExpert program version 1.2 (Beckman Coulter, Brea, CA, USA). DNA index (DI) was determined as the ratio of the mean DNA content of the glioma-derived cells to the mean DNA content of the control diploid cells.

2.5 Western Blotting

Total protein was isolated from monolayer cells at 72 h after plating. Briefly, the cells were washed with PBS and collected in RIPA lysis buffer containing a protease inhibitor cocktail (Sigma). The lysates were clarified by centrifugation at $14,000\times g$ at $4^{\circ}C$ for 15 min. Protein concentrations were determined by the Pierce BCA protein assay kit (Thermo Fisher Scientific) with BSA as a standard, and an equal mass of proteins from the cell samples were resolved on a 10% SDS polyacrylamide gel. In the case of analysis of collagen type I and α -SMA in glioma cell lines 25 μ g of protein per lane was applied, in all other cases - 10 μ g of protein per lane. After that, the proteins were electrotransferred onto a nitrocellulose mem-

brane (Biorad). After blocking in PBS buffer with 0.05% Tween-20 (PBS-T) containing 5% nonfat dry milk at room temperature for 1 hr, blots were incubated overnight at $4^{\circ}C$ with monoclonal anti- α -SMA antibodies (1:1000, Sigma, A5228), or polyclonal anti- β -tubulin antibodies (1:1000, Abcam, AB6046) or polyclonal anti-collagen type I antibodies (1:200, Chemicon, AB745) in blocking buffer. After three washes with PBS-T, the membranes were incubated for 1 hr at room temperature with rabbit peroxidase-labeled anti-mouse-IgG secondary antibodies (1:20000, Thermo Fisher Scientific, #31431) or with goat peroxidase-labeled anti-rabbit-IgG secondary antibodies (1:20000, Sigma, A0545) in blocking buffer. Membranes were washed three times with PBS-T and then were incubated in the chemiluminescent substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Fisher Scientific, #34095) and visualized on Fusion FX imager (Vilber Lourmat). Densitometric analysis of the bands was performed by employing Fusion-FX software (Vilber Lourmat).

3. Results

The stromal cells growing from tumor pieces together with tumor cells are not MSC, microglia/macrophages, or endothelial/subendothelial cells

The cells were isolated from glioma specimens by different approaches and compared cellular morphology and phenotype. The glioma-derived cultures obtained from the fraction of tumor pieces by cell sprouting presented morphologically homogeneous cell populations. At early passages, FAII- and AAIII -derived cells had polygonal epithelial-like shapes whereas ones derived from OligII had elongated fibroblast-like morphology (Figure 1). Further, the cells were enlarged and acquired a flattened shape.

To characterize the cell cultures and estimate their homogeneity we used flow cytometry for cell surface markers which are generally expressed on glioma cells and cells relating to the tumor stroma such as microglia, macrophages, pericytes, endothelial, smooth muscle, and

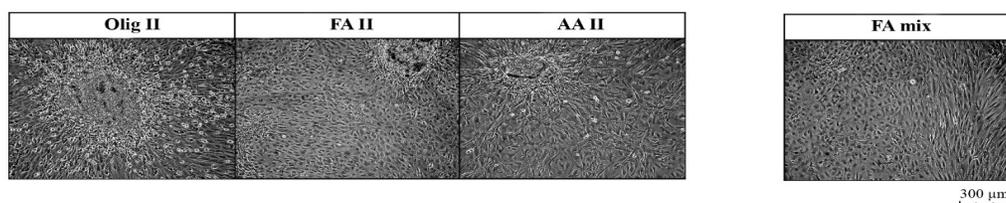


Figure 1. Phase-contrast images of oligodendroglioma grade II (OligII), fibrillar astrocytoma grade II (FAII), anaplastic astrocytoma grade III (AAIII)-derived cells grown out from tumor micro fragments, and cells representing a mixed fraction of single cells and tumor pieces (FAII mix).

mesenchymal stem cells (Table 1).

Table 1. Phenotypic analysis of FAII, FAII mix, AAIII, AAIII mix, and A172 cells by flow cytometry.

	FAII	FAII mix	AAIII	AAIII mix	A172
CD44	99,6	99,8	97,5	94	98,4
CD73	99,9	99,7	99	75	4,8
CD105	99,7	99,7	95,5	71	39,8
CD90	4,4	61	3,6	1,2	21
CD29	99,8	99,9	93	93	98
CD13	99,8	94,6	99,5	96	13
CD9	100	99,8		71	87
CD146	5,8	23	2,9	2,8	97
CD130	1,1	0,4	1,6	1	7,3
CD10	0,2	0,1	3,2	0,7	
CD11b	0,2	1,6		20	
CD45	0,2	0,2			
HLA-DR			3,5	25,5	

Cells were immunostained with antibodies against mesenchymal markers (CD44, CD73, CD105, CD90, CD29, and CD13), endothelial, subendothelial and mesenchymal marker CD146, microglia/macrophages markers (CD11b, CD45 and HLA-DR), CD9, CD130, and CD10.

FAII- and AAIII -derived cells showed a similar surface molecular pattern: high level of mesenchymal markers CD44, CD73, CD105, CD29, CD13, but low CD90; low level of CD146, the laminin receptor, which is typical for pericytes, endothelial, smooth muscle and also mesenchymal stem cells; negligible levels of microglia/macrophages markers CD11b, CD45. For comparison, we analyzed the A172 permanent cell line and revealed a modified pattern of mesenchymal markers, e.g. low percentage of CD73+ and CD13+ cells that may be the result of the long-term culturing and clonal cell selection in permanent cell lines. This assumption is consistent with the detection of high expression of CD146 marker in A172 permanent cell line since this molecule is associated with clonal properties [36].

Flow cytometry analysis of FAII mix and AAIII mix cultures obtained from the mixed fraction of single cells and tumor pieces revealed double peaks on graphs for a number of surface markers whereas only one peak for the corresponding markers was revealed in FAII and AAIII cultures obtained from tumor pieces only (Figure 2A, B). Thus, FAII mix culture contained 61% CD90+ cells, most likely MSCs, though almost no CD90 expressing cells were present in FAII culture (Figure 2A). Apparently, these stromal cells could be detected visually as elongated cells grouped together in FAII mix culture (Figure 1). HLA-DR positive cells presumably representing microglia/macrophages dominated in AAIII mix culture compared to AAIII culture (25% vs 3.5%) (Figure 2B).

Therefore, cells grown intensively from the tumor pieces arise to a morphologically homogeneous cell population depleted for MSC and microglia/macrophages that appear to be a significant part of the cell culture obtained from completely dissociated tumor tissue.

In spite of morphological and surface marker homogeneity of FAII and AAIII cell cultures, the analysis of DNA content by flow cytometry revealed two distinct cell populations: normal stromal diploid and tumor aneuploid (Figure 2C). Aneuploid cell populations in both astrocytoma-derived cell lines were nearly tetraploid (DI was 1,86 (P2) and 1,85 (P6) for FAII, and 1,88 (P5) and 1,89 (P15) for AAIII), the second histogram peak in the PI channel coincides with overlapping aneuploid (G1/G0) tumor cell population and the diploid 4N (G2/M) stromal cell fraction. Interestingly, the proportion of aneuploid and diploid cells remained nearly constant over the passages and was higher in AAIII than FAII cell culture. It may reflect the mutual influence of the tumor and stromal cells and the establishment of a stabilized ratio of the cell types in culture. According to the DNA assay, OligII cells were all diploid (Figure 2C) pointing to the absence of neoplastic component. It is consistent with reports where it has been demonstrated that oligodendroglioma tumor cells are not viable in culture under adherent conditions [16,37].

The stromal cells are α -SMA+ and responsible for the deposition of collagen type I and fibronectin. Alpha-smooth muscle actin (α -SMA) is regarded to be one of the most significant markers of stromal cells in gliomas [2]. Immunostaining with monoclonal antibody to α -SMA confirmed the presence of a stromal component in astrocytoma-derived cell cultures FAII and AAIII (Figure 3A). Numerous cells with well-organized α -SMA-filaments were seen (α -SMA+ cells). Of note, cells without α -SMA-filaments had a diffuse cytoplasmic α -SMA staining. OligII-derived cell culture was a total α -SMA+ stromal cell population according to immunofluorescence (Figure 3A). Western blot analysis revealed the highest level of α -SMA in OligII cells (Figure 3B). Interestingly, FAII mix culture in which MSCs were about 60% of the total cell population showed α -SMA level lower than FAII cells (Figure 3B) in which MSC were only about 4% of the total cell population according to flow cytometry (Figure 1C). This fact suggests that there are neural stem/progenitor-like stromal cells in glioma that produce more α -SMA than MSCs.

In order to estimate the specific fibrotic potential of α -SMA+ cells, the intracellular synthesis of collagen type I and fibronectin and their deposition in ECM were compared between glioma cell cultures by immunofluorescence (Figure 3D).

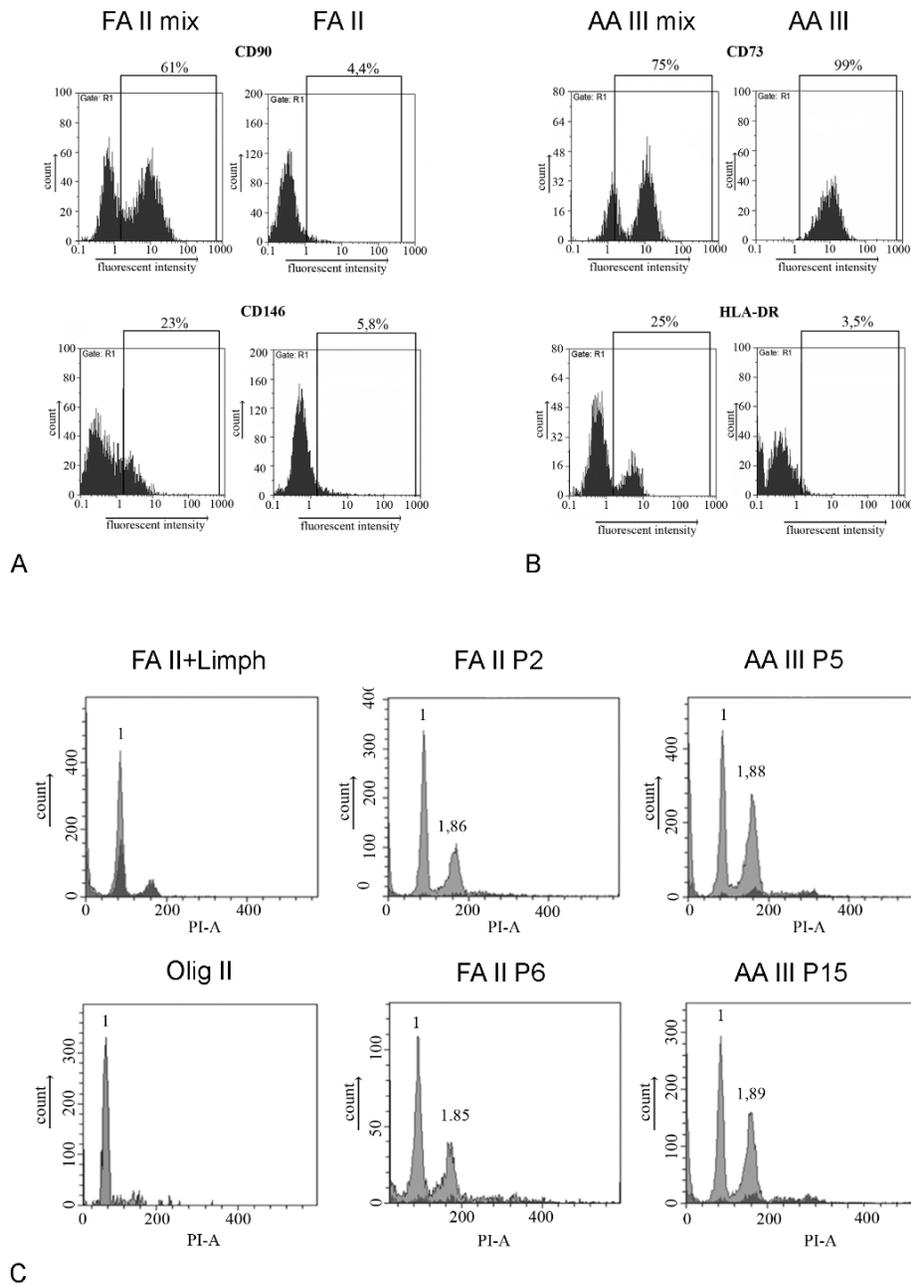


Figure 2. Phenotypic characterization of cells derived from glioma specimens. (A) Flow cytometry assay of CD90 and CD146 expression in FAII mix and in FAII cultures. (B) Flow cytometry assay of CD73 and HLA-DR expression in AAIII mix and AAIII cultures. (C) DNA assays of cells derived from glioma specimens. Flow cytometry histograms showing the DNA content in FAII, AAIII, and OligII cells. Stromal cells and lymphocytes were DNA diploid (DI=1). Tumor cells were aneuploid: FAII (DI=1,86 (2P) and 1,85 (6P)) and AAIII (DI=1,88 (5P) and 1,89 (15P)).

According to immunofluorescence at the 15-th day of culturing the deposition of collagen type I and fibronectin was more abundant in OligII culture (Figure 3D). FAII and AAIII cultures showed resembling the pattern of protein deposition with an increased level in FAII culture. These results suggest that there is a positive correlation between the amount of α -SMA+ cells and collagen type I and fibronectin deposition. These results also show that

the ability of glioma-derived stromal cells to produce ECM may have a tendency to decline with malignancy development.

Glioma cells usually do not express α -SMA and show reduced deposition of collagen type I and fibronectin

As NSC from the subventricular zone and glia progenitors from brain parenchyma are believed to be the cell

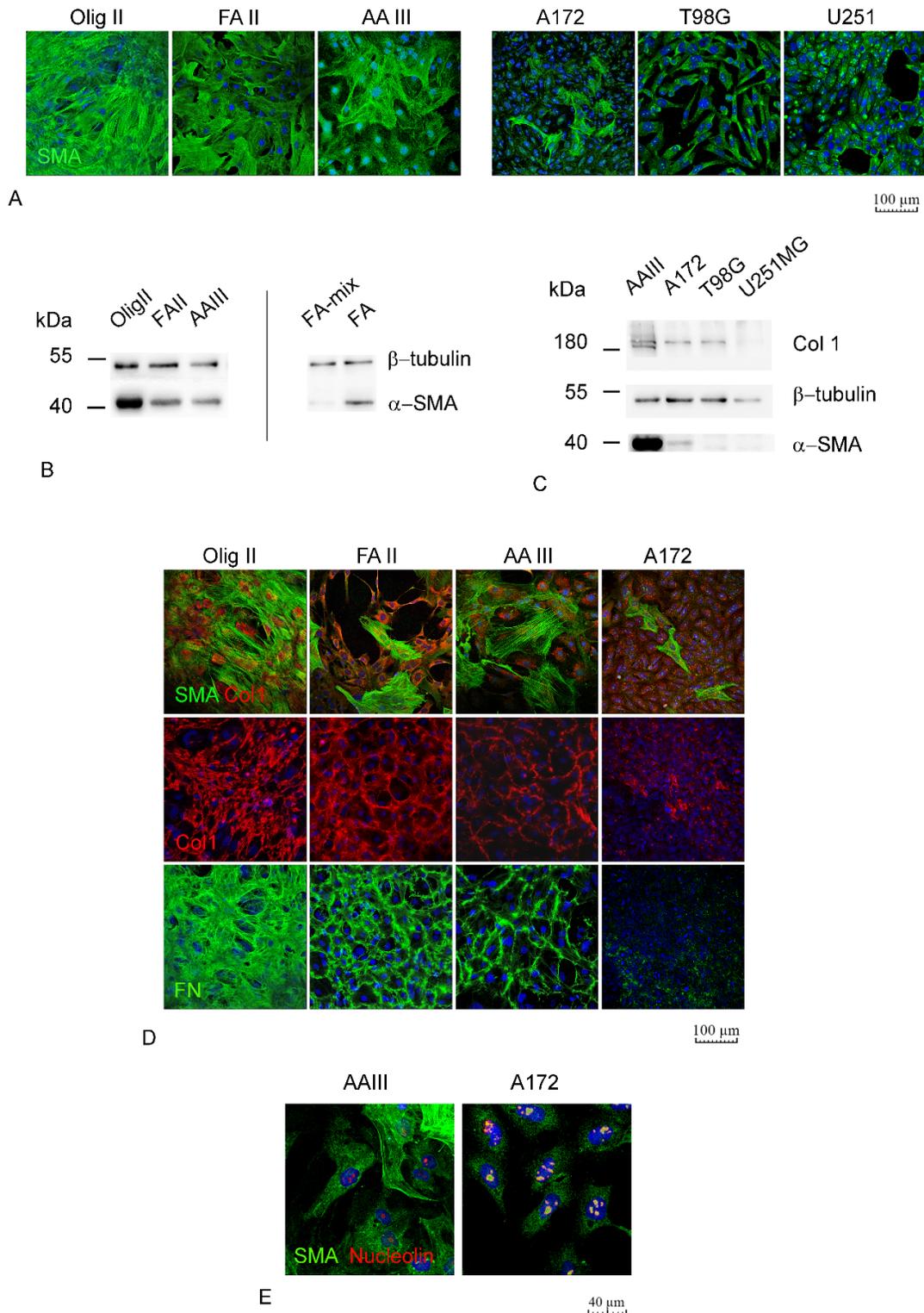


Figure 3. α -SMA⁺ stromal cell component of primary cultures and permanent glioma cell lines, their fibrotic potential. (A) Immunocytochemical staining of α -SMA in OligII, FAII, and AAIII cultures and glioma cell lines. (B) Western blot analysis of α -SMA in OligII, FAII, AAIII, FAI mix cells and glioma cell lines. (C) Western blot analysis of α -SMA and collagen type I in AAIII culture and glioma cell lines. (D) Immunocytochemical staining of α -SMA and collagen type I (upper horizontal line) intracellularly after 3 days in culture, and collagen type I (central horizontal line) and fibronectin (bottom horizontal line) extracellularly after 15 days in culture. (E) Double immunocytochemical staining of α -SMA and nucleolin in AAIII and A172 cells. Localization of α -SMA in nucleoli of A172 and no staining in the AAIII cells.

of origin for glioma^[5-8] and can express α -SMA^[24-26], we attempted to determine whether glioma cells express α -SMA. Immunostaining of α -SMA in three permanent glioma lines A172, T98G, and U251MG revealed that the A172 cell line contained a few α -SMA⁺ cells with their numbers increasing over the time spent in culture (Figure 3A). A172 cells without α -SMA fibers showed negligible α -SMA staining in the cytoplasm and distinct spotted signal in the nucleus. The presence of α -SMA⁺ cells pointed to the basic opportunity of glioma cells to synthesize α -SMA and became additional proof for the neural stem/progenitor cell nature of glioma cells. Although T98G and U251MG lines did not have cells containing α -SMA-filaments they exhibited a homogeneous strong α -SMA staining in the cytoplasm as well as a spotted α -SMA staining in the nucleus (Figure 3A).

Western blot analysis revealed a very weak α -SMA band in A172 cells that apparently correspond to α -SMA⁺ cells, and did not reveal α -SMA full-length protein (43 kDa) in T98G and U251MG lines in which the α -SMA⁺ cells were absent (Figure 3C). Taking into account that T98G and U251MG cells have mutations and A172 cells have a deletion in tumor suppressor PTEN gene^[38] which is located side by side with Acta2 gene encoding α -SMA, we speculate that α -SMA protein could also be altered so that F-actin assembly was disrupted in permanent glioma lines. We speculate further that this altered protein of α -SMA might not be revealed in denatured form by electrophoresis but might be stained in native form by immunocytochemistry.

In order to estimate the fibrotic potential of tumor cells, the intracellular synthesis of collagen type I and fibronectin and their deposition in ECM were compared between A431 cells and A172, T98G, and U251MG cell lines by Western blotting and immunofluorescence (Figure 3C, 3D). Western blot analysis revealed a more reduced intracellular synthesis of collagen type I in the permanent cell lines compared to A431 cells (Figure 3C). The deposition of collagen type I and fibronectin by A172 cells at the 15th day of culturing was also significantly reduced compared with glioma-derived cells (Figure 3D).

Because a strong α -SMA spotted positivity was observed in the nucleolar area in all permanent cell lines, we performed co-immunostaining of α -SMA with nucleolin as a marker for nucleoli (Figure 3E). The results clearly showed that nuclear α -SMA was localized exclusively in the nucleoli. Of note, the α -SMA⁺ A172 cells also exhibited distinct nucleolar α -SMA localization. Nucleolar localization of α -SMA was also detectable in glioma-derived cells, although the fluorescent intensity was much lower, sometimes barely visible. These results suggest that

α -SMA-altered forms could perform cellular functions differently from wild-type α -SMA. Nucleolar localization of α -SMA could be associated with clonal properties of the long-term culturing permanent cell lines and, therefore, with a more proliferative phenotype. Similarly, nucleolar localization of many proteins occurs in highly proliferating cells. Furthermore, the presence of β -actin and non-muscle myosin 1 was shown in the nucleoli of HeLa cells where they were involved in the regulation of RNA synthesis^[39].

The stromal glioma-derived cells express neural stem/progenitor, glial and mesenchymal markers

As α -SMA⁺ stromal cells growing from pieces of tumor tissues together with glioma cells did not have a mesenchymal stem or endothelial/subendothelial cell nature according to FACS analysis (Figure 1B), we assumed their neural stem/progenitor cell origin. Immunocytochemical staining of GFAP and CNPase, astrocyte and oligodendrocyte markers respectively, revealed co-expression of these glia proteins in all cells of glioma-derived cell cultures pointing to their glia precursor cell origin (Figure 4A). CNPase, the enzyme related to multiple cellular functions, displayed a distinct fluorescent signal and was typical for glial cells mitochondrial localization. Well-organized GFAP intermediate filaments were mostly seen in OligII cells. The majority of the astrocytoma cells showed diffuse staining of GFAP. RT-PCR amplification revealed expression of glia progenitor's markers GFAP, NG2, as well as mesenchymal markers vimentin and CD44 in the glioma-derived cell cultures (Figure 4B).

To be sure that α -SMA⁺ stromal cells indeed produce the neural stem/progenitor cell proteins, double immunostaining of α -SMA together with proteins related with low or either high malignant phenotype was performed. GFAP, the astrocyte marker, was stained in all α -SMA⁺ cells of all cultures, and even a strong staining signal was revealed in few cells (data not shown). Co-staining with nestin, the protein associated with high malignancy, revealed ambiguous results (Figure 4C). On the one hand, OligII α -SMA⁺ cells co-expressed nestin highly. The A431 and FAII astrocytoma cells showed an unaltered or decreased level of nestin staining in α -SMA⁺ cells. Cells without α -SMA synthesis found only in A431 culture showed a high level of nestin. This tumor cell population may potentially have more invasive features.

Furthermore, RT-PCR analysis revealed that all glioma-derived cell cultures expressed transcripts encoding CD133 which are specific for cancer and neural stem cells (Figure 4B). We performed magnetic cell sorting of A431 and OligII cultures and revealed that A431-CD133- and

OligII-CD133-enriched fraction reached no less than 10% of the total cell population. Following immunofluorescence showed that the cells displayed typical membranous fluorescent signal as well as distinct atypical nuclear localization of CD133. Unexpectedly, the AAIII-CD133-enriched fraction turned out similar to AAIII culture in terms of the presence of α -SMA+ cells. Double immunostaining of α -SMA and CD133 showed co-expression of these proteins (Figure 4D). However, α -SMA+ cells showed often more weak or negligible dotted signal of CD133 than cells

with diffuse staining of α -SMA. Thus, CD133 expression seems to be inhibited in the α -SMA+ astrocytoma-derived cells. However, CD133+ OligII stromal cells that are total α -SMA+ cell population according to previous results (Figure 3A) showed distinct dotted signal in nuclei. Despite these differences, taken together, these results demonstrate that the stromal cells showed phenotypic mimicry with tumor cells.

4. Discussion and Conclusions

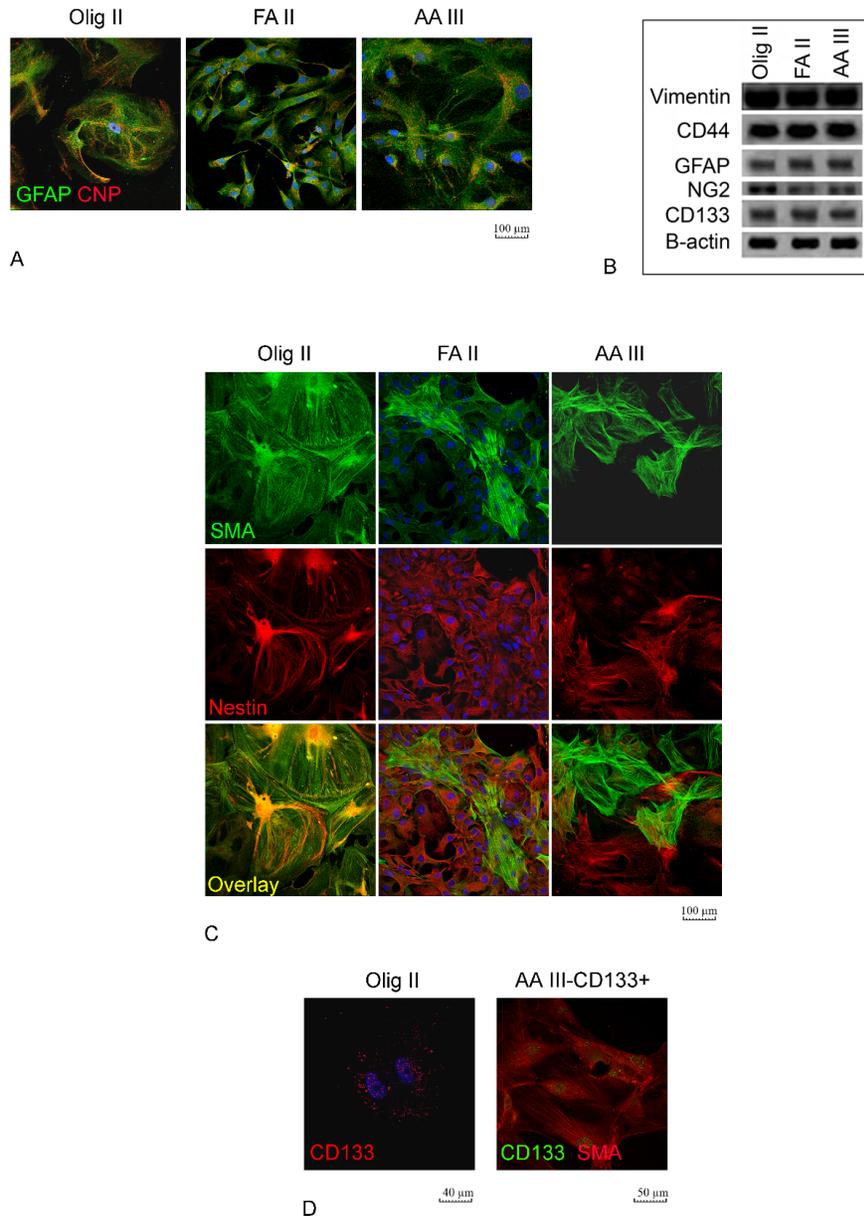


Figure 4. Expression of neural stem/progenitor, glial and mesenchymal markers in glioma-derived cells. (A) Co-expression of GFAP and CNPase in OligII, FAII, and AAIII cells. (B) RT-PCR assay of expression of mesenchymal, glial progenitor's markers and CD133 in the glioma-derived cultures. (C) Co-expression of α -SMA and nestin in glioma-derived cells. α -SMA expression is absent in some the nestin-positive AAIII cells. (D) Expression of CD133 in OligII stromal cells and in α -SMA+ cells from CD133-enriched AAIII culture.

In this study, we isolated a mixed population of tumor and stromal cells which rapidly grew out from the tumor micro fragments. They shared a similar phenotype and were undistinguishable morphologically at the zero passage. As the tumor cells, the stromal cells showed a high expression of mesenchymal markers vimentin and CD44 as well as nestin, the protein associated with neuroepithelial development. Additional expression of both GFAP and CNPase, astrocytic and oligodendrocytic markers respectively, and other glia progenitor protein such as NG2, demonstrated the same discordant phenotype for the stromal cells as glioma cells have^[40]. Moreover, the stromal cells expressed CD133, the protein associated with stemness which is not found in MSC and GASC^[41-43]. All these facts indicate that this type of stromal cells could originate from the same lineage as the tumor cells.

NSCs from the subventricular zone and glia progenitors from subcortical white matter are discussed as the most likely cell of origin for glioma^[5-8]. After an expansion of transformed cells, “normal” neural stem or progenitor cells may be induced to proliferation by glioma cells and migrate together with them to form tumor mass. The presence of NSCs in the tumor bulk has been revealed in PDGF-induced gliomas, along with that tropism of “normal” NSCs and glia progenitors from the adult brain to glioma was demonstrated in orthotopic xenograft models^[12-15]. The stromal cells may constitute a considerable part of the tumor mass. Thus, it has been shown that 90% of glioma specimens are composed of the cells that expressed A2B5, the marker of glia progenitors from the adult forebrain^[6,7]. At the same time, mimicry of many glioma-associated markers can make them poorly distinguishable by immunohistochemical analysis. We first showed that the stromal cells with neural stem/progenitor-like cell phenotype can be easily isolated from tumor specimens together with tumor cells and expanded in culture. Interestingly, the ratio of tumor and stromal cells maintained stable through many passages with the predominance of stromal cells. This could result from the significant growth potential of both cell types maintained by crosstalk between them.

Myofibroblastic phenotype accompanied by α -SMA expression is the main sign of the stromal cells in glioma-derived cultures. This feature of the stromal cells with neural stem/progenitor-like cell phenotype is not contradictory considering that there are glia progenitor cells in the embryonic and neonatal brain that express α -SMA and exhibit myofibroblastic features^[24-26]. Other cells of neural origin capable to produce α -SMA are reactive astrocytes from the adult brain that can gain myofibroblastic phenotype induced by inflammatory cues in response to injury^[27].

A high concentration of the same inflammatory cytokines is detected in glioma bulk and peritumoral space and may stimulate the formation of cells with myofibroblastic phenotype. However, α -SMA immunoreactivity is almost exclusively revealed in the blood vessels on immunohistochemical sections of glioma^[16]. It means that our finding is a cultural phenomenon and *in vivo*, the stromal cells with neural stem/progenitor-like cell phenotype either do not switch on the α -SMA expression or express this protein on a very low level. But we assume that even with the low level of α -SMA expression the stromal cells in glioma may maintain deposition of ECM and contribute to fibrotic processes in glioma.

The expression of α -SMA is thought to be absent in glioma cells, but taking into consideration a possible origin of glioma cells from NSC or glia progenitors we studied whether glioma cells could express this protein. We found that tumor cells showed diffuse α -SMA immunostaining in the cytoplasm from very weak in A172 cells and moderate in astrocytoma-derived tumor cells up to strong in T98G cells. Moreover, a few of the A172 cells demonstrated the ability to form α -SMA filaments. U251MG cells had strong focal α -SMA staining. In addition, distinct spotted staining in the nucleoli was seen in permanent cell lines. The resembling variable diffuse or focal cytoplasmic staining of α -SMA has been revealed in neoplastic cells of gastrointestinal stromal tumors^[43,44]. HeLa cells show a characteristic spot-like pattern of β -actin and non-muscle myosin 1 in the nucleoli where the actin-myosin complex has been shown to regulate mRNA synthesis^[26]. Nevertheless, western blot analysis did not reveal full-length α -SMA (43kD) in T98g and U251MG cell lines in which the α -SMA+ cells are absent. In T98g and U251MG cells, α -SMA could be represented by altered monomeric forms which are not recognized by antibodies against wild-type α -SMA during western blot analysis. This assumption is consistent with the fact that the ACTA2 gene encoding α -SMA is located on a long arm of chromosome 10 in one of the most mutagen regions typical for gliomas^[34,45,46]. Anaplastic astrocytomas and glioblastomas progressing from low-grade astrocytomas often show a reduced number of chromosome segments on 10q^[47]. It is also noteworthy that the gene of tumor suppressor, PTEN, which is the most frequently lost in gliomas^[46,48], is adjacent to the ACTA2 gene^[45]. Like PTEN, the ACTA2 gene must have massive mutagenic pressure. In particular, all three permanent glioma cell lines used in the current work have abnormal PTEN alleles^[38]. Mutations or deletions of α -SMA gene in gliomas could disrupt the filament formation in the same way as it occurs in smooth muscle cells or as it has been demonstrated for β -actin^[50-52].

It is remarkable that the p53 tumor suppressor has the ability to directly activate the transcription of the α -SMA gene [35]. Thus, the mutations in the p53 gene can affect α -SMA expression and they are actually the genetic hallmarks of secondary glioblastomas. At the same time, the entire loss of chromosome 10 containing the ACTA2 gene is typical for primary glioblastomas [34,53-54]. Taken together these facts may imply that the loss or decrease of α -SMA expression with disruption of fiber assembly are factually obligate for glioblastomas and seems to play a critical role in malignant transformation. Indeed, α -SMA inhibits both migration and proliferation of normal and transformed cells due to the forming of focal adhesions and by preventing the activation of small GTPase Rac1 [55-56]. At the same time, the deposition of ECM which is an attribute of α -SMA+ cells in many tissues can also inhibit migration [57-58]. Loss of α -SMA studied in ACTA2 mutant smooth muscle cells induced their proliferation through FAK and Rac1 activation, translocation of p53 from the nucleus to the cytoplasm, and increased expression and ligand-independent activation of PDGF receptor β [59]. On the other hand, in the case of disruption of microfilament bundle assembly due to missense ACTA2 mutations, an increased pool of monomeric actin leads to additional proliferative response through binding G-actin with MRTF-A, a member of the myocardin family of transcriptional coactivators. Thereby it allows SRF to bind to growth responsive genes [52,60]. The proliferative pathways induced by monomeric α -SMA might be the same in malignant cancer cells. In addition, monomeric α -SMA apparently stimulates motility: the presence of α -SMA has been shown in the leading edge of astroglia lamellipodia [25]. Therefore, we hypothesize that the existence of monomeric α -SMA in the tumor cells of anaplastic astrocytomas and benign gliomas could be a significant pathogenetic and prognostic sign.

There is a possibility that similar disruptions of α -SMA expression occur in the stromal component of gliomas. Fomchenko et al. [13] have demonstrated that genetic aberrations typical for glioma cells may be acquired by normal cells recruited into the tumor. Disruptions of α -SMA expression imply that myofibroblastic functions including ECM deposition can be impaired. ECM in turn may restrain tumor spread and so the impairment of its deposition facilitates tumor growth. Growing disturbances of α -SMA expression in stromal cells with increasing malignancy grade would induce their proliferation and, in fact, remove the main differences between stromal cells with neural stem /progenitor-like phenotype and glioma cells.

In this report, we have raised the question about a possible role of α -SMA breakdown in gliomagenesis. Taken

together our findings and published literature data suggest that α -SMA may be one of the main molecule bearing protective functions, and its loss, as well as microfilament assembly disruptions in tumor and stromal cells, seems to be fatal in the way of glioma progression.

Ethics Approval and Consent to Participate

This study was conducted with the approval of the ethics committee of the Polenov Neurosurgical Institute at Almazov National Medical Research Centre and all participants signed an informed consent document.

Consent to Publish

All participants signed an informed consent document.

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Authors' Contributions

IC, IG, VP, ET carried out the study concept, design and analysis and interpretation of all data; VZ did the flow cytometry and analysis of data. IC and AM did the microscopy and histological analysis of data; YL were involved in the treatment of patients and interpretation of clinical data. IG, IC, ET, SK, SP, and AP drafted the manuscript. All authors participated in final approval of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References

- [1] Codrici E, Enciu AM, Popescu ID, Mihai S, Tanase C. Glioma Stem Cells and Their Microenvironments: Providers of Challenging Therapeutic Targets. *Stem Cells Int.*, 2016, 2016: 5728438. DOI: 10.1155/2016/5728438.
- [2] Clavreul A., Menei P. Mesenchymal Stromal-Like Cells in the Glioma Microenvironment: What Are These Cells? *Cancers (Basel)*, 2020, 12(9): 2628. DOI: 10.3390/cancers12092628.
- [3] LeBleu VS, Kalluri R. A peek into cancer-associated fibroblasts: origins, functions and translational impact. *Dis Model Mech.*, 2018, 11(4): dmm029447. DOI: 10.1242/dmm.029447.
- [4] Svensson A., Ramos-Moreno T., Eberstål S., Sched-

- ing S., Bengzon J. Identification of two distinct mesenchymal stromal cell populations in human malignant glioma. *J Neurooncol.*, 2017, 131(2): 245 -254. DOI: 10.1007/s11060-016-2302-y.
- [5] Modrek AS, Bayin NS, Placantonakis DG. Brain stem cells as the cell of origin in glioma. *World J Stem Cells*, 2014, 6(1): 43 -52. DOI: 10.4252/wjsc.v6.i1.43.
- [6] Ogden AT, Waziri AE, Lochhead RA, Fusco D et al. Identification of A2B5+CD133- Tumor-Initiating Cells in Adult Human Gliomas. *Neurosurgery*, 2008, 62: 505 -515. DOI: 10.1227/01.neu.0000316019.28421.95.
- [7] Rebetz J, Tian D, Persson A, et al. Glial progenitor-like phenotype in low-grade glioma and enhanced CD133-expression and neuronal lineage differentiation potential in high-grade glioma. *PLoS One*, 2008, 3(4): e1936. DOI: 10.1371/journal.pone.0001936.
- [8] Lindberg N, Kastemar M, Olofsson T et al. Oligodendrocyte progenitor cells can act as cell of origin for experimental glioma. *Oncogene*, 2009, 28(23): 2266 -2275. DOI: 10.1038/onc.2009.76.
- [9] Bexell D, Gunnarsson S, Nordquist J and Bengzon J. Characterization of the subventricular zone neurogenic response to rat malignant brain tumors. *Neuroscience*, 2007, 147(3): 824-32. DOI: 10.1016/j.neuroscience.2007.04.058.
- [10] Aboody KS, Brown A, Rainov NG, et al. Neural stem cells display extensive tropism for pathology in adult brain: evidence from intracranial gliomas. *Proc Natl Acad Sci U S A*, 2000, 97(23): 12846 -12851. DOI: 10.1073/pnas.97.23.12846.
- [11] Najbauer J, Huszthy PC, Barish ME, et al. Cellular host responses to gliomas. *PLoS One*. 2012;7:e35150. DOI: 10.1371/journal.pone.0035150.
- [12] Hambarzumyan D, Cheng YK, Haeno H, et al. The probable cell of origin of NF1- and PDGF-driven glioblastomas. *PLoS One*, 2011, 6(9): e24454. DOI: 10.1371/journal.pone.0024454.
- [13] Fomchenko EI, Dougherty JD, Helmy KY, et al. Recruited cells can become transformed and overtake PDGF-induced murine gliomas in vivo during tumor progression. *PLoS One*, 2011, 6(7): e20605. DOI: 10.1371/journal.pone.0020605.
- [14] Jackson EL, Garcia-Verdugo JM, Gil-Perotin S, Roy M, et al. PDGFR alpha-positive B cells are neural stem cells in the adult SVZ that form glioma-like growths in response to increased PDGF signaling. *Neuron*, 2006, 51(2): 187 -99. DOI: 10.1016/j.neuron.2006.06.012.
- [15] Assanah M, Lochhead R, Ogden A, et al. Glial progenitors in adult white matter are driven to form malignant gliomas by platelet-derived growth factor-expressing retroviruses. *J Neurosci.*, 2006, 26(25): 6781 -6790. DOI: 10.1523/JNEUROSCI.0514-06.2006.
- [16] Talasila KM, Brekka N, Mangseth K, et al. Tumor versus stromal cells in culture--survival of the fittest? *PLoS One*, 2013, 8(12): e81183. DOI: 10.1371/journal.pone.0081183.
- [17] Kalluri R. The biology and function of fibroblasts in cancer *Nat Rev Cancer*, 2016, 16(9): 582-98. DOI: 10.1038/nrc.2016.73.
- [18] Yoo JE, Kim YJ, Rhee H, et al. Progressive Enrichment of Stemness Features and Tumor Stromal Alterations in Multistep Hepatocarcinogenesis. *PLoS One*, 2017, 12(1): e0170465. DOI: 10.1371/journal.pone.0170465.
- [19] Öhlund D, Handly-Santana A, Biffi G, et al. Distinct populations of inflammatory fibroblasts and myofibroblasts in pancreatic cancer. *J Exp Med.*, 2017, 214(3): 579 -596. DOI: 10.1084/jem.20162024.
- [20] Holm Nielsen S, Willumsen N, Leeming DJ, et al. Serological Assessment of Activated Fibroblasts by alpha-Smooth Muscle Actin (α -SMA): A Noninvasive Biomarker of Activated Fibroblasts in Lung Disorders. *Transl Oncol.*, 2019, 12(2): 368 -374. DOI: 10.1016/j.tranon.2018.11.004.
- [21] Valcz G, Sipos F, Krenács T, et al. Increase of α -SMA(+) and CK (+) cells as an early sign of epithelial-mesenchymal transition during colorectal carcinogenesis. *Pathol Oncol Res.*, 2012, 18(2): 371-6. DOI: 10.1007/s12253-011-9454-z.
- [22] Emon B, Bauer J, Jain Y, Jung B, Saif T. Biophysics of Tumor Microenvironment and Cancer Metastasis - A Mini Review. *Comput Struct Biotechnol J.*, 2018, 16: 279 -287. DOI: 10.1016/j.csbj.2018.07.003.
- [23] Kojima Y, Acar A, Eaton EN, Mellody KT, Scheel C, Ben-Porath I, Onder TT, Wang ZC, Richardson AL, Weinberg RA, et al. Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. *Proc Natl Acad Sci U S A*, 2010, 107(46): 20009 -20014. DOI: 10.1073/pnas.1013805107.
- [24] Abd-el-Basset EM and Fedoroff S. Immunolocalization of the alpha isoform of smooth muscle actin in mouse astroglia in cultures. *Neurosci Lett.*, 1991,

- 125(2): 117-20.
DOI: 10.1016/0304-3940(91)90005-e.
- [25] Abd-El-Basset EM. The effect of dibutyryl cyclic AMP on the expression of actin isoforms in astroglia. *Histochem J.* 2000;32:581-90.
DOI: 10.1023/a:1026738600838.
- [26] Lecain E, Alliot F, Laine MC, Calas B and Pessac B: α Isoform of smooth muscle actin is expressed in astrocytes in vitro and in vivo. *J Neurosci Res.* 1991;28:601-6.
DOI: 10.1002/jnr.490280417.
- [27] Moreels M, Vandenabeele F, Dumont D, Robben J and Lambrechts I. Alpha-smooth muscle actin (α -SMA) and nestin expression in reactive astrocytes in multiple sclerosis lesions: Potential regulatory role of transforming growth factor-beta 1 (TGF- β 1). *Neuropathol Appl Neurobiol.* 2008;34:532-46.
DOI: 10.1111/j.1365-2990.2007.00910.x.
- [28] Frei K, Gramatzki D, Tritschler I, Schroeder JJ, Espinoza L, Rushing EJ, Weller M. Transforming growth factor- β pathway activity in glioblastoma. *Oncotarget.* 2015;6(8):5963-77.
DOI: 10.18632/oncotarget.3467.PMID: 25849941.
- [29] Pogoda K., Janmey P. A. Glial Tissue Mechanics and Mechanosensing by Glial Cells *Front Cell Neurosci.* 2018; 12: 25.
DOI: 10.3389/fncel.2018.00025.
- [30] Leavitt J, Gunning P, Kedes L, Jariwalla R. Smooth muscle alpha-actin is a transformation-sensitive marker for mouse NIH 3T3 and Rat-2 cells. *Nature.* 1985;316(6031):840-2.
- [31] Kumar CC, Bushel P, Mohan-Peterson S, Ramirez F. Regulation of smooth muscle alpha-actin promoter in ras-transformed cells: usefulness for setting up reporter gene-based assay system for drug screening. *Cancer Res.* 1992;52(24):6877-84.
- [32] Bushel P, Kim JH, Chang W, Catino JJ, Ruley HE, Kumar CC. Two serum response elements mediate transcriptional repression of human smooth muscle alpha-actin promoter in ras-transformed cells. *Oncogene.* 1995;10(7):1361-70.
- [33] Okamoto-Inoue M, Kamada S, Kimura G, Taniguchi S. The induction of smooth muscle alpha actin in a transformed rat cell line suppresses malignant properties in vitro and in vivo. *Cancer Lett.* 1999;142(2):173-8.
- [34] Fujisawa, H., Reis, R., Nakamura, M. et al. Loss of Heterozygosity on Chromosome 10 Is More Extensive in Primary (De Novo) Than in Secondary Glioblastomas. *Lab Invest.* 2000;80:65 -72. doi.org/10.1038/labinvest.3780009.
- [35] Comer, K., Dennis, P., Armstrong, L. et al. Human smooth muscle α -actin gene is a transcriptional target of the p53 tumor suppressor protein. *Oncogene.* 1998;16:1299 -1308. <https://doi.org/10.1038/sj.onc.1201645>.
- [36] Russell KC, Phinney DG, Lacey MR, Barrilleaux BL, et al. In vitro high-capacity assay to quantify the clonal heterogeneity in trilineage potential of mesenchymal stem cells reveals a complex hierarchy of lineage commitment. *Stem Cells.* 2010;28:788-98.
DOI: 10.1002/stem.312.
- [37] Klink B, Miletic H, Stieber D, et al. A novel, diffusely infiltrative xenograft model of human anaplastic oligodendroglioma with mutations in FUBP1, CIC, and IDH1. *PLoS One.* 2013;8:e59773.
DOI: 10.1371/journal.pone.0059773.
- [38] Adachi J, Ohbayashi K, Suzuki T and Sasaki T: Cell cycle arrest and astrocytic differentiation resulting from PTEN expression in glioma cells. *J Neurosurg.* 1999;91:822-30.
DOI: 10.3171/jns.1999.91.5.0822.
- [39] Fomproix N and Percipalle P. An actin-myosin complex on actively transcribing genes. *Exp Cell Res.* 2004 10;294:140-8.
DOI: 10.1016/j.yexcr.2003.10.028.
- [40] Vogel W, Grünebach F, Messam CA, Kanz L, et al. Heterogeneity among human bone marrow-derived mesenchymal stem cells and neural progenitor cells. *Haematologica.* 2003;88:126-33.
- [41] Bourkoula E, Mangoni D, Ius T, et al.: Glioma-associated stem cells: A novel class of tumor-supporting cells able to predict prognosis of human low-grade gliomas. *Stem Cells.* 2014;32:1239-53.
DOI: 10.1002/stem.1605.
- [42] Bataller R, Paik YH, Lindquist JN, et al. Hepatitis C Virus Core and Nonstructural Proteins Induce Fibrogenic Effects in Hepatic Stellate Cells. *Gastroenterology.* 2004;126:529-40.
DOI: 10.1053/j.gastro.2003.11.018.
- [43] Hafner S, Harmon BG and King T. Gastrointestinal stromal tumors of the equine cecum. *Vet Pathol.* 2001 Mar;38:242-6.
DOI: 10.1354/vp.38-2-242.
- [44] Muravnick KB, Parente EJ and Del Piero F: An atypical equine gastrointestinal stromal tumor. *J Vet Diagnostic Investig.* 2001;38:689-97.
DOI: 10.1354/vp.38-6-689.
- [45] Ueyama H, Bruns G and Kanda N. Assignment of the vascular smooth muscle actin gene ACTSA to human chromosome 10. *Jpn J Hum Genet.* 1990;35:145-50.
DOI: 10.1007/BF01876459.

- [46] Maier D, Zhang Z, Taylor E, et al. Somatic deletion mapping on chromosome 10 and sequence analysis of PTEN/MMAC1 point to the 10q25-26 region as the primary target in low-grade and high-grade gliomas. *Oncogene*. 1998 ;16:3331-5.
DOI: 10.1038/sj.onc.1201832.
- [47] Weber RG, Sabel M, Reifenberger J, et al. Characterization of genomic alterations associated with glioma progression by comparative genomic hybridization. *Oncogene*. 1996;13:983-94.
- [48] Tohma Y, Gratas C, Biernat W, et al. PTEN (MMAC1) mutations are frequent in primary glioblastomas (de novo) but not in secondary glioblastomas. *J Neuro-pathol Exp Neurol*. 1998;57:684-9.
DOI: 10.1097/00005072-199807000-00005.
- [49] Hossain A, Gumin J, Gao F, et al. Mesenchymal Stem Cells Isolated From Human Gliomas Increase Proliferation and Maintain Stemness of Glioma Stem Cells Through the IL-6/gp130/STAT3 Pathway. *Stem Cells*. 2015;33:2400 -2415.
DOI: 10.1002/stem.2053.
- [50] Konopka G, Bonni A. Signaling pathways regulating gliomagenesis. *Curr Mol Med.*, 2003, 3(1): 73-84.
DOI: 10.2174/1566524033361609.
- [51] Guo DC, Papke CL, Tran-Fadulu V, et al. Mutations in smooth muscle alpha-actin (ACTA2) cause coronary artery disease, stroke, and Moyamoya disease, along with thoracic aortic disease. *Am J Hum Genet*. 2009;84:617 -627.
DOI: 10.1016/j.ajhg.2009.04.007.
- [52] Posern G, Sotiropoulos A, Treisman R. Mutant actins demonstrate a role for unpolymerized actin in control of transcription by serum response factor. *Mol Biol Cell*. 2002;13(12):4167-78.
- [53] Kleihues P, Ohgaki H. Primary and secondary glioblastomas: from concept to clinical diagnosis. *Neuro Oncol*. 1999;1:44 -51.
DOI: 10.1093/neuonc/1.1.44.
- [54] Ichimura K, Schmidt EE, Miyakawa A, Goike HM and Collins VP. Distinct patterns of deletion on 10p and 10q suggest involvement of multiple tumor suppressor genes in the development of astrocytic gliomas of different malignancy grades. *Genes Chromosom Cancer*. 1998;22:9-15.
DOI: 10.1002/(sici)1098-2264(199805)22:1<9::aid-gcc2>3.0.co;2-1.
- [55] Chen L, DeWispelaere A, Dastvan F, et al. Smooth Muscle-Alpha Actin Inhibits Vascular Smooth Muscle Cell Proliferation and Migration by Inhibiting Rac1 Activity. *PLoS One*. 2016;11:e0155726.
DOI: 10.1371/journal.pone.0155726.
- [56] Rønnev-Jessen L and Petersen OW. A function for filamentous α -smooth muscle actin: Retardation of motility in fibroblasts. *J Cell Biol*. 1996;134:67-80.
DOI: 10.1083/jcb.134.1.67.
- [57] Sabari J, Lax D, Connors D, et al. Fibronectin matrix assembly suppresses dispersal of glioblastoma cells. *PLoS One*. 2011;6:e24810.
DOI: 10.1371/journal.pone.0024810.
- [58] Shannon S, Vaca C, Jia D, et al. Dexamethasone-Mediated Activation of Fibronectin Matrix Assembly Reduces Dispersal of Primary Human Glioblastoma Cells. *PLoS One*. 2015;10:e0135951.
DOI: 10.1371/journal.pone.0135951.
- [59] Papke CL, Cao J, Kwartler CS, et al. Smooth muscle hyperplasia due to loss of smooth muscle α -actin is driven by activation of focal adhesion kinase, altered p53 localization and increased levels of platelet-derived growth factor receptor- β . *Hum Mol Genet*. 2013;22:3123 -3137.
DOI: 10.1093/hmg/ddt167.
- [60] Zaromytidou AI, Miralles F, Treisman R. MAL and ternary complex factor use different mechanisms to contact a common surface on the serum response factor DNA-binding domain. *Mol Cell Biol*. 2006;26:4134 -4148.
DOI: 10.1128/MCB.01902-05.