Spontaneous *In Vitro* Senescence of Glioma Cells Confirmed by an Antibody Against IDH1^{R132H}

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Abstract. *Background: We have recently suggested that glioblastoma cells become spontaneously senescent in cell culture conditions. The antibody specific against IDH1^{R132H} offers the perfect opportunity to verify this hypothesis. Materials and Methods: We analyzed the features of senescence in 8 glioma cell cultures showing the IDH1^{R132H} mutation based on combination of immunocytochemistry, enzymocytochemistry, BrdU incorporation assay and real-time microscopic observation. Results: We report that glioma cells showing the IDH1^{R132H} mutation become rapidly and spontaneously senescent in vitro. Senescence was observed in both classical and novel serum-free cell culture conditions. Importantly, the senescent IDH1^{R132H}-positive cells showed the expression of stemness marker (SOX2). Conclusion: In vitro senescence appeared to be the main reason of the difficulties in any kind culturing of glioma cells. 3D cell cultures prolonged the survival and in vitro proliferation of neoplastic IDH1^{R132H}-positive cells, however, did not enhance the stabilization efficiency. Senescence of glioma cells is spontaneously triggered in vitro, which offers the opportunity of potential new therapeutic strategies based on this phenomenon.*

This article is freely accessible online.

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Key Words: IDH1 gene, glioma, glioblastoma, astrocytoma, senescence, 3D cell culture.

Senescence is currently under detailed investigation from the perspective of tumor biology. The role of this process is enigmatic; from one perspective, an irreversible inhibition of proliferation may potentially be anti-tumorigenic; on the other hand, some data suggest a proinvasive role of senescent cells (1-4). We have recently reported that glioblastoma cells become easily (spontaneously in cell culture conditions) senescent *in vitro* (5). These results inspired the question of senescence in cultured *IDH1*-mutant glioma cells. We based our analyses of senescence in tumor cells on the combination of immunocytochemistry, enzymocytochemistry, BrdU incorporation assay and real-time microscopy. The cells were recognized as neoplastic based on TP53 nuclear accumulation, EGFR overexpression and MAP2/GFAP coexpression. Senescent neoplastic cells showed, apart from the listed markers, the features of senescence such as SA-β-Gal activity and morphological changes. Additionally, supportive data may be extracted from nucleic acid analysis, which, however, were not performed at the single-cell level (5). Such an approach leaves the limited spectrum for uncertainty aside. Moreover, IDH1^{R132H}-specific antibody offers the uncommon possibility to reliably differentiate neoplastic cells and, therefore, is the perfect tool to test the analyzed hypothesis. Additionally, in the present study we performed a comparison of 2D and 3D cultures for the purpose of culturing of IDH1^{R132H}-positive cells isolated from glial tumors.

Materials and Methods

Tumor samples. The analyzed group consisted of 8 consecutive glioma specimens with *IDH1* mutation (R132H) obtained from patients treated at the Department of Neurological Surgery, The Maria

Sklodowska-Curie Regional Specialist Hospital in Zgierz and at the Clinical Department of Neurosurgery, The Voivodal Specialistic Hospital in Olsztyn. All samples were collected using the protocol approved by the Bioethical Committee of the Medical University of Lodz (Approval No RNN/9/10/KE). Written informed consent was obtained from every patient and their data were processed and stored according to the principles expressed in the Declaration of Helsinki. The patients were diagnosed according to the World Health Organization criteria for brain tumor classification; the diagnosis was glioblastoma (3 cases), anaplastic astrocytoma (1 case), diffuse astrocytoma (3 cases) and anaplastic oligodendro-glioma (1 case).

Irrespective of the cell culture type, the isolation of cells from fresh specimens started within 3 h after the neurosurgical operation. Neurosurgical specimens were shipped in 1x Hank's Balanced Salt Solution (PAA, The Cell Culture Company, Austria).

Cell cultures

Classical monolayer conditions. Fresh tissue samples were washed twice with 1× Hank's BSS and centrifuged for 90 s at 80 × *g* each time. Then, the sample was transferred to a 10 cm dish, where it was cut into <1 mm^3 fragments, washed with 1× Hanks' BSS. Tumor cells were dispersed with collagenase type IV (200 U/mL, 37˚C for 6 h; Sigma-Aldrich, USA). The cells were then cultured in αMEM medium (PAA) containing NEAA and supplemented with 10% FBS (Gibco). The total time of isolation and establishment of cell cultures was about seven hours. Depending on the rate of proliferation, the cells were passaged with Trypsin-EDTA (0.05% trypsin; Gibco) to a new culture dish every 7-14 days.

Monolayer conditions in serum-free medium. Fresh tissue samples were washed twice with 1× Hank's BSS and centrifuged for 90 sec at 80 × *g*. Then, the sample was transferred to a 10-cm dish, where it was cut into <1 mm^3 fragments and washed with 1× Hanks' BSS. Tumor cells were dispersed with collagenase type IV (200 U/mL, 37˚C for 6 h). The total time of isolation and establishment of cell cultures was approximately 7 h. The cells were cultured in Neurobasal Medium supplemented with N2 and B27 (0.5× each; Invitrogen), human recombinant bFGF (50 ng/mL; Invitrogen), EGF (50 ng/mL; Invitrogen) and NEAA (1×; Gibco). For monolayer cultures, the plates were pre-coated with a poly-L-lysine/laminin mixture (Invitrogen) as previously reported (6). Monolayer cells were passaged with accutase (Invitrogen) (7). The cells under these conditions were passaged less often (every 3-4 weeks) due to their lower proliferation rate.

3D conditions in serum-free medium. Spheroid cell cultures were performed as neurospheres and as adherent spheres (8-10). Fresh tissue samples were washed twice with 1× Hank's BSS) and centrifuged for 90 s at 80 × *g* each time. Then, the sample was transferred to a 10 cm dish, where it was cut into <1 mm^3 fragments, washed with 1× Hanks' BSS, digested with collagenase type IV/dispase (200 u/mL; Sigma-Aldrich) for 30 min at 37˚C and then filtered using a 70-μm cell strainer (BD Biosciences, USA). Filtered cells were washed twice with 1× Hank's BSS and centrifuged 90 s at 80 × *g* each time and then seeded into 6-well plates at 2,500-5,000 cells/cm^3. Having prepared the medium and reagents before tissue samples processing, the total time of isolation and establishment of spheroid cell cultures was about 1 h.

3-D cultures could not be constructed from astrocytomas as easily as from glioblastomas. Astrocytoma cells did not proliferate efficiently enough to develop spheroids and, therefore, they were cultured as explants, which were then propagated as spheroids.

The culture consisted of Neurobasal Medium with B27 supplement (20 μl/mL; Invitrogen), Glutamax (10 μl/mL; Invitrogen), fibroblast growth factor-2 (20 ng/mL; Invitrogen), NEAA and heparin (2 μg/mL; StemCell Technologies). Growth factors and heparin were renewed twice a week. For the adherent sphere conditions Matrigel covered plates were used and medium was not supplemented with heparin. The spheres were split by mechanical dissociation and transferred to a new dish when they reached the size of 200-500 μm. Cells were cultured at 37˚C in 5% CO_2, 95% humidity and without O_2 control.

$IDH1^{R132H}$ subcloning and transient transfection. RNA from human mammary gland MCF7 cell line was isolated by AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed by QuantiTect Rev. Transcription Kit (Qiagen). Q5® Hot Start High-Fidelity DNA Polymerase (NEB, Massachusetts, USA) was used to amplify *IDH1* gene with Gateway® specific primers: GGGGAC AAGTTTGTACAAAAAAGCAGCGTATGTCCAAAAAAAT CAGTGGCG (forward) and GGGGACCACTTTGTACAAGA AAGCTGGGTTAAAGTTTGGCCTGAGCT AGT (reverse), and Gateway® BP Clonase® II Enzyme mix (Life Technologies, Carlsbad, USA) was used to introduce *IDH1* to pENTR/zeo vector. The sequence was confirmed with Applied Biosystems 3130 Genetic Analyzer. R132H mutation in *IDH1* was inserted by PCR (Q5® Hot Start High-Fidelity DNA Polymerase) with *IDH1* specific primers: ATCATAGGTCATCATGCTTATG (forward) and GATAGGTTTTA CCCATCCAC (reverse). The resulting linear plasmid was ligated by T4 DNA Ligase (NEB) and One Shot® OmniMAX™ 2 T1R Chemically Competent *E. coli* (Life Technologies) were transformed with 3 μl of ligation mix. The plasmid was isolated with NucleoSpin Plasmid (Macherey-Nagel GmbH&Co. KG, Duren, Germany) and sequenced, then Gateway® LR Clonase® II Enzyme mix (Life Technologies) was used to move the IDH1 cassette to pLV/puro-DEST vector. Finally, AD293 cells were transfected with pLV/puro-$IDH1^{R132H}$ using Lipofectamine® LTX (Life Technologies) (Figure 1A-B).

Western blotting. Total cellular protein was isolated from cell cultures using RIPA Lysis and Extraction Buffer supplemented with Halt Protease Inhibitor Cocktail (Thermo Scientific, USA), suspended in 4× Laemmli Sample Buffer (Bio-Rad Laboratories, USA) with β-mercaptoethanol (Sigma, USA) and boiled (95˚C, 5 min). After the separation in 10% SDS-polyacrylamide gel (Rotiphorese Ready-to-Use Gel Solutions; Carl Roth GmbH + Co. KG, Germany) the protein was transferred onto PVDF membrane (Bio-Rad Laboratories, USA) and 5% Skim Milk Powder (Sigma, USA) diluted with TBS-T buffer was used to block nonspecific binding sites (1h, room temperature). PVDF membrane was incubated with the mouse antibody against $IDH1^{R132H}$ (1:1000, overnight, 4˚C; Dianova, USA) and subsequently with the goat anti-mouse IgG-HRP antibody (1:5,000; Santa Cruz Biotechnology, USA). After washing with TBS-T buffer (4×10 min), Opti-4CN Substrate Kit (Bio-Rad Laboratories, USA) was used for HRP visualization (Figure 1C).

Immunocytochemistry. For immunocytochemical analyses cell cultures were fixed for 10 min in 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 for 10 min at room

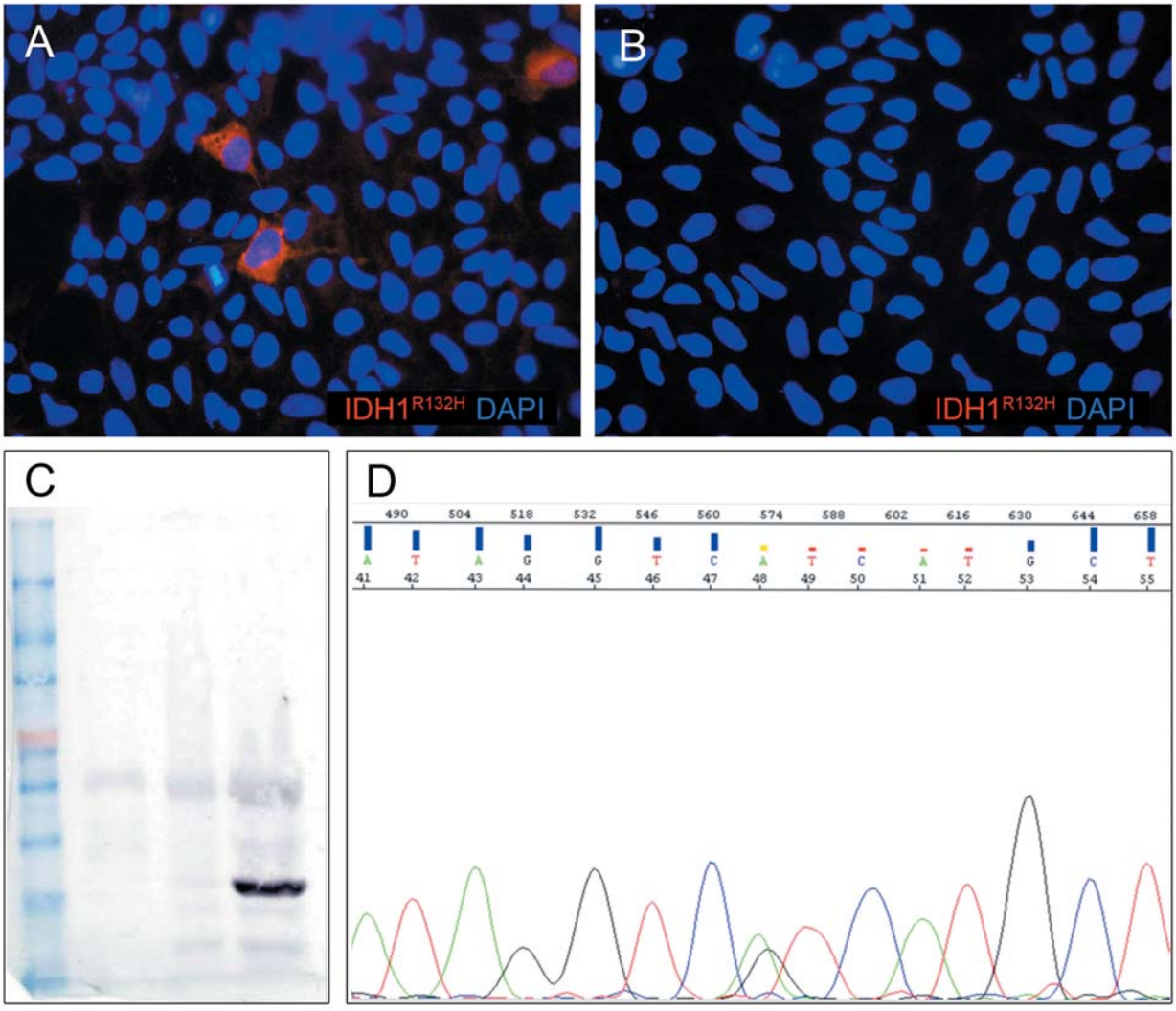

Figure 1. *Verification of the specificity of the antibody against IDH1^{R132H}; exemplary western blot and sequencing results. Positive (A) and negative (B) control for IDH1^{R132H} antibody after transient transfection of AD293 cells with pLV/puro-IDH1^{R132H} plasmid and empty pLV/puro-DEST plasmid, respectively. (C) Exemplary western blot result with IDH1^{R132H} specific antibody: line 1 – ColorPlus™ Prestained Protein Ladder, Broad Range (10-230 kDa) (#P7711S, NEB); line 2 and 3 – negative controls; line 4 – sample with IDH1^{R132H} mutation (46.5 kDa). (D) Exemplary IDH1^{R132H} sequencing result.*

temperature. Non-specific binding sites were blocked by incubation with 2% donkey serum (Sigma) in PBS for 1 h. For double or triple immunolabeling, the fixed cells were subsequently incubated with the following antibodies: (1:50) mouse antibody against IDH1R132H (DIA-H09; Dianova), (1:200) rabbit antibody against TP53 (sc-6243; Santa Cruz Biotechnology), (1:50) goat antibody against GFAP (sc-6171, Santa Cruz Biotechnology) and (1:1000) mouse antibody against αSMA (MAB1420, RD Systems), (1:500) rabbit antibody against SOX2 (AB-5603; Millipore), (1:50) rabbit antibody against Nestin (19483-1-AP; Proteintech Europe) for 1 h at room temperature. Double or triple labeling was visualized by simultaneous incubation with a combination of species-specific fluorochrome-conjugated secondary antibodies: (1:500) donkey anti-rabbit AlexaFluor®488; (1:500) donkey anti-mouse Alexa-Fluor®594 and (1:500) donkey anti-goat Alexa-Fluor® 350 (Molecular Probes, Invitrogen) for 1 h at room temperature. The control samples were incubated with the secondary antibodies-alone or with the matched isotype controls instead of the primary antibody and were otherwise processed identically. The slides were mounted with ProLong® Gold Antifade Reagent or ProLong® Gold Antifade Reagent with DAPI (Molecular Probes), coverslipped and examined using a Nikon fluorescence microscope.

5-Bromo-2'-deoxyuridine incorporation assay (BrdU co-staining). To assess the proliferation rate, 10 μM BrdU was added to the cultures. After 48 h-14 days of incubation the tested cultures were processed for immunocytochemical BrdU co-staining. First, an

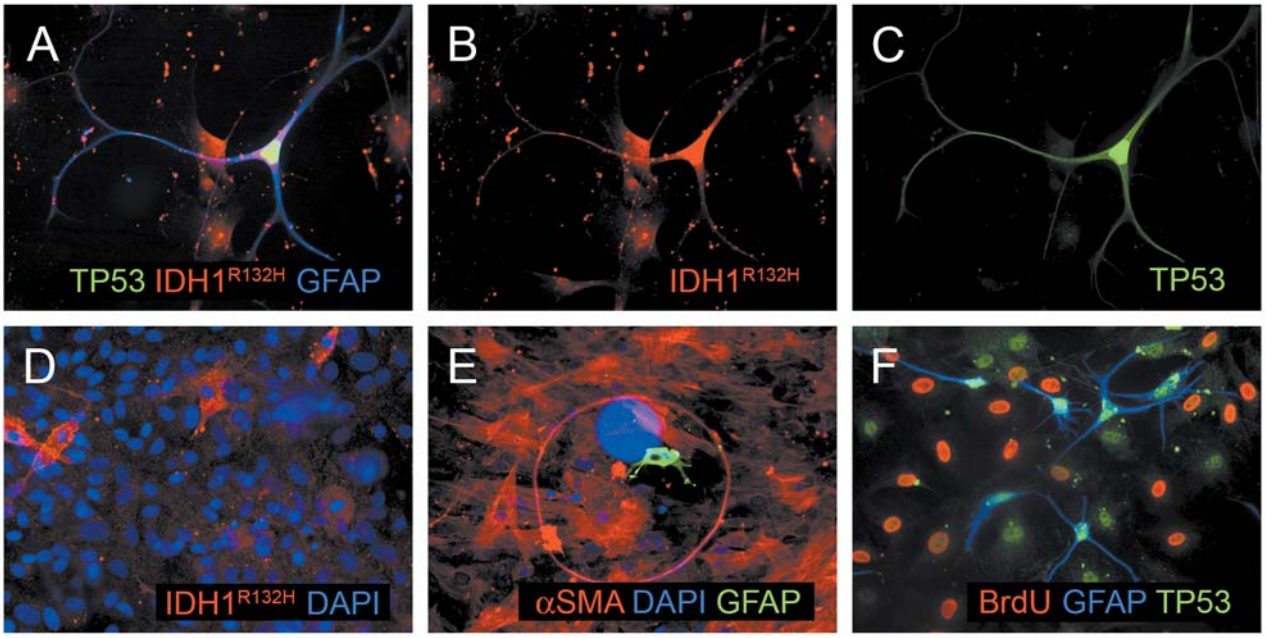

Figure 2. *The classical monolayer conditions. (A-C) IDH1^{R132H}(+) cells accumulate TP53 and show GFAP expression; (D) IDH1^{R132H}(+) cells constitute a minority even before the 1st passage; (E) αSMA-positive cells constitute the majority at passage 1st, few GFAP-positive cells can be observed. (F) GFAP-positive cells do not incorporate BrdU even before the 1st passage.*

immunocytochemical staining for other markers was performed as described, up to the step of PBS washing after the incubation with the secondary antibodies. Next, the cells were post-fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. Non-specific binding sites were blocked by the incubation with 2% donkey serum (Sigma) in PBS for 30 min. After blocking, the cells were treated with 2N HCl in 37˚C for 40 min and then with 0.1 M borate buffer (pH 8.5) at room temperature for 12 min. Then, cells were incubated with mouse anti-BrdU antibody (1:500; B 8434, Sigma-Aldrich) for 1 h, washed with PBS and incubated with the appropriate secondary antibodies at room temperature for 1 h. Finally, cells were mounted with ProLong® Gold Antifade Reagent (Molecular Probes), coverslipped and examined using Nikon fluorescence microscope. For each analysis 200 nuclei were examined.

Senescence-associated (SA)-β-Gal staining. SA-β-Gal staining was performed following the protocol by Dimri *et al*. (11). Cells were washed three times with PBS and fixed with cold 3% paraformaldehyde for 5 min. The cells were than washed two times with PBS for 5 min. Next, a fresh senescence-associated staining solution (1 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, X-Gal in dimethylformamide (stock 20 mg/mL)/40 mM citric acid/sodium phosphate, pH 6.0/5 mM potassium ferrocyanide/5 mM potassium ferricyanide/150 mM NaCl/2 mM $MgCl_2$), pre-warmed to 37˚C, was added and the cells were incubated in 37˚C (no CO_2) for 12 h. After the incubation, the cells were washed two times with PBS for 5 min and photographed using Olympus CKX41 microscope. The percentage of the stained cells was calculated.

DNA/RNA isolation and reverse transcriptase-PCR. Total DNA and RNA were isolated from frozen tissue samples (stored at –80˚C), the corresponding cell cultures and frozen leukocytes from peripheral blood obtained from patients and healthy volunteers using an AllPrep DNA/RNA Mini Kit (Qiagen), according to the manufacturer's protocol. RNA and DNA concentrations were measured spectrophotometrically. 100 ng of total RNA was reverse-transcribed into a single-stranded cDNA using a QuantiTect Rev. Transcription Kit (Qiagen) according to the manufacturer's protocol.

IDH1 sequencing analysis. Exon 4, including codon 132 of the *IDH1* gene, was amplified by PCR and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The primers used for PCR amplification of the DNA sequence were: GGCACCCA TCTTCTGTGTTT (forward) and ATATATGCATTTCTCAATTTCA (reverse). The sequencing primer used was: GCAAAAAT ATCCCCCGGCTT (forward). In order to confirm the result of the sequencing a second primer was used: CGGTCTTCAGA GAAGCCATT (reverse). The sequences were analyzed with the ABI 3130 Genetic Analyzer and DNA Sequencing Analysis Software (Applied Biosystems, Foster City, CA, USA) (Figure 1D).

TP53 sequencing analysis. Exons 5 to 8 of the *TP53* gene were subjected to the sequencing analysis. The primers used for the PCR amplification of cDNA sequences were: GTGCAGCTGTG GGTTGATT (exons 5-8, forward) and GCAGTGCTCGCTTA GTGCTC (exons 5-8, reverse); annealing temperature was 53˚C. The sequencing primers were GCCATCTACAA GCAGTCACA

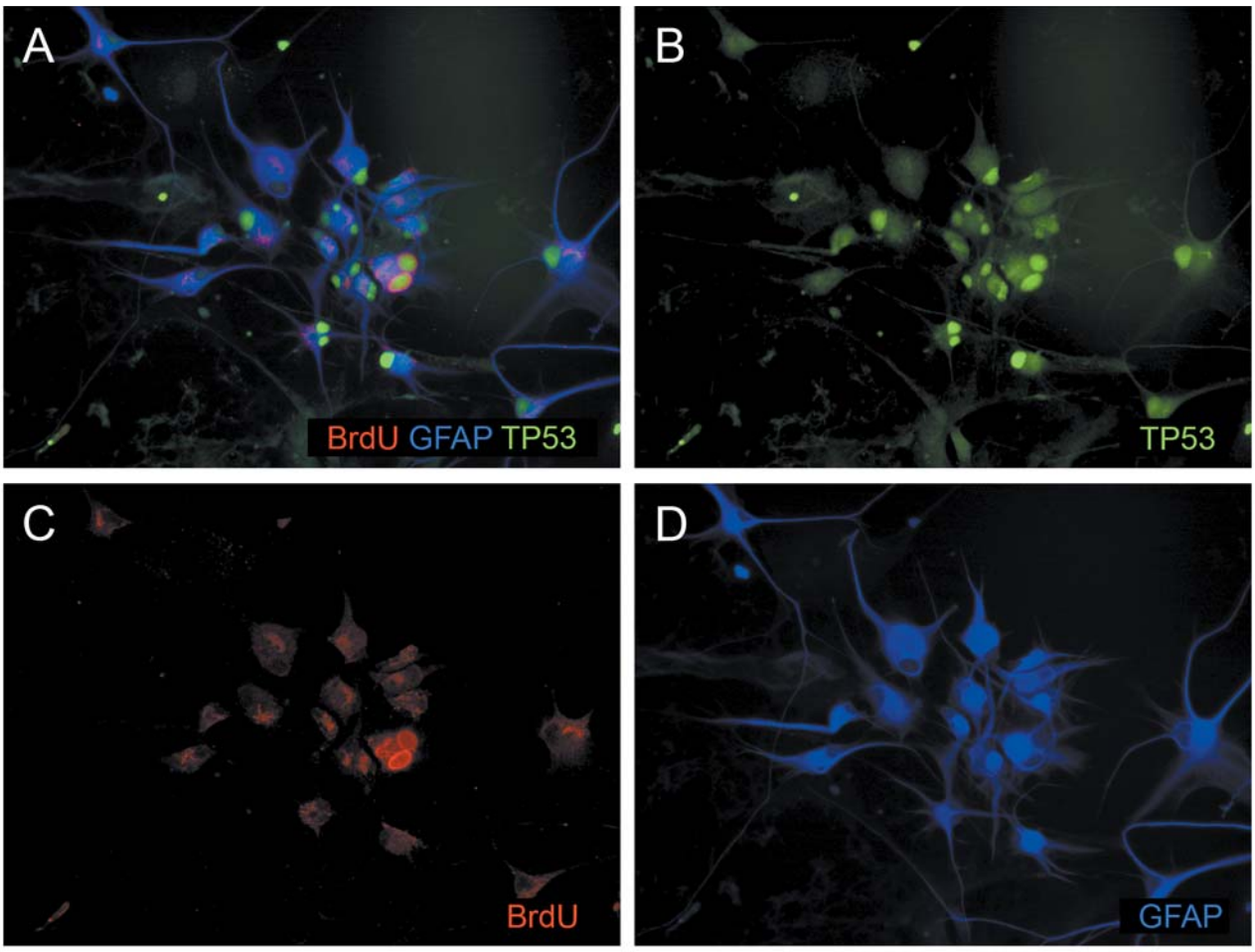

Figure 3. *Monolayer serum-free conditions. (A-D) GFAP(+)/TP53(+) cells constitute the majority of cells, but rarely incorporate BrdU. Similarly, GFAP(–)/TP53(–) cells do not proliferate efficiently.*

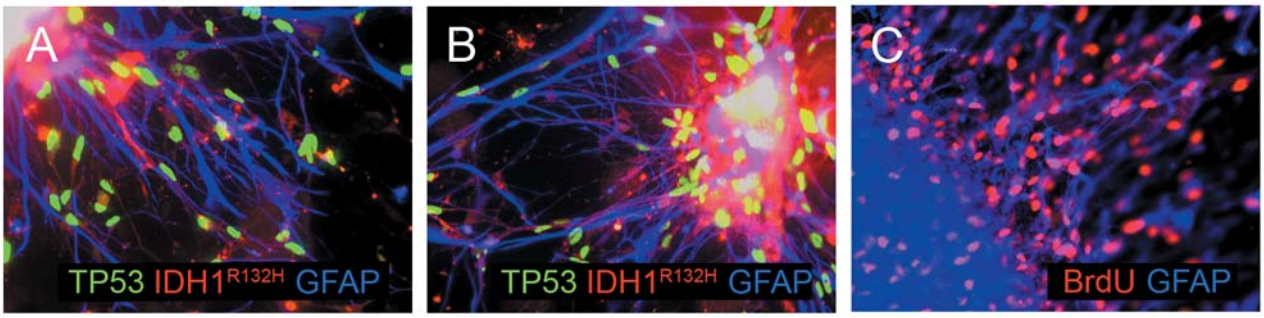

Figure 4. *3-D culture conditions. (A-C) The majority of GFAP(+)/IDH1^{R132H}(+)/TP53(+) cells in early spheroids incorporate BrdU.*

(exons 5-8, forward) and CCCTTTCTTGCGGA GATTCT (exons 5-8, reverse). cDNA sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequences were analyzed with the ABI 3130 Genetic Analyzer and DNA Sequencing Analysis Software (Applied Biosystems, Foster City, CA, USA).

Results

Firstly, we performed the screening of the 8 cases with *IDH1* mutation for other concurrent molecular alterations, which resulted in *TP53* mutation detection in 4 out of 8 cases. Next,

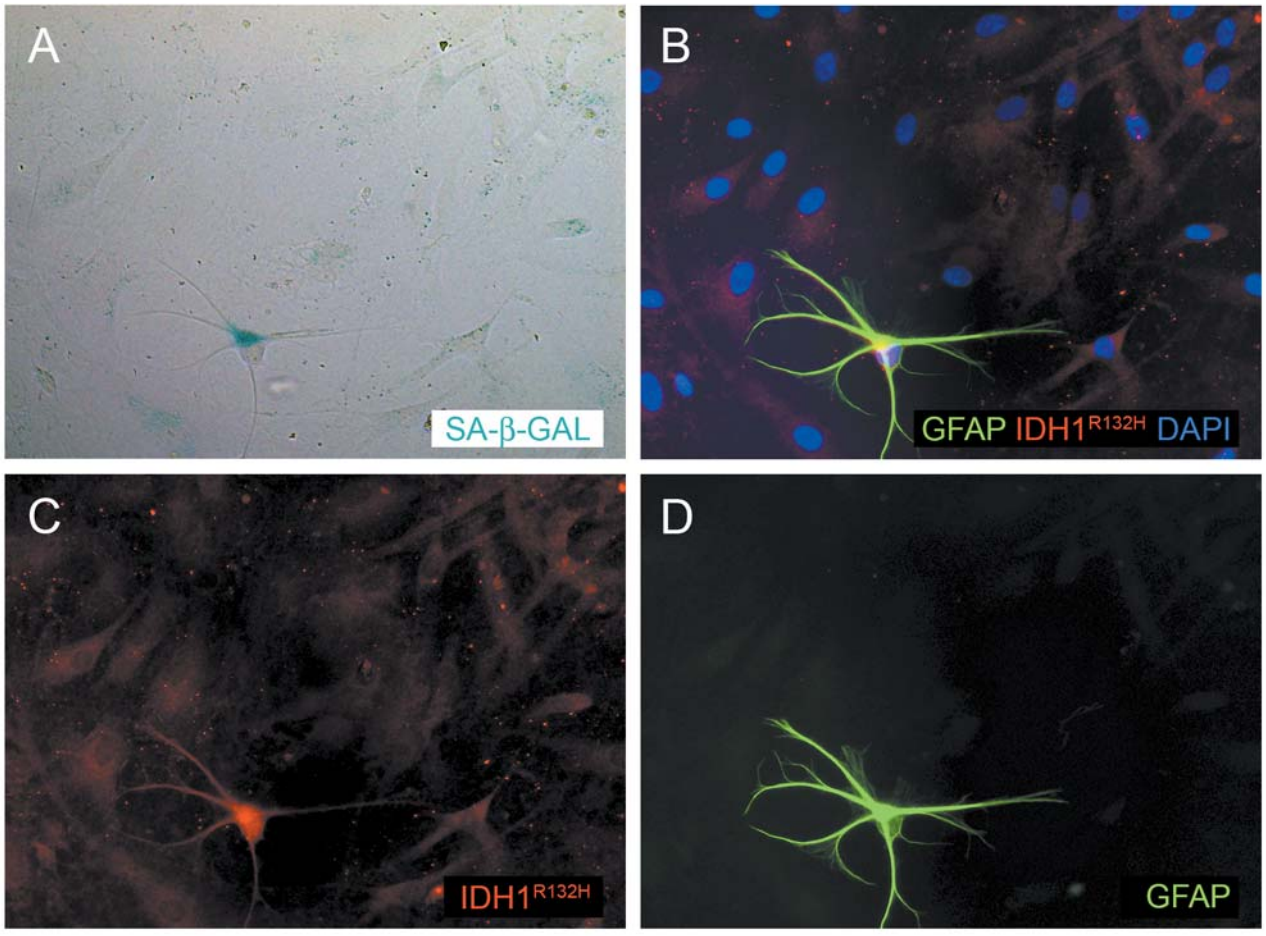

Figure 5. *IDH1^{R132H}(+)/GFAP(+) cells under monolayer conditions show SA-β-Gal activity (A-D).*

we analyzed the cellular subpopulations for the co-expression of glial markers. IDH1^{R132H}-positive cells expressed GFAP and accumulated nuclear TP53 (the cases with mutation) (Figure 2A-D). The remaining (IDH1^{R132H}-negative) cells were also negative for GFAP and TP53; αSMA was expressed by a sub-population of these cells (Figure 2E). Sequencing analysis confirmed that αSMA-positive cells were negative for *TP53* and *IDH1* mutations, which indicates their stromal/non-neoplastic origin.

Next, we analyzed the cellular sub-populations under different culture conditions. Under the classical conditions IDH1^{R132H}-positive cells did not proliferate efficiently and their number decreased rapidly. In GB such cells were detectable up to the 4th passage, while in grade II/III gliomas no longer than for 2 passages. On the other hand, the remaining (IDH1^{R132H}-negative) population proliferated easily and, subsequently, overgrew the neoplastic cells (Figure 2F). The proliferation of GFAP(–)/TP53(–) cells and its lack in GFAP(+)/TP53(+) cells was verified with BrdU incorporation assay (Figure 2F).

The behavior of IDH1^{R132H}-positive cells under the serum-free monolayer conditions was similar to the classical conditions in their lack of proliferation and their disappearance from the culture within the first two passages (Figure 3A–D). Conversely, such conditions inhibited the proliferation of IDH1^{R132H}-negative cells, which did not overgrow the tumor cells (Figure 3A-D). In general, the cells proliferated more slowly under the serum-free conditions, which prolonged the periods between each passage and, therefore, the presence of the IDH1^{R132H}-positive cells was seemingly maintained for a longer time. BrdU incorporation was rarely observed in tumor cells even after a long incubation.

In contrast to the monolayer cultures, the 3D conditions maintained the prevalence of the IDH1^{R132H}-positive cells for 1-2 months, suppressing the stromal cells (Figure 4A-C). The proliferation of the IDH1^{R132H}-positive cells was detectable by means of BrdU incorporation assay, which, however, required longer incubation periods; in general, 50% of fresh explant cells incorporated BrdU within about 10 days (7 days for GB spheroids), whereas the older explants

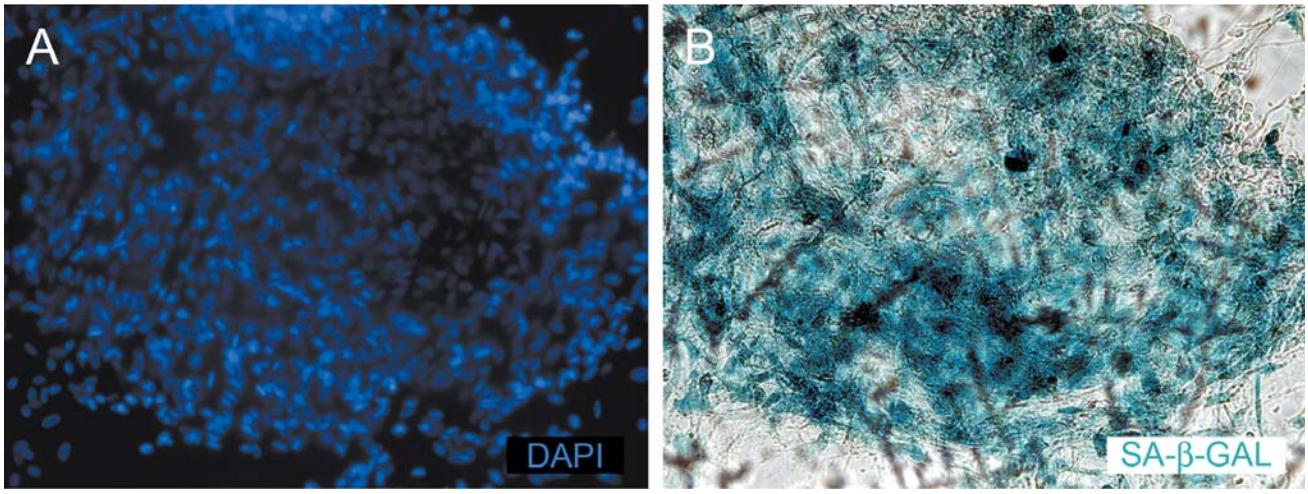

Figure 6. *3D (spheroid) culture from a glioblastoma specimen with IDH1^{R132H} mutation. The majority of cells are SA-β-Gal-positive (A, B).*

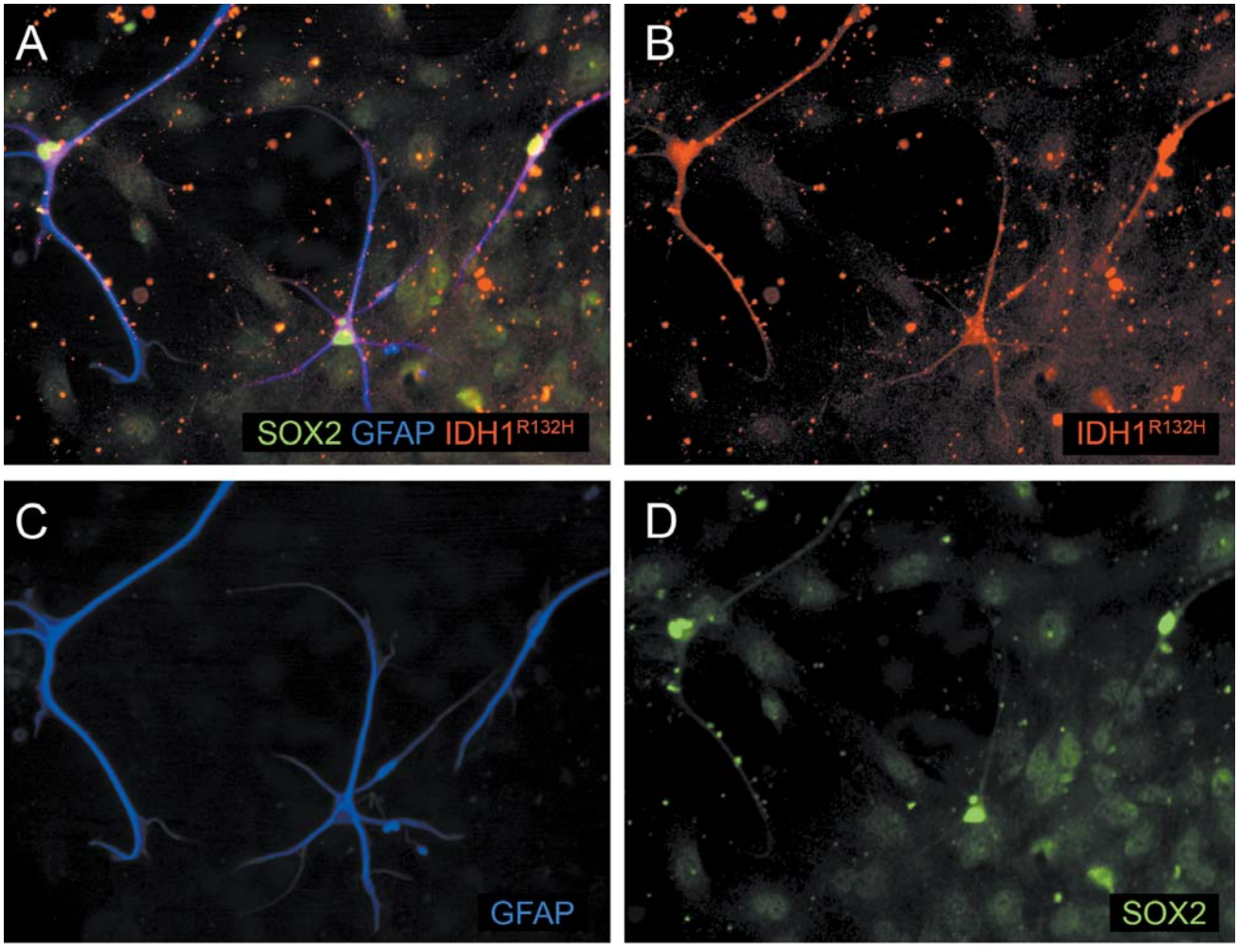

Figure 7. *Co-expression of glial and stemness markers in IDH1^{R132H}-positive cells. (A-D) IDH1^{R132H}(+), GFAP(+), SOX2(+) cells are present.*

(2-3 weeks old) required about 20-day incubation (14 days for GB spheroids) for the same effect. Nonetheless, even spheroids/explants from the same sample differed in terms of proliferation. Intriguingly, in cases with mutant *TP53* not all IDH1^{R132H}-positive cells accumulated TP53, and conversely, not all TP53-accumulating cells expressed IDH1^{R132H} (Figure 4A-B). In contrast to the previously described glioblastoma spheroids, the IDH1^{R132H}-positive ones did not allow for several month-long cultivation.

We observed the SA-β-Gal activity and the classical morphological changes in the most of IDH1^{R132H}-positive cells under the monolayer conditions (Figure 5A-D), which together with their lack of proliferation detected both by BrdU incorporation assay and by real-time *in vitro* observation may be considered as the typical features of senescence. These features were also observable under the 3D conditions, especially in late spheroid transfers (Figure 6A-B), but even the earliest spheroids contained senescent IDH1^{R132H}-positive cells. Additionally, we also detected the apoptosis of glioma cells under all cell culture conditions.

Finally, we analyzed the IDH1^{R132H}-positive cells for the coexpression of neuronal, glial and stemness markers. We detected the nuclear accumulation of TP53 as well as the expression of GFAP and SOX2 together with the features of senescence (Figure 7A-D).

Discussion

The role of senescence in gliomas requires a close investigation. Thus far, it is unclear whether this process serves an anti-neoplastic role, a pro-neoplastic one or both, depending on other factors. Additionally, it may be potentially employed as the target of new therapeutic methods. Finally, senescence may be responsible for the failure in glioma cell line stabilization experiments.

Most likely, glioma cells become senescent during the classical and novel monolayer as well as 3D *in vitro* cell culturing, as we have recently reported (5). In this study we analyzed cell cultures originating from 8 specimens with *IDH1*^{R132H} mutation. The antibodies recognizing the IDH1^{R132H} protein offer the unmatched opportunity to identify the neoplastic cells. In general, under all conditions we observed two cellular subpopulations (varying in their proportions): IDH1^{R132H}(+)/TP53(+)/GFAP(+)/MAP2(+)/αSMA(–), which were concluded as the neoplastic cells, and the IDH1^{R132H}(–)/TP53(–)/GFAP(–)/MAP2(–), among which a portion was αSMA(+) and which were most probably the stromal cells. As we previously reported, the cells with the *IDH1* mutation could be shortly (at most for 2 passages) observed under the classical conditions, due to their almost complete lack of proliferation (12). Here, we report data clearly linking this issue with the phenomenon of *in vitro* senescence: only the IDH1R132H-positive cells presented the SA-β-Gal activity and the classical morphological changes. The serum-free monolayer conditions were no more efficient in stimulating the proliferation and in protecting against senescence of such cells. In accordance with Luchman *et al*., we observed that under the 3D conditions the IDH1^{R132H}-positive cells could be preserved in the proliferative state for 3-4 weeks, which was, however, not sufficient for the cell line stabilization (13). What is important, the observed senescence of neoplastic cells is not induced by any specific chemical or physical factors other than the typical (classical or novel) cell culture conditions themselves. Therefore, we refer to the observed process as spontaneous senescence. The other (non-senescent) population lacked the *IDH1* and *TP53* mutations (verified by both immunocytochemical and sequencing analyses). A population of non-neoplastic, stromal, αSMA-positive cells was reported in glioblastoma by Clavreul *et al*. (14). Apparently other glial tumors (including low grade astrocytomas) are also infiltrated by these cells, and thus, one might consider to re-define the GASC abbreviation as the Glioma-Associated Stromal Cells. The infiltrating stromal cells were easily adaptable to the classical monolayer conditions and, as a result of the expansive proliferation, overgrew the neoplastic cells. Under the monolayer serum-free conditions the proliferation of αSMA-positive cells was suppressed, which moderately prolonged the original cellular composition. Under the 3-D conditions the stromal cells constituted a minor subpopulation with a low proliferation rate. It should be also noted that other processes such as apoptosis may be observed under *in vitro* conditions and should not be neglected in future analyses.

Moreover, the senescent IDH1^{R132H}-positive cells also expressed stemness markers such as SOX2. Importantly, all IDH1^{R132H}-positive cells were SOX2-positive as well as all SOX2-positive cells were IDH1^{R132H}-positive. Therefore, it suggests that glioma cells with the features of stemness become senescent *in vitro* along with other tumor cells. Interestingly, not all TP53-positive cells were IDH1^{R132H}-positive and *vice versa*, but we would be hesitant to suspect genetic heterogeneity as the underlying mechanism.

Conclusion

In conclusion, the anti-IDH1^{R132H} antibody was used to specifically confirm the *in vitro* senescence of glioma cells. The IDH1^{R132H}-positive cells both were senescent and showed the expression of stemness marker (SOX2). The recognition of the mechanisms responsible for *in vitro* senescence of glioma cells is important from the perspective of future medical research and potential application.

Acknowledgements

This study was sponsored by National Science Center Grants No. 2012/05/B/NZ4/02623 (IDH1 analyses and cell cultures), No. 2011/03/N/NZ1/06534 (senescence analyses) and No. 2011/01/B/NZ1/01502 (TP53 analyses).

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Received February 18, 2014
Revised March 27, 2014
Accepted March 28, 2014