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Immunohistochemical detection of EGFRvIII in glioblastoma – Anti-EGFRvIII antibody validation for diagnostic and CAR-T purposes

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ABSTRACT

The emergence of therapies such as CAR-T has created a need for reliable, validated methods for detecting EGFRvIII in patient tumor cells. Particularly so since previous studies have already suggested that some anti-EGFRvIII antibodies may be non-specific. The present paper evaluates the use of the L8A4 antibody in the immunohistochemical (IHC) and immunocytochemical (ICC) detection of EGFRvIII in 30 glioblastoma specimens, and compares it with other methods such as RT-PCR, MLPA, and FISH. The results indicate that Real-time PCR appears to be a very specific and sensitive method of EGFRvIII detection. ICC analysis with L8A4 also appears specific but requires cell culture. IHC analyses of EGFRvIII returned a number of false positives when using L8A4. Due to the growing need for an effective diagnostic tool before starting immunotherapy methods, such as the CAR-T anti-EGFRvIII or SynNotch CAR-T recognizing EGFRvIII, it is necessary to identify a more reliable and simple method of EGFRvIII detection or improve the specificity of the anti-EGFRvIII antibody, until then, immunocytochemistry may temporarily replace immunohistochemistry.

1. Introduction

The role of CAR T-cell therapy is progressing rapidly due to its strong efficacy against leukemia and lymphoma, as well as the growing prevalence of cancer cases. To date, drug regulatory agencies have approved six different CAR-T therapies, all for the treatment of patients with hematologic malignancies [1]. Despite numerous limitations, CAR-T in solid tumors in clinical and preclinical trials is also developing [2]. However, one of the main problems related to the effectiveness of CAR-T therapy in solid tumors is its suboptimal therapeutic target [3].

A number of immunotherapies are currently being developed for glioblastoma (GB), mainly due to current lack of effective treatments. GB patients treated with surgical resection, radiation therapy and chemotherapy, still can only expect a median survival of approximately 15 months, with fewer than 25% of patients surviving up to two years and fewer than 10% surviving up to five years. A novel potential treatment option for this disease is immunotherapy with CAR-T cells [4,5].

In glioblastomas, the most commonly considered CAR-T targets are EGFRvIII, IL13Ra2 and HER [6–8].

The EGFRvIII oncogene encodes a protein with a specific epitope localized in the extracellular domain, therefore, in theory, it seems to be an ideal target for CAR-T therapy. The use of CAR-T appears promising in patients expressing EGFRvIII at the protein level. Their cells are, in a sense, "addicted" to the oncogene: they would lose a critical part of their cancer potential if the oncogene were silenced (antigen), i.e. they will not easily become resistant to CAR-T.

However, developing a suitable diagnostic method to qualify

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patients for therapy presents an interesting challenge in the case of EGFR variant III. Firstly, the protein is very stable [9]. Secondly, in some cases, the occurrence of the oncogene at the DNA level is not accompanied by the appearance of a protein [10]. It is most likely epigenetically silenced. This opens the debate on the importance of EGFRvIII in the late stages of the cancer process, as well as on the value of its therapeutic targeting and diagnostic methodology based on DNA analysis [11]. Only the presence of the EGFRvIII protein within an appropriate percentage of cells or their type (cancer stem cells) may give a chance for therapeutic success. Therefore, reliable immunocytochemistry and immunohistochemistry methods appear to be the key to diagnosis: mRNA analysis does not indicate the number of positive cells and the antigens these cells express. CAR-T treatment appears quite tolerant of antigen density, being effective against antigens in small concentrations on large numbers of cells. The location of EGFRvIII-positive cells within the tumor may also be significant.

The present study evaluates the potential of immunohistochemistry based on L8A4 EGFRvIII-specific antibodies for diagnosing and stratifying patients conducted on primary glioblastoma material obtained directly from patients and glioblastoma cell lines. The obtained results were compared with results from other molecular biology techniques to evaluate EGFRvIII expression at the DNA, mRNA, and protein level.

2. Materials and methods

2.1. Clinical material and primary cell cultures

Glioblastoma primary cell cultures were derived from tissue samples obtained from patients treated at the Clinical Department of Neurosurgery, The Regional Specialist Hospital in Olsztyn. The patients were diagnosed according to the latest World Health Organization Criteria for Brain Tumor Classification [12]. All procedures were carried out based in accordance with the ethical standards and protocol approved by the Bioethics Committee of the Medical University of Lodz (Approval No. RNN/156/20/KE). Written informed consent was obtained from all patients before taking part, and their data were processed, and stored according to the principles expressed in the Declaration of Helsinki. Neurosurgical specimens were collected in aseptic conditions, shipped in 1X Hank's Balanced Salt Solution (Biowest, Cat. No. 21-022-CVR) and cell isolation was started as soon as possible after surgery (usually within 3 h).

Primary GB cell cultures were established according to Xie et al. [13]. Briefly, the cells were washed twice with PBS and centrifuged between washes (80×*g*, 2 min). The tissue was fragmented with a scalpel into pieces smaller than 1 mm^3, enzymatically dispersed with a mixture of StemPro Accutase (Gibco, Cat. No. A1110501) and TrypLe (Gibco, Cat. No. 12563011) and incubated with gentle shaking for 10 min at 37°C. Cell suspension was passed through two cell strainers (100 μm and 70 μm) and then washed twice in the medium. To establish a spheroidal culture, the cell pellet was suspended in the following mixture: Neurobasal (Gibco, Cat. No. 21103049) and DMEM/F12 medium (Corning, Cat. No. 10-090-CV) (1:1, supplemented with N2 (1X; Gibco, Cat. No. 17502048), B27 (1X; Gibco, Cat. No. 12587010), GlutaMAX (1X; Gibco, Cat. No. 35050061), Non Essential Amino Acids (1X; Biowest, Cat. No. X0557), Antibiotic-Antimycotic (5 μl/ml; Gibco, Cat. No. 15240062), bFGF2 (20 ng/ml; Peprotech, Cat. No. AF-100-18B), EGF (10 ng/ml; Peprotech, Cat. No. AF-100-15). After approximately five to seven days, the formed spheres were treated with StemPro Accutase, centrifuged, and washed with fresh culture medium. The spheres were then transferred to plates previously coated with poly-L-ornithine (10 μg/ml; 2 h; room temperature; Sigma-Aldrich, Cat. No. P4957) and with mouse laminin (10 μg/ml; 30 min; 37°C; Sigma Aldrich, Cat. no L2020). Depending on the proliferation rate, cells were passaged using StemPro Accutase every seven to fourteen days.

2.2. Stable cell lines

DK-MG (Cat. No. ACC277) and GMS10 (Cat. No. ACC 405) cell lines were purchased from DSMZ – German Collection of Microorganisms and Cell Cultures GmbH (Cat. No. ACC 277). DK-$MG^{extra\text{-}high}$, DK-MG^{high}, and DK-MG^{low} sublines were obtained by single cell cloning and cultured in RPMI 1640 medium (Biowest, Cat. No. L0500).. For GMS10, the basal medium was DMEM with L-glutamine (Biowest, Cat. No. L0102). The Fibroblast Cell Line of the Human Foreskin (Cat. No. CRL-2522), U-87MG (Cat. No. HTB14), T98G (Cat. No. CRL-1690) and MDA-MB-468 (Cat. No. HTB-132) were purchased from the ATCC – American Type Culture Collection. AD293 (parental) cells were purchased from Agilent Technologies (Cat. No. 240085) and cultured in complete DMEM High Glucose (Biowest, Cat. No. L0102-500) such as Human Fibroblast cell line. The AD293wt and AD293vIII cell lines were created as described previously [14]. The U-87MG and T98G cell lines were cultured in EMEM (ATCC, Cat. No. ATCC-30-2003). Both primary and stable cell lines were cultured in 5% CO_2, 37°C in a humidified atmosphere [14, 15]. Characteristic features of the stable cell lines used in experiments are listed in Supplemental Table S1.

2.3. Immunofluorescence

For immunofluorescence analyses, cells were fixed for 10 min in 4% paraformaldehyde in PBS. After fixation, samples were washed three times with PBS and permeabilized for 10 min in PBS/0.1% Triton X-100 (Sigma-Aldrich, Cat. No. T8787). After three washing steps with PBS, the samples were incubated with gentle agitation for 1 h in a blocking solution, 2% donkey serum (Sigma-Aldrich, Cat. no D9663) in PBS. The cells were then incubated with the appropriate primary antibodies for 1 h and visualized by incubation with the appropriate species-specific fluorochrome-conjugated secondary antibodies for 1 h in the dark. Control samples were incubated with the secondary antibodies alone and otherwise processed identically. Each step of the procedure was performed at room temperature. Slides were mounted using the ProLong Gold Antifade Reagent with DAPI (Invitrogen, Cat. no P36931), coverslipped and examined with a Nikon Eclipse Ci-S fluorescence microscope, and images were taken using Nikon's NIS – Element F. GB sample numbers were assigned after any sensitive patient data was coded. Antibodies used for immunocytochemistry are listed in Supplemental Table S2.

2.4. Western blotting

Total cellular protein was isolated from cultures using cell lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM Sodium Orthovanadate, 10 μM β-glycerophosphate, 5 μM Sodium Pyrophosphate and 0.5% Triton X-100) freshly supplemented with Protease Inhibitor Cocktail (Thermo Scientific, Cat. No. 78442) at 4°C. Lysates were clarified at 7000×*g* for 6 min at 4°C and suspended in 4X Laemmli Sample Buffer (Bio-Rad Laboratories, Cat. No. 1610747) with β-mercaptoethanol (Sigma-Aldrich, Cat. no M3148) and boiled (98°C, 3 min). Equal amounts of protein extracts, 25 μg per lane, were separated in 8% SDS-polyacrylamide gel and transferred onto PVDF membrane. After membrane blocking with 5% skim milk (Millipore, Cat. No. 70166), the primary and then the secondary antibodies listed in Supplemental Table S3 were added. Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Cat. No. RPN2232) was used for HRP visualization. Densitometry measurements were performed in ImageJ and normalized to Actin bands.

2.5. Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tissue specimens taken from 20 patients diagnosed with glioblastoma IDH-wild type were deparaffinized and rehydrated by passing them through xylene and a

graded series of ethanol. The tissue sections were placed in a 10 mM citric acid solution (pH 6.0) and boiled at 96°C for 10 min followed by cooling for 30 min. To block the endogenous peroxidase activity, the sections were incubated with 0.5% hydrogen peroxide for 10 min. The sections were then washed in water and TBST and blocked for 75 min in 10% normal goat serum in TBST at room temperature. The slides were then incubated for 16 h at 4°C with anti-EGFRvIII [L8A4] antibody (Kerafast, Cat. No. EDK002, in a concentration of 1:100–1:500), A10 antibody (Santa Cruz Biotechnology, Cat. No. sc-373746, in a concentration of 1:200) or anti-GFAP (glial fibrillary acidic protein) antibody (Sigma-Aldrich, Cat. no MAB360, in a concentration of 1:50) followed by 30 min incubation with SignalStain Boost IHC Detection Reagent (Cell Signaling Technology, Cat. no 8125P) in a humidified chamber at room temperature. Staining was developed using DAB substrate-chromogen solution (SignalStain DAB Substrate Kit, Cell Signaling Technology, Cat. No. 8059P), and the slides were counterstained with Harris hematoxylin. The sections were dehydrated and coverslipped using DPX mounting medium (Sigma-Aldrich, Cat. No. 1.00579) and visualized with Nikon ECLIPSE Ts2 microscope. GB sample numbers were assigned after coding sensitive patient data.

The following steps of the procedure were optimized: heating time and temperature, incubation time in xylene, antigen retrieval conditions, concentration of hydrogen peroxide, wash repeats, serum concentration, time of incubation with blocking solution, concentration, conditions and time of incubation with antibodies, incubation time with chromogen and hematoxylin, discoloration time and rising in ethanol, acetone and xylene. Following this, specific results were obtained for control samples and the final immunohistochemical staining protocol was established.

2.6. Cell block preparation for DK-MG^{high} cells

Cell blocks were prepared according to Cattoretti (https://www.ihcworld.com/_protocols/histology/cell_block.htm). To obtain a EGFRvIII-positive control for IHC analysis, a cell block was prepared from the pellet of centrifuged DK-MG^{high} cell suspension. For this purpose, 30 million cells were trypsinized, centrifuged, and resuspended in a cell culture medium. The agar was then boiled to make a 1% solution in iso-osmotic PBS and cooled to 50°C. The cells were mixed with the agar solution and quickly placed on ice to solidify the agar. Slides for IHC staining were prepared by cutting the prepared cell suspension in agar on a microtome (Sliding Microtome Microm HM 430, Thermo Scientific).

2.7. Real-time qRT-PCR for cDNA and DNA

Genomic DNA and RNA were isolated from fresh tissues or cell cultures and formalin-fixed paraffin-embedded tissue sections, using an AllPrep DNA/RNA Mini Kit (Qiagen, Cat. No. 80204) or AllPrep DNA/RNA FFPE kit (Qiagen, Cat. No. 80234), respectively, according to the manufacturer's instructions. Fresh tumor tissues were placed on dry ice, and a section of approximately 4 mm diameter was cut and placed in a lysis buffer (100:1 mixture of RLT buffer with β-mercaptoethanol). The tumor sections were homogenized and centrifuged at 14 000×*g*, after which the supernatant was collected and applied to a nucleic acid isolation column from the AllPrep DNA/RNA Mini Kit. Subsequent isolation steps were performed according to the manufacturer's protocol using the AllPrep DNA/RNA Mini Kit (Qiagen, Cat. No. 80204). The stained paraffin sections were incubated for five days in xylene, and scraped from the slides into the tubes containing fresh xylene. After mixing and centrifugation, ethanol (99%) was added to the pellet to remove residual xylene from the sample. Again, the solution was mixed and centrifuged. The sections were then allowed to air dry at room temperature until all the ethanol had evaporated. The dry pellet was treated with Proteinase K and incubated at 56°C for 15 min, followed by centrifugation. The obtained precipitate was used as input for DNA extraction, while the supernatant was used for RNA extraction. The concentrations of DNA and RNA isolated from FFPE sections and from fresh tissues or cell cultures were determined with a NanoPhotometer N50 (Implen) and their OD260/OD280 ratios were assessed for nucleic acid purity. Reverse transcription was performed at 42°C for 2 min, 42°C for 15 min, and then 95°C for 3 min. For this reaction, cDNA was synthesized using the QuantiTect® Reverse Transcription Kit (Qiagen, Cat. no 205311) according to the manufacturer's protocol.

Real-time PCR was performed using the Kapa SYBR Fast Universal (Kapa Biosystems, Cat. No. KK4601) in duplicate in a final volume of 12 μl. The reaction mixture was prepared according to the ratio: Kapa SYBR Fast Universal (2X), 200 nM of each of the reaction primers (sequences of reaction primers are summarized in Supplemental Table S4), and 12.5 ng cDNA/DNA (depending on the experiment). Then, 11 μl of the reaction mixture and 1 μl of the matrix were pipetted onto a 48-well plate. Real-time PCR was conducted using the Fast Real-Time PCR System (Illumina Eco, USA, Cat. No. 1010180) with 48-well plates. The reaction was performed according to the scheme: 5 °C (2 min; UDG activation), 95°C (10 min; polymerase activation), and 40 cycles: 95°C (15 s; denaturation), 60°C (30 s; primer annealing), 72°C (30 s; chain extension). The Ct values were obtained from the Illumina ECO and adjusted for TBP (cDNA) or RPP25 (DNA) Ct values for each sample to determine ΔCt and relative expression of the target mRNA or DNA copy number. Results were obtained using equation $2^{-\Delta\Delta Ct}$ [16,17]. Trials were classified as positive after detection of the reaction product below threshold cycle number 37 ($Ct \leq 37$).

Pooled cDNA from EGFRvIII-positive samples was used as a positive control of EGFRvIII expression [9]. To generate the pool, mRNA isolated from 15 tumor samples expressing the mutated receptor was pooled and diluted 50 times. The expression of EGFRvIII was normalized using the pooled cDNA, while EGFRwt was normalized using cDNA from human foreskin fibroblasts. Selected samples were divided into three groups. If the normalized expression of EGFRvIII was 0, selected samples were marked as negative, between 0 and 1 were marked as low, and above 1 as high, if the amplification product was detected above 35 reaction cycles, the samples were marked '<35 cycles'.

Quantitative Real-time PCR was used for the indirect determination of relative DNA copy number (CN) of EGFRvIII [18–20]. For this reaction, two types of primers were designed: EGFRtotal and EGFRwt. Primers (forward and reverse) of EGFRtotal are based on a partial fragment of intron 24–25, and those of EGFRwt are based on a partial fragment of exon 2 of the EGFR gene.

If both EGFRtotal and EGFRwt in the sample exhibited the same Ct value, this result indicated an absence of DNA copies: the deletion of exons 2–7 is characteristic of EGFRvIII. If, on the other hand, the EGFRwt gene yielded a higher Ct value than EGFRtotal, this would indicate the presence of the mutated EGFRvIII gene in the sample as the EGFRwt primers did not flank the amplified fragment in exon 2 (due to its absence) in the EGFR gene.

The relative quantification of EGFRtotal and EGFRwt was assessed based on Ct values collected using an autotreshold and automatic baseline. Human foreskin fibroblasts provided a negative control for EGFRvIII detection. The CN assessment was conducted by normalizing the number of EGFRtotal and EGFRwt gene copies to the RPP25 gene. The copy number of EGFRtotal and EGFRwt analysis was carried out using a comparative (ΔΔCt) method.

All primers used were purchased from Merck/Sigma-Aldrich. The primer sequences of the tested genes are summarized in Supplemental Table S4.

Due to the high level of degradation of RNA in paraffin samples, the manufacturer of the RNA isolation kit only guarantees the isolation of RNA up to 70 nucleotides. Therefore, new primers (short) for the EGFRwt, EGFRvIII, and TBP genes were designed, whose amplification products do not exceed 72 base pairs.

Each of the selected primer pairs was checked for PCR efficiency with the LinRegPCR program, which showed a mean minimum PCR

efficiency of 85–90% [21]. For each reaction, the correctness of the obtained products was routinely verified by making a melting curve of the reaction products, where a single peak with the correct melting point was always visible depending on the size of the resulting PCR product.

2.8. Multiplex ligation-dependent probe amplification (MLPA)

The MLPA reactions were performed as described previously using commercially available probe mixes (P105) and dedicated kits (MRC-Holland) [22]. The fragments were separated by capillary electrophoresis using an ABI 3500 Genetic Analyzer (Applied BioSystems). The comparative analyses were performed using Coffalyzer.Net software (MRC-Holland). DNA isolated from peripheral blood from healthy volunteers was used as a control sample. The resultant ratio for a given gene was interpreted as follows: normal copy number (0.7–1.3), deletion of one allele (0.35–0.65), deletion of both alleles (0), gain of one allele (1.35–1.55) or gain of more than one allele (1.6–2.2). Results in the range of 0.1–0.3 were considered to result from the analysis of heterogenous material in which deletion was detected.

2.9. Fluorescence in situ hybridization (FISH)

FISH analysis for the EGFR gene was performed as described previously [20]. Briefly, a commercially available probe set (Vysis LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen; Abbott Molecular, Cat. No. 05J48-001) was used to simultaneously detect the copy numbers of the EGFR gene and chromosome 7. The samples were examined with a Nikon Eclipse Ci-S fluorescence microscope, and images were taken using Nikon's NIS – Element F. EGFR copies were assessed by the number of red signals visible upon binding to the EGFR specific probe, while the chromosome 7 copy number was assessed by the number of green signals caused by CEP7 probe binding.

2.10. Preparation of the EGFRvIII-recognizing antibody

The synthesis of antibody variable chains was performed on the basis of the deposited scFv sequence (GenBank, U76382, Mus musculus epidermal growth factor receptor antibody MR1scFv mRNA). The light chain variable fragment recognizing EGFRvIII epitope was fused to the constant light chain (kappa) fragment of the murine IgG1a antibody, the N-terminal interleukin-2 secretion signal was added and inserted into the pENTR/zeo construct. The heavy chain variable fragment containing the mutant receptor recognition paratopes was ligated with the CH1–CH3 fragments of the heavy chain of the murine IgG1a antibody and the IL-2 secretion signal, and the resulting construct was then cloned into the pENTR/zeo plasmid. Expression vectors were obtained from the above-described ENTRY constructs and the pLenti-PGKhygro-DEST and pLEX_307 plasmids. The obtained expression vectors were used to generate lentiviral particles, which were used to transduce AD293 parental cells. Transduced cells were simultaneously selected for puromycin and hygromycin for 10 days. Following this, six clones were isolated, selected, and expanded.

2.11. Dot-blot assay

The analysis was performed to assess the degree of antibody production by the cells. Cells were seeded in equal amounts in 6-well culture vessels and incubated for five days. The expression of the antibody and its secretion into the culture medium were assessed by direct application of the protein to a PVDF membrane activated with methanol. The membrane was incubated with a blocking solution (5% milk). The membranes were then incubated with horseradish peroxidase-conjugated anti-mouse secondary antibodies for 1 h at room temperature, washed with TBST. Any protein was then detected, using horseradish peroxidase substrate as a chemiluminescent reagent. A detection solution was prepared consisting of 24 ml 100 mM Tris-HCl (pH 8.5), 7.6 μl 30% hydrogen peroxide, 120 μl 250 mM luminol (Sigma-Aldrich, dissolved in DMSO), 2 μl 90 mM para-coumaric acid (Sigma-Aldrich, dissolved in DMSO). Membranes were incubated for 60 sec with freshly prepared reagents.

Clones showing the highest protein expression were selected. The cells were grown to 50% confluence, after which the medium was replaced with fresh medium (DMEM/10% FBS), and the culture was maintained for another five days. After this time, the medium was centrifuged, separated from the cell debris and concentrated by ultrafiltration at the 100 kDa cut-off limit. Alternatively, cells were cultured and maintained in FreeStyle F17 medium during production to obtain bovine serum-free antibodies. After five days, the medium was separated from the cells and their debris, and the immunoglobulin fraction was precipitated with saturated ammonium sulfate. The purified antibody (DA-01) was dissolved in 150 mM sodium chloride solution and stored at 4 °C.

3. Results

3.1. The occurrence of EGFRvIII in selected cell line models

Appropriate controls for verifying the specificity of anti-EGFR antibodies were selected based on DNA and mRNA isolated from the selected models. Firstly, based on Real-time PCR analysis, primary GB cultures (with and without EGFRvIII expression) were selected as study controls, together with DK-MG^{high} and DK-MG^{low} stable cell lines with different levels of EGFRvIII expression. Some analyses also included cell lines with exogenous EGFR expression (AD293wt and AD293vIII).

EGFR gene copy number was determined using MLPA analysis (Fig. 1A). In both EGFRvIII positive glioblastomas and the DK-MG^{high} cell line, EGFR exon 1 and exons 8 to 23 demonstrated a gain of more than two copies, while exons 2 to 7 exhibited a normal copy number. Although exons 2–7 were deleted in EGFRvIII, they were still detected due to the co-occurrence of EGFRvIII with EGFRwt and chromosome 7 trisomy. All exons in EGFRvIII-negative glioblastomas and in DK-MG^{low} were characterized by normal copy number: no gain or loss was noted.

The results of the EGFRvIII mRNA expression analysis (Fig. 1B) are consistent with the above-mentioned DNA-based results (Fig. 1A). Analyses of EGFR mRNA expression from tumor specimens and their derived cell lines were consistent both for EGFRwt and EGFRvIII. The mRNA analyses confirmed the expression of exogenously-introduced EGFRwt in AD293wt and EGFRvIII in AD293vIII. FISH analysis detected EGFRvIII amplification in EGFRvIII-positive glioblastoma and DK-MG^{high}, but not in DK-MG^{low} (Fig. 1C).

3.2. Development of a specific anti-EGFRvIII antibody

An attempt was made to obtain an antibody that specifically recognizes the EGFRvIII epitope. The applied procedure obtained six clones of AD293 cells, these were assessed for the degree of antibody production using the dot-blot technique, based on a secondary anti-mouse antibody conjugated with horseradish peroxidase (Fig. 2A). The analysis showed that clone 5 had the highest expression, and the produced antibody was given the working name DA-01.

To evaluate the specificity of the developed antibody, immunocytochemical staining was performed on AD293 cells with exogenous expression of EGFRwt (AD293wt) or EGFRvIII (AD293vIII) according to a standard procedure. It was found that the DA-01 antibody binds to both EGFRvIII and (nonspecifically) to the unmutated form of the EGFR receptor present in AD293wt cells (Fig. 2B), however, a much stronger signal was observed for the antibody binding to EGFRvIII.

In Western Blot, both DA-01 antibodies and a commercially-available anti-EGFR antibody (sc-03) bound to domains present in both EGFRwt and EGFRvIII, this indicates that the developed tool lacks specificity (Fig. 2C). A densitometric evaluation of the EGFRvIII and EGFRwt bands normalized to actin, analyzed with ImageJ is given in

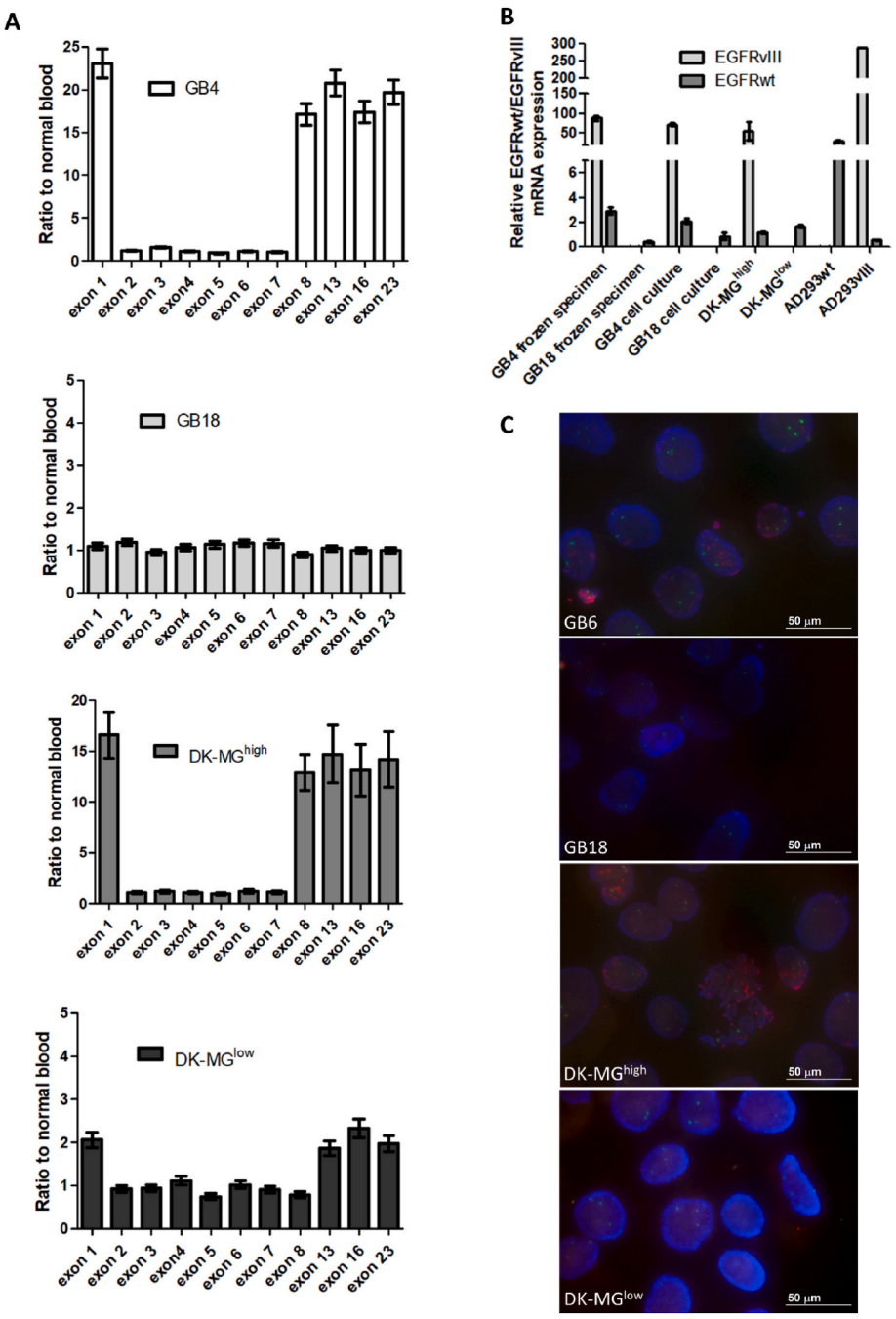

Fig. 1. Molecular analyses at the DNA and mRNA level confirm the selection of appropriate cell line controls for EGFRvIII detection. (**A**) MLPA analysis for exemplary either EGFRvIII-positive or -negative primary glioblastoma cells and for DK-MG^{high} and DK-MG^{low} cell lines. (**B**) Evaluation of EGFRwt and EGFRvIII mRNA expression in primary and stable glioblastoma cells and in AD293 cells with EGFRwt or EGFRvIII introduced. (**C**) Verification of increased *EGFR* copy number (either by amplification or polysomy) by FISH. Red signals indicate the EGFR gene and green signals indicate the centromere of chromosome 7. Error bars indicate SD for MLPA results, and SEM for RT-PCR. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

Fig. 2D and E. Our findings indicate that the DA-01 antibody non-specifically binds to EGFRwt, which unfortunately excludes its further use for the specific detection of EGFRvIII in cells.

3.3. L8A4 antibody specificity in immunofluorescence and Western blotting analysis

Both immunocytochemical and Western Blot analyses used two antibodies for EGFR detection. The A10 antibody allows the detection of both the mutated (EGFRvIII) and the wild type form of EGFR (EGFRwt) by binding to the C-terminal cytoplasmic domain of EGFR, while the L8A4 antibody allows the detection of EGFRvIII by binding to an amino acid sequence at the fusion junction site.

Immunocytochemical analysis of EGFRvIII positive GB indicated the presence of both GFAP-expressing and non-expressing cells that showed positive staining with A10 and L8A4 antibodies (Fig. 3A). Although cells from the DK-MG^{high} cell line also showed positive staining with the A10 and L8A4 antibodies, a strong signal gradient was noticed. This heterogeneity was due to the fact that EGFRvIII was only expressed by a subpopulation of cells.

In EGFRvIII negative glioblastoma and DK-MG^{low} cells, a very weak signal for total EGFR protein was observed, while staining for EGFRvIII

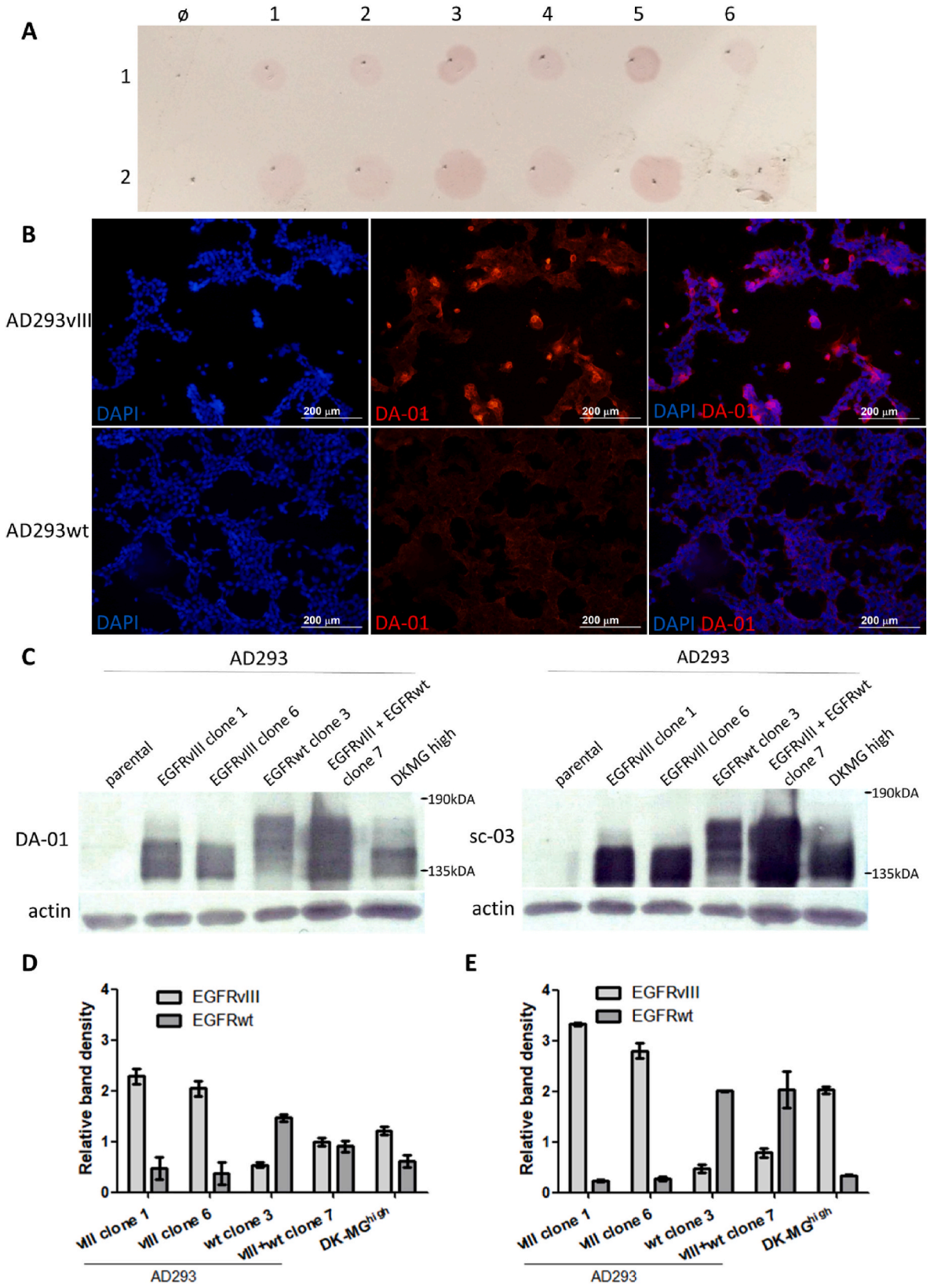

Fig. 2. Results of the examination of antibody DA-01 in the AD293 and DK-MG cell lines. (**A**) Antibody expression and secretion into the culture medium in six AD293 cell clones (described as 1–6 at the top of the membrane) based on dot-blot technique. ø means blank. The test was performed in duplicate (described as 1 and 2 on the left side of the membrane). (**B**) Immunocytochemical staining of AD293wt and AD293vIII cells with the DA-01 antibody. (**C**) Results of the analysis of the specificity of antibodies DA-01 and sc-03 antibodies by Western Blot in five clones of AD293 and DK-MG^{high} cell lines. (**D**, **E**) Densitometric evaluation of the results obtained during Western Blot analysis with DA-01 (D) or sc-03 antibody (E). The intensity of EGFRwt and EGFRvIII bands normalized to actin. Error bars indicate SEM.

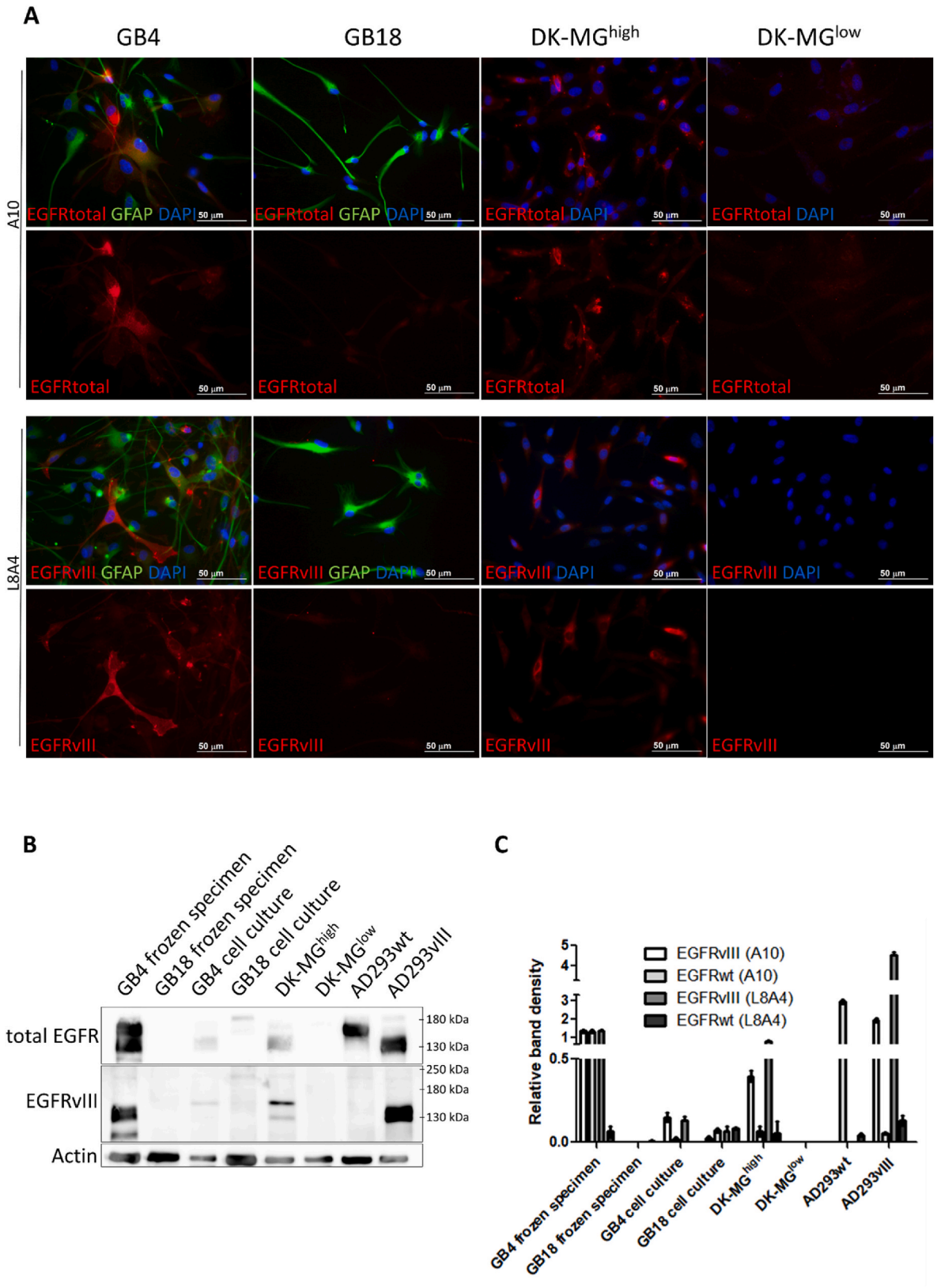

Fig. 3. Analysis of the binding specificity of the L8A4 antibody to EGFRvIII protein in immunofluorescence and Western Blot analysis. (A) Immunocytochemical staining of GB4 EGFRvIII+, GB18 EGFRvIII-, DK-MG^{high} and DK-MG^{low} cell lines with the A10 and L8A4 antibodies. Antibody A10 can detect both EGFRwt and EGFRvIII, while antibody L8A4 should only bind to EGFRvIII. (B) Results of the analysis of the specificity of EGFRvIII detection with the L8A4 antibody by Western Blot in GB specimens, primary and stable cancer cell lines. (C) Densitometric evaluation of the results obtained during Western Blot analysis with A10 or L8A4 antibody. Error bars indicate SEM.

was negative, suggesting that the L8A4 antibody may allow specific detection of cells expressing EGFRvIII in immunocytochemistry. In the AD293wt and AD293vIII cell lines, L8A4 only bound to EGFRvIII in AD293vIII cells (Supplemental Fig. S1).

Western Blot analysis showed less total EGFR protein in EGFRvIII positive GB cells compared to GB EGFRvIII positive tissue (Fig. 3B), however, this is typical for primary GB cell lines [23]. The anti-EGFRvIII antibody appeared to obtain specific results in the tested cell lines, however, in some samples, a faint band was observed at about 200 kDa, i.e. slightly higher than expected for unmodified EGFRwt. This was observed after using an antibody that detected both this form of the receptor and the mutated receptor; however, it is worth noting that such a very faint band appeared. Densitometric analysis was performed to carefully check the differences in the amount of analyzed EGFR proteins between cell lines (Fig. 3C).

3.4. L8A4 antibody specificity in immunohistochemical analysis

FFPE slides prepared and obtained from 20 patients diagnosed with glioblastoma-IDH wild type were analyzed immunochemically with the L8A4 anti EGFRvIII antibody. The A10 antibody was used to stain the total EGFR in the slides, while the anti-GFAP antibody was used to confirm the identity of the stained cells. Of the 20 qualified samples, seven were negative, three were negative if less than 35 cycles in real-time PCR were used, three were low positive, and the remaining seven were strongly positive for EGFRvIII expression (Table 1). Slides obtained from the DK-MG^{high} cell blocks were used as positive controls, and slides from normal brain tissues as negative controls (Fig. 4A and B).

Immunohistochemical staining indicated that among the seven EGFRvIII mRNA negative samples, two slides, with clearly visible gemistocytes, were EGFRvIII positive when the L8A4 antibody was used, and the other five were negative (Fig. 4C and D; Table 1). In the group where the EGFRvIII amplification product was detected above cycle 35, all three samples were negative for IHC staining with L8A4.

Interestingly, of the three samples which were low-positive for EGFRvIII mRNA expression, only slides from one FFPE were EGFRvIII positive, while the other two were negative (Fig. 4E and F; Table 1). In contrast, of the seven highly positive EGFRvIII samples, four were found to be immunohistochemically positive with L8A4, while the remaining three were negative (Fig. 4G,H,I; Table 1). In the EGFRvIII-positive glioblastomas, both a heterogeneous signal (GB7) and a membrane-cytoplasmic signal (GB2) could be observed. For each glioblastoma specimen, the IHC analysis was conducted on at least three slides: each time, the result was the same.

To verify whether the sections used for the IHC tests with L8A4 corresponded directly with the GB fragments analyzed at DNA and/or mRNA level in the frozen/fresh specimens, nucleic acids were isolated from the slides after analysis and then further analyzed in Real-time PCR. Unfortunately, despite the use of primers dedicated for short sequences, no mRNA products were obtained in real-time PCR, possibly due to degradation. Fortunately, however, DNA, was successfully isolated from the slides and subjected to real-time PCR. Results indicate that the stained sections correspond with the frozen or fresh specimens regarding EGFRvIII detection (Fig. 4J), therefore, the cause of the false-negative results of the IHC was not the use of different tumor fragments in the analysis. IHC with A10 antibody confirmed that EGFR was expressed in the stained specimens (Fig. 4K). In addition, it was confirmed that cells expressing the glial fibrillary acidic protein (GFAP) were present in the samples (Fig. 4L).

3.5. L8A4 antibody specificity in immunocytochemical analysis

Immunocytochemical analyses with the anti-EGFRvIII L8A4 antibody were conducted on primary cell cultures obtained from 10 patients diagnosed with IDH wild-type glioblastoma and on seven stable cell lines: DK-MG$^{extra-high}$, DK-MG^{high}, DK-MG^{low}, MDA-MB-468, U87-MG, T98G and GMS-10. Of the 10 qualified samples and seven stable cell lines, six were strongly positive and two were low positive, in addition, EGFRvIII amplification products were detected above cycle 35 in four samples, and EGFRvIII expression was negative in six samples (Table 2).

Immunocytochemical staining indicated that all of the six EGFRvIII mRNA strongly-positive samples were also positive when the L8A4 antibody was used. All of the low positive samples were negative for L8A4. No protein was detected by ICC in any sample where expression of EGFRvIII mRNA was detected above cycle 35 of Real-time PCR. Each sample that was EGFRvIII negative in Real-time PCR was also negative for immunocytochemical analysis.

Table 1
Summary of clinical data (age, sex), Real-time PCR and immunohistochemical detection of EGFRvIII for 20 patients diagnosed with glioblastoma IDH wild type. For the purpose of IHC method, L8A4 antibody (Absolute Antibody) was used. Both Real-time PCR and IHC was conducted in triplicates. If the normalized expression of EGFRvIII was 0, selected samples were marked negative, if the product of amplification was detected above 35 cycles of reaction, the samples were marked '<35 cycles' and between 0 and 1 as 'low', above 1 as 'high'. GB sample numbers were as-signed after coding sensitive patient data.

Sample name	Sex	Age	EGFRvIII mRNA expression		EGFRwt mRNA expression	IHC with L8A4 Ab
GB1	M	54		43.546 ± 1.010	0.588 ± 0.009	negative
GB2	F	52		11.046 ± 2.618	5.186 ± 0.387	positive
GB3	M	35		52.484 ± 1.821	2.746 ± 0.329	negative
GB4	M	39	high	88.112 ± 4.923	2.937 ± 0.231	positive
GB5	M	48		28.703 ± 1.680	12.511 ± 0.102	negative
GB6	F	66		15.610 ± 2.349	2.857 ± 0.089	positive
GB7	M	54		25.584 ± 6.270	6.220 ± 0.410	positive
GB10	F	82		0.396 ± 0.016	13.144 ± 1.925	negative
GB11	M	39	low	0.447 ± 0.266	9.841 ± 0.342	positive
GB13	F	50		0.229 ± 0.061	7.333 ± 0.747	negative
GB8	M	69	<35 cycles	0.018 ± 0.002	15.970 ± 0.747	negative
GB9	F	74		0.009 ± 0.004	2.074 ± 0.119	negative
GB12	M	53		0.061 ± 0.010	36.614 ± 1.008	negative
GB14	M	68		0.000	39.762 ± 0.946	negative
GB15	M	55		0.000	0.376 ± 0.013	negative
GB16	M	63		0.000	9.113 ± 0.443	negative
GB17	F	75	negative	0.000	0.271 ± 0.009	positive
GB18	F	64		0.000	0.795 ± 0.028	negative
GB19	M	63		0.000	0.165 ± 0.012	negative
GB20	M	59		0.000	0.453 ± 0.064	positive

4. Discussion

Raising antibodies specifically recognizing EGFRvIII, but not EGFRwt, is a great challenge. EGFRvIII has a specific epitope: compared to the native EGFR, 273 amino acids are deleted, and a number of non-adjacent sequences are joined by an additional glycine at the junction site. Multiple analyses of antibodies indicate that immunization with the same epitope containing the unique glycine does not always result in the production of antibodies binding only to EGFRvIII [24]. It is possible that some antibodies also recognize EGFRwt by binding to amino acid sequences flanking the EGFRvIII glycine at the junction site. Several

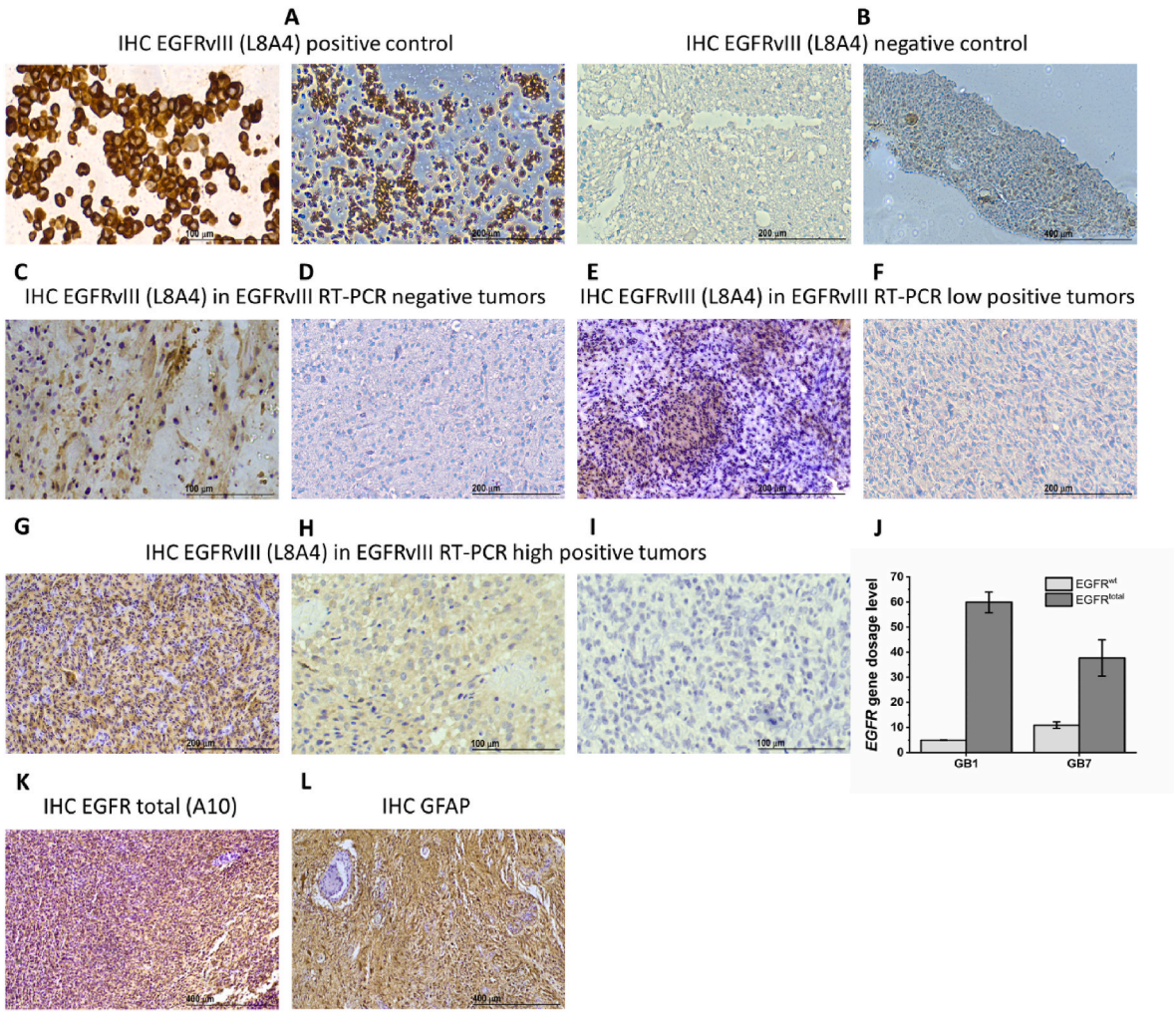

Fig. 4. Exemplary results of immunohistochemical detection of EGFRvIII with L8A4 antibody in glioblastoma slides. (**A**) Cell block sections from DK-MG cells serve positive control while sections from normal brain serve as negative controls (**B**). Five out of the seven EGFRvIII RT-PCR negative samples were also IHC negative for EGFRvIII. (**C**) Despite being EGFRvIII mRNA negative, the sections from GB20 were IHC positive with clearly visible gemistocytes (**D**), while those from GB19 were negative in both IHC and RT-PCR EGFRvIII. In the group of EGFRvIII RT-PCR low-positive samples, only 2/6 were IHC positive for EGFRvIII. (**E**) GB11 was low positive for EGFRvIII in RT-PCR and IHC positive, while GB10 low positive for EGFRvIII in RT-PCR but IHC negative (**F**). In the group of EGFRvIII RT-PCR positive samples 4/7 were also IHC positive for EGFRvIII (**G**) EGFRvIII positive and negative cells were detected in GB7 sections. (**H**) Both cell membrane and cytoplasmic staining for GB2 were detected. (**I**) EGFRvIII positive in RT-PCR GB1 was immunohistochemically negative. (**J**) RT-PCR analysis of DNA stained IHC with L8A4 antibody found samples isolated from paraffin sections to demonstrate a similar occurrence of EGFRvIII to fresh/frozen glioblastoma specimens. As controls, slides were stained for total EGFR (**K**) and GFAP (**L**) proteins expression with the use of A10 and MAB360 antibody, respectively. GB sample numbers were assigned after coding sensitive patient data.

antibodies designed to detect EGFRvIII are commercially available, such as ABT-806, DH8.3 and L8A4. Literature data show that even antibodies with the suggested specificity for EGFRvIII also detect EGFRwt. One such antibody is ABT-806, which binds to EGFRwt upon receptor overexpression or amplification, regardless of EGFRvIII expression Moreover, other commercially available antibodies to detect EGFRvIII were non-specific in immunocytochemical analyses, only L8A4 turned out to be specific and was used for immunohistochemical analyses.

Based on the available in literature data, it appears that the L8A4 antibody is most likely to specifically detect the mutated EGF receptor, and for this reason this antibody was chosen to perform our analyses.

These is a pressing need to obtain and analyze such antibodies as they have great potential in diagnostics and research, as well as in immunotherapies such as CAR-T. The present study examined antibodies that should specifically recognize EGFRvIII. While one of these antibodies turned out to be completely non-specific, i.e. recognizing EGFRwt and EGFRvIII with similar affinity (Fig. 2), a commercially-available antibody (L8A4; Absolute Antibody) showed highly promising initial results. The specificity of antibodies against EGFRvIII was verified by testing glioblastoma specimens' DNA and mRNA levels as well as against positive and negative control samples. The analyses showed that L8A4 can be successfully used for immunocytochemistry and Western blotting, but not for immunohistochemistry assays. In the WB and ICC analyses, the antibody gave interpretable results.

Unfortunately, ICC and WB are rarely used in diagnostics due to the difficulties in quick analysis. In such cases, specimens require the establishment of, for example, a cell culture. In addition, the general research approach is that IHC has been dominant for years, and staining is conducted in parallel with a typical histochemistry. It is worth noting that WB allows EGFRwt to be distinguished from EGFRvIII based on molecular weight, as such, even non-specific antibodies binding both EGFRwt and EGFRvIII could be used in this case.

Our ICC and WB analyses found that L8A4 demonstrated a weak interaction with EGFRwt (Fig. 3). However, paraffin blocks are not suitable for Western blotting. Furthermore, a band of EGFRvIII could be confused with typical EGFRwt degradation products. More importantly,

Table 2
Summary of clinical data (age, sex), Real-time PCR and immunocytochemical detection of EGFRvIII for 10 patients diagnosed with glioblastoma IDH wild type as well as seven stable cell lines. For the purpose of ICC method, L8A4 antibody (Absolute Antibody) was used. Both Real-time PCR and ICC were conducted in triplicates. In samples described as 'low', EGFRvIII mRNA expression was greater than 0 but less than 1, above 1 samples were marked as 'high'. If the EGFRvIII Real-time PCR amplification product was detected above 35 cycles of the reaction, the samples were assigned to the '<35 cycles' group. GB sample numbers were assigned after coding sensitive patient data.

Sample name	Sex	Age	EGFRvIII mRNA expression		EGFRwt mRNA expression	ICC with L8A4 Ab
GB21	F	51		16.713 ± 0.102	9.059 ± 0.164	positive
GB22	F	73		105.573 ± 0.063	4.914 ± 0.279	positive
GB23	F	77	high	9.083 ± 0.050	23.277 ± 0.151	positive
DKMG$^{extra-high}$	n.a.			232.912 ± 0.163	0.974 ± 0.134	positive
DKMG^{high}	n.a.			122.532 ± 0.007	2.284 ± 0.014	positive
DK-MG^{low}	n.a.			4.83 ± 0.127	2.157 ± 0.021	positive
GB27	M	82	low	0.231 ± 0.025	7.433 ± 0.185	negative
DKMG$^{extra-low}$	n.a.			0.243 ± 0.276	00.912 ± 0,028	negative
GB24	M	59		0.012 ± 0.035	1.906 ± 0.158	negative
GB25	M	55	<35 cycles	0.025 ± 0.039	0.423 ± 0.079	negative
GB26	M	55		0.002 ± 0.001	0.780 ± 0.032	negative
GB28	M	68		0.023 ± 0.008	0.455 ± 0.197	negative
GB29	M	76	negative	0.000	0.222 ± 0.294	negative
GB30	M	55		0.000	0.853 ± 0.791	negative
MDA-MB-468	n.a.			0.000	75.946 ± 11.953	negative
U-87 MG	n.a.			0.000	7.119 ± 0.279	negative
T98G	n.a.			0.000	12.461 ± 0.902	negative
GMS-10	n.a.			0.000	6.309 ± 0.312	negative

n.a. – not applicable.

in the case of EGFRvIII, it has been shown that, due to the low percentage of EGFRvIII-positive cells in the tumor, Western blotting gives false negative results when analyzing tumor specimens [9]. This fact, together with protein degradation in frozen sections, reduces the analytical sensitivity of the test.

The greatest difficulties by far associated with the use of L8A4 were associated with immunohistochemical staining. Despite the use of a panel of protocols to optimize each step, diagnostic sensitivity and specificity remained insufficient. In IHC analysis, the sensitivity of detecting EGFRvIII with the L8A4 antibody was found to be 50% and the specificity 80%, assuming that negative staining results of GB slides in which the Real-time PCR product is detected >35 of the reaction cycle are true negative results (Table 1). However, assuming that these results are false negatives, the sensitivity of detecting EGFRvIII with this method drops to 38.46% and specificity to 71.43. In either case, the values are too low, i.e. the test would incorrectly determine the expression of the oncogene in too many cases.

The real-time PCR analyses of EGFRvIII negative sections allowed the detection of cells showing ICC signal after the application of L8A4 (Table 1). The RT-PCR technique used to detect EGFRvIII is characterized by very high sensitivity and specificity, allowing the detection of even single mRNA molecules of EGFRvIII [9]. Therefore, it is unlikely that the discrepancies between IHC and RT-PCR analyses can be explained by the false-negative RT-PCR results. Thus, it was considered whether L8A4 binds to tumor cells more easily than to normal cells, or to tumor cells with a higher EGFRwt expression than to normal cells, however, no such correlation was observed (Table 1). Also, previous studies have suggested that anti-EGFRvIII antibodies including L8A4 may be non-specific when used in IHC staining [25,26]. For example, some studies have indicated detection of EGFRvIII in about half of analyzed HNSCC cases, however, the RT-PCR did not allow for the detection of EGFRvIII mRNA in all EGFRvIII-positive immunohistochemical specimens [25,26]. In addition, IHC testing with the G100 antibody, theoretically specific against EGFRvIII, detected EGFRvIII in normal lungs; however, this is not consistent with most studies of these cancers and these results seem to be false positive [27].

The reasons for the false positive results associated with the L8A4 antibody during IHC remains unclear. Undoubtedly, histochemical processing has a greater effect on protein epitopes than WB or ICC, which also allows the L8A4 antibody to interact with EGFRwt (Fig. 3). It seems that this interaction may be enhanced under different experimental conditions. It was found that the L8A4 antibody also did not show any diagnostic value in ICC analysis: the diagnostic sensitivity of the method was 75% and the specificity 100% when an RT-PCR Ct value of about 35 was assumed as true negative, and a sensitivity of 50% and specificity of 100% for false negatives (Table 2). Although ICC achieved much better results than the IHC, these values are still not sufficient for the diagnostic analysis of clinical samples. Hence, despite the enormous advantage of L8A4 over other antibodies designed against EGFRvIII, the antibody does not appear to demonstrate absolute specificity.

Most diagnostic tests are based on IHC than ICC. Cytological analyses, particularly those based on *in vitro* processing, are rarely used to diagnose tumors commonly associated with EGFRvIII, such as glioblastomas. An exception could be the use of L8A4 in the cytology of cerebrospinal fluid. Immunocytochemical analyses allow the simultaneous detection of more than one protein that without the need for a confocal microscope, this is important in combined therapy (polytherapy), but this procedure has not been seriously considered.

Western blotting is not an option due to its lower diagnostic sensitivity resulting from the low percentage of EGFRvIII-positive cells, and hence its lower analytical sensitivity [9]. As such, the sensitivity and specificity of L8A4 in immunohistochemistry should be carefully assessed before it can be used in oncological diagnostics. Our research so far suggests that these parameters are unsatisfactory.

More importantly, L8A4 scFv is also the basis of CAR-T therapy against EGFRvIII-positive GB [28]. Such therapies can also have an effect on EGFRwt positive but EGFRvIII negative cells, and thus also on normal cells. Our findings indicate that no antibody is currently suitable for IHC testing for therapies targeting EGFRvIII (or generally EGFRvIII-positive cells), and if protein testing is to be useful, such specific antibodies are difficult to obtain and hence L8A4 has no competition so far (Fig. 2). It seems that only cell culture can allow for the effective use of such antibodies,; however, in such cases, cytological methods are more appropriate than histological ones. Therefore, the information about localization of EGFRvIII positive cells within the tumor is lost.

On the other hand, ICC can be used to determine the percentage of EGFRvIII-positive cells. Our long-term analyses show that such tests must be conducted as soon as possible, because under *in vitro* conditions, EGFRvIII-positive glioblastoma cells quickly become senescent and are eliminated from the cell culture during subsequent passages. It is difficult to predict whether such analyses will be applicable due to the unclear role of EGFRvIII in tumorigenesis.

The percentage of EGFRvIII positive cells in a single tumor can be very low, and it is yet not clear why [29]. It is possible that EGFRvIII is a marker of cancer stem cells, which is a promising suggestion from a

therapeutic standpoint [30]. This is an optimistic hypothesis because the removal of such cells would result in the elimination of the entire tumor. However, previous ICC studies found both EGFRvIII positive and EGFRvIII negative cells to express SOX-2 [31]. Unfortunately, in some cases, EGFRvIII cells are not detected in GB recurrences, despite their presence in primary tumors [32].

Furthermore, the mechanism of EGFRvIII oncogenic activity is unclear. Although it initially appeared to be a potent autonomous and constitutive oncogene, it is difficult to confirm whether this receptor has any clear kinase activity, suggesting it has nuclear rather than membrane function [33,34].

It may be possible to induce EGFRvIII expression by means of small molecule blockers of EGFR kinase [35]. However, it is not explained whether this can significantly increase the percentage of positive cells, or rather the receptor density in the positive cells themselves. The latter option may be of little importance in the case of CAR-T.

Clearly this topic merits further study. It is important to confirm whether samples showing trace amounts of EGFRvIII mRNA and demonstrate negative ICC and IHC test results are indeed false negative. From an analytical point of view, it cannot be expected that the ICC test would give positive result if the analogous RT-PCR Ct value is 35. This would indicate that the tested sample contains several EGFRvIII mRNA molecules and the ICC has insufficient analytical sensitivity to confirm this [9].

Does it have any value from diagnostic point of view? If the detection of trace amounts of mRNA had any importance, like with the discovery of BCR/Abl translocations, then of course the result of such a test would be wrong. However, it seems that in this case, the use of targeted therapy in EGFRvIII will not make sense. Therefore, the present study included two separate interpretations of ICC and IHC predictive values. In one interpretation, ICC or IHC tests with negative results, for samples for which the Ct value of Real-time PCR was about 35 were defined as false negative in other as true negative. Unfortunately, nothing can able to defend false positive IHC results. These data discredit IHC for EGFRvIII detection.

It is very difficult to find a good example comparing the use of such antibodies as L8A4 with known diagnostic antibodies. For example, while IDH1R132H antibodies play a critical role as a diagnostic tool, IDH1R132H is present in all tumor cells. It is also not a good target for CAR-T but for small chemical compounds, and can be detected by Sanger sequencing, IHC and NGS. Furthermore, RT-PCR is not optimal for detecting IDH1 mutation, which are heterozygous, and 20% of tumor cells in a sample give ambiguous results after Sanger sequencing. NGS seems to be too complicated to be used for one gene. Most importantly, IDH1R132H antibodies do not detect IDH1wt after either ICC or IHC. As such, ICC seems to be adequate for analyzing IDH1 mutated brain tumors.

One recent proposal involves the use of CAR-T with SynNotch. Here EGFRvIII is not a target for CAR but is recognized by SynNotch, this results in the expression of CAR against an antigen other than EGFRvIII, which is presented on a high percentage of cells, including even normal ones [36]. This assumes that this would limit inflammation.

It remains unclear whether methods based on mRNA and DNA analysis may yield satisfactory diagnostic results, the biology of GB tumors is complex and the role of EGFRvIII remains poorly understood, therefore, research into GB continues.

We believe that our publication yields two key points. Firstly, it cautions against the routine use of L8A4 in diagnostic analyses based on IHC. We recommend the use of ICC if, for some reason, they would actually be preferable to genetic tests, particularly mRNA analyses. Secondly, it indicates that there are currently no grounds for replacing genetic tests with ICC methods requiring *in vitro* culture, however, the use of SynNotch to recognize EGFRvIII may yield improvements, especially if ICC analysis combines EGFRvIII with an antigen recognized by CAR (induced by SynNotch) by double staining. We recommend that only EGFRvIII ICC-positive tumors yielding samples with lower RT-PCR Ct values should be considered for such therapies.

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Informed consent statement

All primary samples were collected using the protocol approved by the Bioethical Committee of the Regional Medical Chamber in Lodz (Approval No. RNN/156/20/KE). All the experiment protocol for involving human data was in accordance with the guidelines of national/international/institutional or Declaration of Helsinki. The consent obtained from study participants was written.

Data availability statement

All data generated or analyzed during this study are included in this published article and its supplementary information files E.S.-F. should be contacted if someone wants to request the data.

CRediT authorship contribution statement

Adrianna Rutkowska: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Writing – original draft, Writing – review & editing. **Tadeusz Strózik:** Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Writing – original draft, Writing – review & editing. **Krystyna Jędrychowska-Dańska:** Conceptualization, Formal analysis, Investigation. **Alicja Zamerska:** Conceptualization, Formal analysis, Investigation. **Dorota Jesionek-Kupnicka:** Conceptualization, Formal analysis, Investigation, Resources. **Tamara Kowalczyk:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources. **Waldemar Och:** Conceptualization, Formal analysis, Investigation, Resources. **Błażej Szóstak:** Conceptualization, Formal analysis, Investigation, Resources. **Cezary Tręda:** Conceptualization, Formal analysis, Investigation, Resources. **Aneta Włodarczyk:** Conceptualization, Formal analysis, Investigation, Resources. **Amelia Kierasińska-Kałka:** Conceptualization, Formal analysis, Investigation, Resources. **Tomasz Wasiak:** Conceptualization, Formal analysis, Investigation, Resources. **Damian Ciunowicz:** Conceptualization, Formal analysis, Investigation, Resources. **Piotr Rieske:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. **Ewelina Stoczyńska-Fidelus:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2023.149133.

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