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All-Trans Retinoic Acid Fosters the Multifarious U87MG Cell Line as a Model of Glioblastoma

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Abstract: Glioblastoma multiforme (GBM) is a primary brain cancer of poor prognosis, with existing treatments remaining essentially palliative. Current GBM therapy fails due to rapid reappearance of the heterogeneous neoplasm, with models suggesting that the recurrent growth is from treatment-resistant glioblastoma stem-like cells (GSCs). Whether GSCs depend on survival/proliferative cues from their surrounding microenvironmental niche, particularly surrounding the leading edge after treatment remains unknown. Simulating human GBM in the laboratory relies on representative cell lines and xenograft models for translational medicine. Due to U87MG source discrepancy and differential proliferation responses to retinoic acid treatment, this study highlights the challenges faced by laboratory scientists working with this representative GBM cell line. Investigating the response to all trans-retinoic acid (ATRA) revealed its sequestering of the prominin-1 stem cell marker. ICAM-1 universally present throughout U87MG was enhanced by ATRA, of interest for chemotherapy targeting studies. ATRA triggered diverse expression patterns of long non-coding RNAs *PARTICLE* and *GAS5* in the leading edge and established monolayer growth zone microenvironment. Karyotyping confirmed the female origin of U87MG sourced from Europe. Passaging U87MG revealed the presence of chromosomal anomalies reflective of structural genomic alterations in this glioblastoma cell line. All evidence considered, this study exposes further phenotypic nuances of U87MG which may belie researchers seeking data contributing towards the elusive cure for GBM.

Keywords: chromosome; lncRNA; CD54; prominin-1; ATRA; brain cancer

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1. Introduction

Transformation of benign astrocyte glial cells instigates the development of fast-growing infiltrative glioblastoma multiforme (GBM), predominantly within the human cerebral cortex [1]. Such proliferating brain cancer activates adjacent normal stromal tissue via the release of pro-tumorigenic factors into their surrounding microenvironment [2]. A dearth of molecular tests has been recognized as an obstacle for evolving clinical practices related to GBM treatment which to date remains non-curative. Scientific evidence is heavily reliant on in vitro cultured cell lines (e.g., human-derived U251, U87, murine GL261 and rat 9L/LacZ, F98, RG2, CNS-1, and C6) [3] and xenografted animal models, to simulate human GBM for translational medicine yet clinical trials have still proven ineffective [4]. Variance exists when attempting to utilize cell line data and animal models due to differences between the in vivo microenvironment and the artificial conditions generated in vitro when growing cells in suspension or in adhesion to plastic surfaces. Appropriate and predictive preclinical animal glioblastoma models generated from xenografted glioma cell lines require optimized implantation sites for assisting the development of innovative GBM therapies [3,5,6].

Therapeutic retinoids operating on a feedback inhibition basis [7], recently sparked a promising GBM treatment strategy utilizing in vitro poly (diol citrate) wafers to ensure the gradual release of all-trans retinoic acid (ATRA) [8]. Cultured astrocytes have been shown to actively synthesize ATRA, the most biologically active metabolite of vitamin A [9], for potential neuronal differentiation [10]. Data revealed that U87MG (Uppsala 87 Malignant Glioma) differentiation levels varied depending on whether ATRA was released from a polymeric wafer or added directly to culture media [8]. Contingent to retinoic acid concentration, opposite proliferation effects on human glioblastoma cell lines were noted [8].

The human derived cell line U87MG was originally obtained from a 44-year-old female patient in 1966 at Uppsala University, Sweden. This cell line has been a hallmark of scientific investigation resulting in thousands of publications offering contributions to glioblastoma research [11]. A comparison of gene-expression profiles between the U87MG cell line distributed by American Type Culture Collection (ATCC) cell repository and the original tumor tissue began after a higher proliferation rate was noted in the former as well as the identification of a Y chromosome [12]. In recognition of the discrepancy, ATCC currently marks U87MG with the identifier HTB-14^{TM} and the description that the cell line is likely to be a glioblastoma of male CNS origin [12].

Operated by Public Health England, U87MG is also available from the European Collection of Authenticated Cell Cultures (ECACC). They indicate that U87MG is derived from a female patient with malignant glioma. This study aimed to investigate the proliferative characteristics, gene expression profile and karyotype of U87MG (ECACC 89081402) post ATRA exposure to highlight subtle challenges associated with this widely utilized laboratory cell line. Given the discrepancy that evidently occurred previously between suppliers and research institutes, it was deemed prudent to characterize U87MG (ECACC 89081402) given recent calls by the neuro-oncology community for the relegation of this cell line as a prerequisite for more representative GBM models.

2. Materials and Methods

2.1. Propagation and Maintenance of U87MG

U87MG (ECACC; cat. #89081402) was grown in Dulbecco modified eagle medium (DMEM; 4.5 g/L D-glucose, 4 µM L-glutamine and 1 mM sodium pyruvate additive), fetal bovine serum (FBS) (10%; Life Technologies, Prague, Czech Republic), penicillin and streptomycin (1%; Sigma-Aldrich®, St. Louis, MO, USA). Cultures were routinely checked for mycoplasma contamination using a LookOut Mycoplasma PCR Detection Kit (Sigma Aldrich, St. Louis, MO, USA, cat. #IMP0035-1KT). In general, cells were grown (with media change every 3 days) in a humidified incubator with 5% CO_2 at 37 °C, to 80% confluency prior to removal from the dish using trypsin (0.25%)/EDTA (0.02%) and sub-culturing (passaging) or harvesting.

2.2. All Trans-Retinoic Acid (ATRA) Preparation

As ATRA (cat. #44540.77, Thermo-Fisher Scientific, Waltham, MA, USA) is extremely sensitive to UV light and air, the entire contents of the purchased ampoule were dissolved in dimethyl sulfoxide as a stock solution. A stock (10 mM) was dissolved in DMEM for a final concentration of 100 µM. U87MG were exposed to an ATRA range of 10^{-7} M–10^{-4} M for up to 7 days.

2.3. Wound Healing Assay

U87MG were seeded at 1×10^5 cells/well and cultivated in 6-well dishes to 80% confluency. Cells were removed with a scraper to create a gap from a demarcated central 1 cm zone in the well and washed twice with PBS. Media was changed to include a range of ATRA concentrations (0–10^{-4} M) per dish for up to 7 days when wound closure was complete. The central or peripheral zones of the dish represented the leading edge (LE) and established cellular monolayer (ECM) of the U87MG culture. Cellular proliferation

into the previously cleared region (LE) and within the ECM was monitored over 7 days using an upright light microscope (Nikon Eclipse TS100, Nikon, Tokyo, Japan) equipped with mounted camera (Canon, Tokyo, Japan) and finally removed for total RNA extraction, gene expression assessment or flow cytometry.

2.4. Determination of Proliferation with EdU Flow Cytometry

A Click-iT™ Plus EdU flow cytometry assay kit (Thermo-Fisher Scientific, Waltham, MA, USA cat #C10634) was utilized to measure U87MG proliferation in the LE and ECM zones of the culture exposed to ATRA concentrations (0–10^{-4} M) as outlined above. EdU (5-ethynyl-2′-deoxyuridine) is a nucleoside analog of thymidine which is incorporated into DNA during active DNA synthesis. The detection is based on a copper catalyzed covalent reaction between picolyl azide coupled to Alexa Fluor™ 647 dye and an alkyne provided by the ethyl moiety of the EdU. The procedure was followed as per manufacturer's instructions with EdU (1 µM per well) exposure for 48 h during wound closure. Cells were removed from the LE and ECM zones with a cell scraper which was rinsed in 1% BSA in PBS. Cells were pelleted by centrifugation at 4000× *g* and resuspended in 4% paraformaldehyde in PBS (100 µL). Following incubation for 15 min in the dark, cells were washed in 1% BSA in PBS (3 mL) and pelleted by centrifugation at 4000× *g*. Cells were resuspended in a saponin-based permeabilization and wash reagent (1×) with incubation for 15 min. A Click-iT Plus reaction cocktail (500 µL per sample) was prepared according to instructions which included PBS, copper protectant, Alexa Fluor 647 picolyl azide and a buffer additive. The reaction mixture was incubated for 30 min at RT with protection from the light. Cells were washed in saponin-based permeabilization and wash reagent (1×) and transferred to polystyrene round bottom tubes (cat. #352054, Corning, New York, NY, USA) for analysis on a BD FACSVerse flow cytometer. Sample measurements were taken using allophycocyanin channel settings (Ex-Max: 650 nm; Em-Max: 660 nm) due to the near identical spectra to that of Alexa Fluor 647 picolyl azide. Fluorescence intensity, cell counts and side scatter profiles were determined through cloud-based capabilities and histogram determination.

2.5. Total RNA Isolation and cDNA Synthesis

Total RNA was isolated from U87MG using a MirVana™ miRNA Isolation Kit (Ambion RNA Life Technologies, Prague, Czech Republic, cat. #AM1560). It should be noted that this protocol extracts total RNA with the option for further purification of RNA enriched for small RNAs which was not done for this study. In brief, 1×10^6 cells were disrupted using lysis solution, with RNA extracted via phenol/chloroform and ethanol precipitation. RNA purification was carried out with solid phase filter cartridges using appropriate washing solutions with final elution in 95 °C ultrapure water. Concentration and purity assessment of total RNA was achieved using O.D. 260/280 ratio determination (NanoDrop 1000, Thermo-Fisher Scientific, Waltham, MA, USA). Total RNA was stored at −80 °C. Total RNA (100 ng) from ±ATRA exposed U87MG cells was converted into first strand cDNA using standard protocol procedures (with the inclusion of random hexamers) and reagents (Thermo Fisher Scientific, cat. #18091050).

2.6. Real Time PCR Quantification of Long Non-Coding RNA and Endogenous Control Genes

Pre-designed single Taqman gene expression assays were purchased (Thermo-Fisher Scientific, Waltham, MA, USA) for the assessment of long non-coding RNAs (*PARTICLE* [13–17] and *GAS5* [18–20]) and GAPDH as an endogenous control gene. The reaction conditions for single gene assays were as reported [15,20]. In brief: cDNA (50–100 ng), 1× Taqman universal PCR master mix (no AmpErase UNG; Life Technologies, Prague, Czech Republic, cat. #4324018), forward and reverse primers (10 pmol), specific fluorescent probe (5 pmol), nuclease-free water up to 25 µL. Negative controls were represented as samples with the absence of template. StepOne™ Real-time PCR systems (Life Technologies, Prague, Czech Republic) enabled holding (50 °C, 2 min; 95 °C, 10 min) and cycling (95 °C, 15 s; 60 °C 1 min; 40 cycles). Cycle threshold values were extracted and fold changes in gene

expression determined by $2^{(-\Delta\Delta Ct)}$. Naïve samples (non ATRA exposed) were normalized to a value = 1 with test samples relatively compared.

2.7. Protein Extraction

Protein extraction from U87MG cells was performed using a T-PER reagent (cat. #78510, Thermo-FisherScientific, Waltham, MA, USA) with addition of protease inhibitor cocktail tablets (cat. #04693116001, Roche, Basel, Switzerland). 100 µL of the reagent was added to the 1×10^6 cells followed by homogenization and sonication (20 s) for membrane disruption. Cells were centrifuged for 5 min at 10,000× *g* to remove cell debris. Protein concentration was determined using a bicinchoninic acid (BCA) assay (cat. #23227, Thermo-FisherScientific, Waltham, MA, USA).

2.8. Electrophoresis and Western Blotting

Cell lysates (25 µg, 10 µL) were mixed with 4× NuPage LDS Sample Buffer (Life Technologies, Prague, Czech Republic) (2.5 µL) and heated for 5 min at 70 °C before loading onto 12% Bis Tris NuPage gels (cat. #NP0342BOX, Novex Life Technologies, Prague, Czech Republic) with electrophoresis in 1× MOPS—SDS running buffer at 180 V in 4 °C. Separated proteins were transferred onto Nytran membranes under standard conditions followed by blocking in 5% bovine serum albumin in TBS-T. Detection of prominin 1/CD133 or ICAM-1/CD54 were determined with over-night incubation at 4 °C (1:1000 in blocking reagent) using rabbit monoclonal anti-prominin 1/CD133 (cat. #ab216323, abcam, Cambridge, United Kingdom,) or rabbit monoclonal anti-CD54/ICAM-1 (cat. #4915, Cell Signaling Technology, Danvers, MA, USA) respectively. GAPDH was also detected as a comparative control using a mouse anti-GAPDH mAb (cat. #C2514, Santa Cruz Biotechnology, Dallas, TX, USA). Following extensive washing with TBS-T, Nytran membranes were exposed to alkaline phosphatase-conjugated goat anti-rabbit (1:5000) (cat. #A-3687, Sigma-Aldrich®, St. Louis, MO, USA) or goat-anti-mouse (1:5000) (cat. #A-3562, Sigma-Aldrich®, St. Louis, MO, USA) secondary antibody for 1 h at RT. Specific proteins (Prominin1/CD133, ICAM1/CD54 and GAPDH) were visualized using a mix of 5-bromo-4-chloro-3′-indolyl-phospate p-toluidine and nitro-blue tetrazolium chloride solution (cat. #1001973039, Sigma-Aldrich®, St. Louis, MO, USA). Western blots were photographed using a FluorChem HD2 gel visualization system (Alpha Innotec, Kasendorf, Germany) with specific protein band intensities quantified using ImageJ (NIH, Bethesda, Rockville, MD, USA).

2.9. Cytogenetic Harvesting of U87MG

Cells were grown to 60–70% confluence with exposure to ATRA (10^{-4} M), washed in phosphate buffered saline and exposed for 17 h to growth medium (outlined above) containing colchicine (250 µg/mL) in standard incubation conditions [21]. Cells were removed with trypsin/EDTA and treated with warm (37 °C) hypotonic solution (ultra-pure water and growth medium, 3:1 ratio respectively) for 20 min at RT. Following centrifugation (1000× *g* for 15 min), the pellet was gently resuspended in ice cold fixative (10 mL; methanol: glacial acetic acid, 3:1 ratio respectively). Centrifugation was repeated as previously done and pellet resuspended as before but in a reduced volume of ice-cold fixative (2 mL) followed by incubation at 4 °C for 20 min. After a final centrifugation (1000× *g* for 15 min), the chromosomal pellet was resuspended in acetic acid (200 µL). Ultra-clean microscope slides were prepared with overnight treatment in hot (65 °C) concentrated HCl, followed by three rounds of sonication in sterile distilled water. Cell suspension solution (25 µL) was applied from a height to cold wet ultra-clean microscope slides and dried on a hotplate (50 °C) for 15 min.

2.10. Wright Staining and Banding of U87MG Chromosomes

Following cell suspension attachment, microscope slides were heated to 95 °C for 25 min. Then slides were immersed for 20 s in Sörensen phosphate buffer (SPB) containing

trypsin (2 µg/mL; pH 6.8). The action of trypsin was stopped with subsequent immersion of slides for 30 s into SPB containing fetal bovine serum (10%; pH 6.8), rinsed in SPB and allowed to air dry. Wright staining solution (WSS: Hydrion buffer capsules diluted in dH_20, pH 6.8 (cat. #60784–226, Micro Essential Laboratory, New York, NY, USA) and Wright stain, 3:1 ratio) was prepared. The slides were placed in a horizonal position on a staining rack and entirely covered with WSS for 5 min. After washing with distilled water, slides were drained and allowed to air dry. At least 10 metaphase spreads of chromosomes per passage were viewed under an Olympus BX43F light microscope at 1000× magnification.

2.11. Giemsa Staining for Structural Chromosomal Abberations

Microscopic slides of U87MG cell suspension were heated as outlined above, allowed to come to RT and immersed in a vertical position into a coplin jar containing Giemsa staining solution (GSS: Giemsa stain in Sörensen phosphate buffer, pH 6.8, 1 in 20 dilution) for 5 min. Slides were transfered to distilled water for 2–3 s to remove excess stain and air dried. At least 10 metaphase spreads of chromosomes per passage were viewed under an Olympus BX43F light microscope at 1000× magnification with images obtained via green pseudo-coloration.

2.12. Immunofluorescence

U87MG were cultivated as previously described above on glass coverslips. Having reached ~80% confluence, the media was removed and the cells were washed two times for 5 min with PBS. Cellular fixation occurred upon exposure to 4% paraformaldehyde for 1 h, followed by washing for 5 min with PBS. Cells were permeabilized in 1× TBST (1× TBS including 0.5% Triton™ X-100 (cat. #X100–5ML, Sigma-Aldrich®, St. Louis, MO, USA)) for 1 h. Following one wash for 5 min in 1× TBS, U87MG were placed in blocking solution (1× TBS containing 5% bovine serum albumin and 0.5% Triton™ X-100) for 1 h at room temperature (RT). Cells were then exposed to antibody representing rabbit monoclonal anti-prominin 1/CD133 (cat. #ab216323, abcam, Cambridge, United Kingdom, 1:200 in blocking solution) or rabbit monoclonal anti-CD54/ICAM-1 (cat. #4915, Cell Signaling Technology, New York, NY, USA, 1:200 in blocking solution) with o/n incubation at 4 °C. Samples were washed three times for 15 min in 1× TBST and incubated in Alexa fluor® 488 goat anti-rabbit IgG (H + L) (1:500; in blocking solution) for 1 h at RT in the dark. This was followed by washing three times for 15 min with 1× TBST and air drying in the dark. To prepare for microscopy, cells on coverslips were mounted in ProLong® Gold Antifade reagent (cat. #8961S, Cell Signaling Technology, New York, NY, USA) containing DAPI, and placed on a glass slide. The images were acquired with a Leica TCS SP8X confocal system (Leica Microsystems, Mannheim, Germany) using an HCX PL APO 40×/1.30 Oil objective and appropriate excitation 405 and 488 nm lasers, which were analyzed in LAS AF software (Leica Microsystems, Mannheim, Germany), and ImageJ 1.47 software (Bethesda, NIH). The brightness and contrast of images have been adjusted in a standardized manner for all of the images as described [22]. All images and graphs were generated and assembled in figures using Adobe Illustrator (Adobe Systems, San Jose, CA, USA).

2.13. Statistical Analysis

Two tailed Student's t-test was employed for comparative purposes between groups. Data is presented as mean ± standard error with significance determined at $p \leq 0.05$. Experiments were replicated $n = 3$.

2.14. Data Availability

All material and data will be available upon request.

3. Results

3.1. Reduced Expression of the Stem Cell Marker Prominin-1 (CD133) in U87MG in Response to ATRA

The prognostic value and distribution of prominin 1/CD133 in U87MG has proven to be controversial due to reported inconsistencies in the literature [23]. Availing of recently generated, more specific anti-CD133 antibodies, our results support the widespread distribution (92.4 ± 3%, CD133+ cells per field of view (FoV = 20)) of this protein within the cytoplasm of U87MG but not within the perinuclear space (Figure 1, Supplementary Materials Figures S1 and S2).

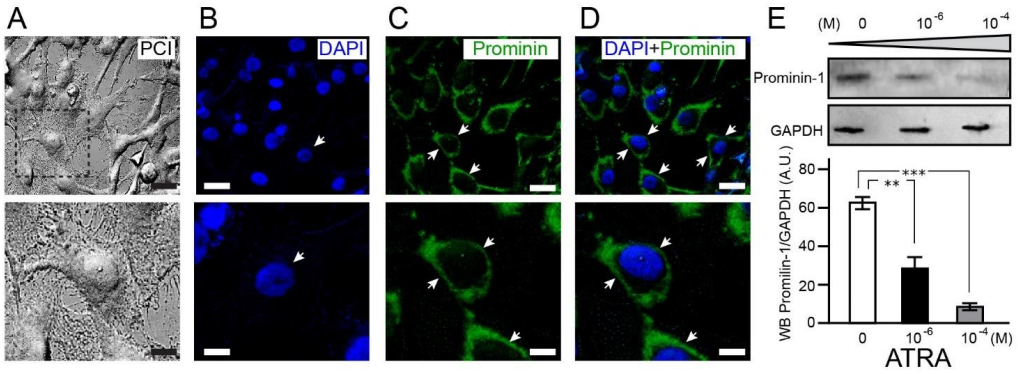

Figure 1. The widely expressed stem cell marker prominin-1 in U87MG is curtailed in a dose dependent manner by ATRA. Phase contrast image (PCI) of early passaged U87MG grown to 8090% confluency ((**A**), upper). Boxed region shown in higher magnification ((**A**), lower) showing a representative U87MG cell with large nucleus and cytoplasmic projections. Confocal micrographs with U87MG nuclear staining (arrow; DAPI—Blue; (**B**)), stem cell marker prominin-1 revealing strong cytoplasmic expression pattern (arrow; Alexa fluor 488; Green; (**C**)) without ATRA treatment. Merged images (**D**) showing lack of prominin-1 peri-nuclear expression. Scale bars: 20 µm (upper) and 10 µm (lower). Representative Western blot highlighting decreased prominin-1 expression in U87MG with increasing ATRA exposure and GAPDH as a loading control ((**E**), upper). Histograms depicting Western blot analysis and significant inverse relationship between prominin-1 expression and ATRA exposure ((**E**), lower). ** $p < 0.005$, *** $p < 0.0005$, $n = 2$.

Prominin 1/CD133 revealed an inverse expression response to increased ATRA concentrations after 7 days exposure as shown by Western blotting analysis. Prominin 1/CD133 significantly decreased in U87MG exposed to ATRA (10^{-6} M, 1-fold, $p = 0.003$; 10^{-4} M, 1.7-fold, $p = 0.000024$) in comparison to non-treated cells (Figure 1). This data supports the role of ATRA in the inhibition of stem cell characteristics as seen with other cancer types, e.g., thyroid [24]. As glioblastoma has a sub-population of stem cells capable of self-renewal and radiotherapy resistance, the response of prominin-1/CD133 to ATRA in U87MG may potentially serve as a GBM cell line model for targeted differentiation strategies.

3.2. The Intercellular Adhesion Molecule (ICAM-1; CD54) Is Distributed throughout U87MG with Increased Expression in Response to ATRA

Research suggests that ICAM-1 is potentially an important mediator of tumor migration and invasion in chemotherapy resistant glioblastoma [25]. The findings from this study provide evidence for the presence of ICAM-1/CD54 in the nucleus and cytoplasm of U87MG (Figure 2) and its even distribution throughout the cell culture population. With increasing ATRA concentrations, U87MG show enhanced expression of ICAM-1/CD54 as shown by Western blot analysis. In comparison to non-treated U87MG, exposure to ATRA (10^{-6} M) caused a significant 1.2 ± 4—fold increase ($p = 0.005$) (Figure 2, Supplementary Materials Figure S3).

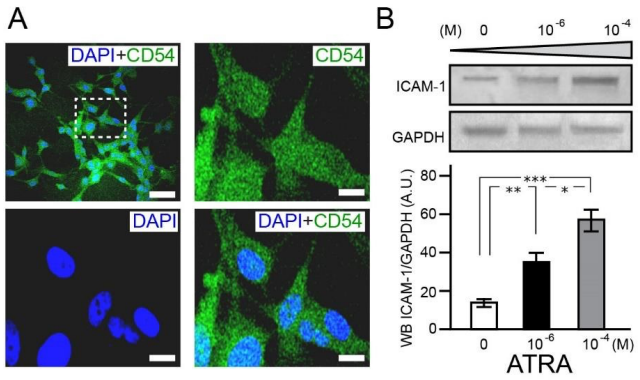

Figure 2. Intercellular adhesion molecule 1 is located in the nucleus and cytosol with increased expression in response to ATRA. Confocal micrographs of U87MG showing immunohistochemical detection of ICAM-1 (CD54; Alexa fluor 488; green) and nuclear stained DAPI (blue) (**A**) after 7 days of ATRA (10^{-4} M) treatment. Scale bar 100 µm. White dashed box region ((**A**), upper left) is shown in higher magnification (×40, (**A**)) to highlight the presence of ICAM-1 in the nucleus and cytosol. Scale bar 20 µm. Representative Western blot (upper (**B**)) and analysis (lower (**B**)) indicates the incremental increased expression of ICAM-1 in response to higher ATRA concentrations. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, $n = 2$.

When similarly compared against non-treated U87MG, further elevation in ATRA concentration (10^{-4} M) resulted in even higher ICAM-1/CD54 expression levels (3.3 ± 3-fold increase, $p = 0.0004$) (Figure 2). Targeting ICAM-1 may provide a strategy for enhancing the efficacy of anti-angiogenic therapy against GBM and prevent the invasive phenotype of this disease. Hence, U87MG could serve as a model for ICAM-1 manipulation studies and chemotherapeutic testing experimentation.

3.3. ATRA Increases Proliferation of U87MG in the Leading Edge in Comparison to the Established Cellular Monolayer

The effects of ATRA on the migration and invasiveness of glioma remains poorly understood, although it is universally accepted that it can induce apoptosis and inhibit proliferation in GBM [26–29]. Nevertheless, the association between the concentration and effects of ATRA and GBM proliferation remains unclear [30]. U87MG is regarded as a proliferative GBM cell line that forms fast-growing tumors when inoculated orthotopically into a mouse [31]. This study chose to examine the proliferative activity of U87MG (passage 5) following ATRA exposure in two regions of an in vitro culture environment i.e., LE and ECM. Five days after wound formation, exposure to ATRA and EdU labelling, U87MG cells were removed and processed for flow cytometry. Results showed that the ECM and LE cells differed slightly in the forward scatter pattern (Figure 3A) indicative of the presence of mature cells with alternative structural conformation in the former zone. Gating analysis for the selection of cells in G2 phase enabled the effects of plus/minus ATRA exposure to be determined (Figure 3B,C).

The percentage of G2 cells per total events (cell number) was similar in the ECM and LE zones ($p = 0.21$, $n = 3$; Figure 3B,C). These findings revealed that ATRA did not significantly alter the proliferative status of cells grown in the ECM zone when in the presence of 10^{-6} or $10^{-}4$ M ATRA ($p = 0.108$; $p = 0.64$ respectively) when compared to non-exposed controls. In contrast U87MG in the LE showed significantly increased proliferation upon ATRA exposure (10^{-4} M) in comparison to controls ($p = 0.028$, $n = 3$). This effect was not found at a lower ATRA concentration (10^{-6} M). Results of these experiments, highlight the differential effects of ATRA within an in vitro culture of U87MG. The concentrations of ATRA used in our study did not inhibit proliferation in U87MG or induce apoptosis in

contrast to previous findings [30] but may influence late passaged cells (see below). Results show the importance of testing a range of ATRA concentrations and the potential influence of the microenvironment when considering this U87MG cell line in proliferation studies.

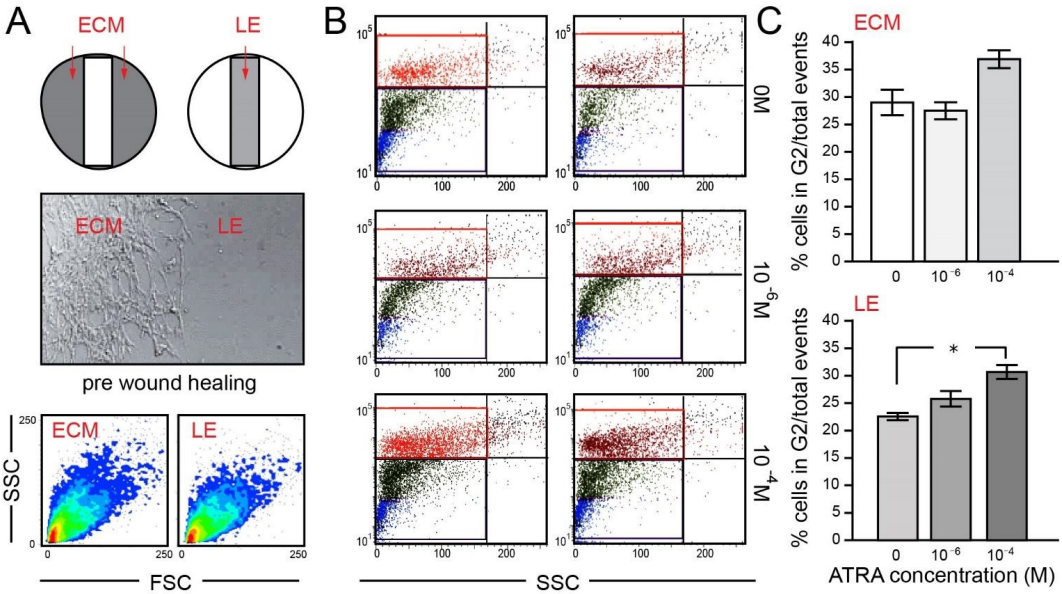

Figure 3. ATRA influences U87MG proliferation only in the leading edge. Schematic of the location of the established cellular monolayer (ECM) and leading edge (LE) zones in a tissue culture in vitro environment post wound formation (**A**); upper). Confocal micrograph of U87MG cells in the ECM and LE before would healing (**A**); lower). Dot blot density cytogram of side scatter (SSC) versus forward scatter (FSC) of U87MG in the ECM (left) and LE (right), providing an indication of the size and granularity of cells respectively. The red/yellow/green/blue hot spots represent increasing numbers of events resulting from discrete cell populations ((**A**); bottom). Side scatter plot of Click-iT Plus EdU AlexaTM 647 fluorescence incorporated cells in ECM and LE zones plus/minus ATRA with gating to demarcate the population in G2 growth phase (upper left (UL); red). Populations in G1 and S phase are indicated in blue and green respectively (**B**). Histograms showing the percentage of cells in G2 per total events (assessed cell population) indicating significance of ATRA (10^{-4} M) in U87MG cells in the LE zone. * $p < 0.05$, $n = 3$, (**C**).

3.4. Long Non-Coding RNAs (lncRNA) at the Leading Edge Respond to ATRA Treatment in Early Passaged U87MG

Next, this investigation sought to determine whether the lncRNAs *PARTICLE* and *GAS5* are expressed in U87MG given their recognized role in tumor activation and repression respectively [14,32,33]. Dose dependent decreased expression of *PARTICLE* and *GAS5* was identified in the LE of U87MG cultures post exposure to ATRA for 7 days when compared to non-treated controls. Significantly reduced expression of these lncRNAs occurred within the ATRA 10^{-7}–10^{-4} M range (Figure 4A,B). When compared to non-treated controls, *PARTICLE* expression showed a significant 22–52% reduction following exposure to ATRA (10^{-7} M, $p = 0.03$; 10^{-6} M, $p = 0.000019$; 10^{-5} M, $p = 0.000018$; 10^{-4} M, $p = 0.011$; Figure 4A). Upon comparison to non-treated controls, *GAS5* expression showed a significant 25–92% lower level after exposure to ATRA (10^{-7} M, $p = 0.04$; 10^{-6} M, $p = 0.03$; 10^{-5} M, $p = 0.027$; 10^{-4} M, $p = 0.004$; Figure 4B). Intriguingly, decreases in *PARTICLE* and *GAS5* were not apparent in the ECM of the culture following ATRA within an identical concentration range and treatment time (Figure 4C,D). Upon passaging, significantly decreased expression of *PARTICLE* (0.4-fold reduction, $p = 0.029$) occurred (Figure 4E), in

contrast to *GAS5* which showed elevated levels (0.9-fold increase, $p = 0.019$) when passage #5 was compared to passage #15 following ATRA treatment (10^{-4} M) (Figure 4F). Results highlight the altered expression of lncRNAs within the microenvironment of the U87MG in vitro culture and the influence of passaging on transcription profiles.

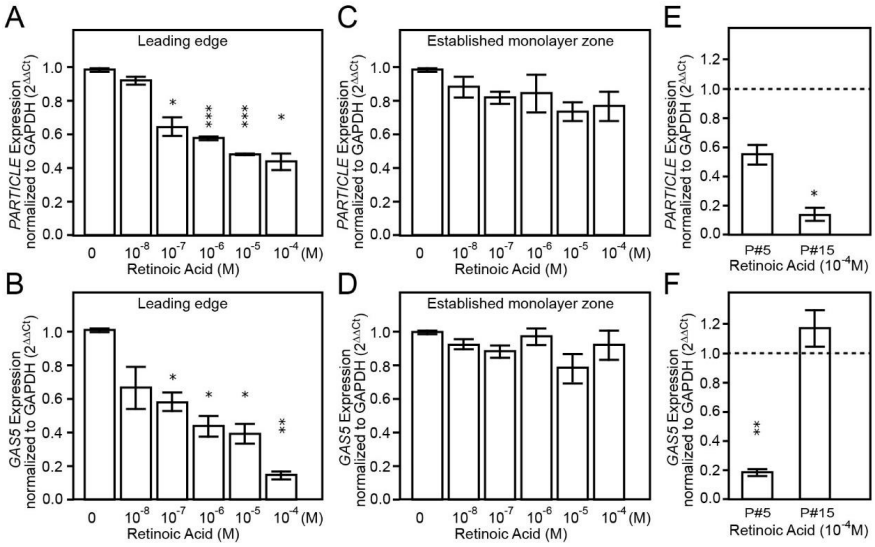

Figure 4. Long non-coding RNA *PARTICLE* and *GAS5* respond to ATRA in U87MG leading edge cells and not in the established cellular monolayer zone with differential passage effects. Histograms of quantitative real time PCR data of *PARTICLE* and *GAS5* showing decreased expression profiles in leading edge cells in response to increased ATRA concentrations (**A**,**B**). Neither *PARTICLE* nor *GAS5* show differential expression in response to increased ATRA concentrations in the established cellular monolayer zone of U87MG (**C**,**D**). Histograms highlight reduced levels of *PARTICLE* with passaging (**E**) in contrast to *GAS5* which shows significantly elevated expression in later passages (**F**). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, $n = 3$.

3.5. Increased Prevalence of Chromosomal Aberrations with Passaging in U87MG Exposed to ATRA

Microscopic evaluation of chromosomal spreads revealed varying susceptibility to aberrations across the 22 autosomes and X gonosome. Frequent chromosomal anomalies included potential inter-chromatid interchanges, interstitial deletions, break discontinuities and dicentric occurrence in U87MG passaged 10–16 times (Figure 5A 1–6). Monosomy was evident at passage 10 with chromosomes 11, 13, 14 and 21 represented as single non-paired chromosomes (Figure 5B). The lack of chromosome pairs became more prevalent by passage 14 onwards (Figure 5C) affecting chromosome 5, 6, 8, 9, 11, 13, 14, 15, 17 and 18. Chromosomes 3, 4, 20, 22 and X were stable up until late passaging (passage 16) was reached by which time polyploidy was evident across the entire karyotype of the U87MG line (Figure 5C). Chromosome 21 was only found as a single chromosome in 70% of chromosomal spreads examined from passage 10 (R.O.I. = 20). Given that U87MG were exposed to ATRA (10^{-4} M) and subsequently passaged, the chromosomal aberrations are potentially the effect of prolonged passaging rather than lengthy ATRA exposure.

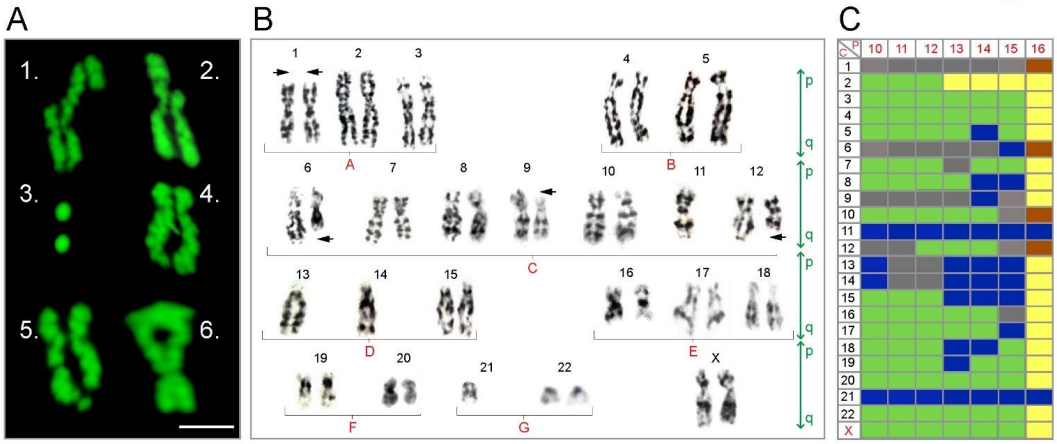

Figure 5. Chromosomal aberrations with passaging in U87MG exposed to ATRA. Representative examples of pseudo-colored chromosomes after Giemsa staining showing various chromosome aberrations in U87MG cells post passage 10–16. Chromosome 6 alignment with chromosome 13 with potential inter-chromatid interchanges (**A1**), chromosome 3 with chromatid p arm deletion (**A2**), interstitial deletions (**A3**), chromosome 12 with chromatid protrusion (**A4**), chromosome 4 with potential q-arm breakage (**A5**), chromosome 6 telomeric alignment with chromosome 18 or dicentric chromosome (**A6**). Representative karyotype spread of Wright stained chromosomes from U87MG passage 10 cells. Monosomy indicated by absence of chromosome. P and q represent short and long chromosomal arms. Chromosomal groups represented by letters A–G, and chromosomes numbered according to standard practice for a female karyotype. Arrows point to deletions (black arrows) (**B**). Illustrative table summarizing chromosomal anomalies arising with passaging U87MG. Passage (P) number shown across the top, chromosome (**C**) number shown along the side of the table. Colors represent chromosomal deletions (grey), stability (green), anomalies (red), polyploidy (yellow) and monosomy (navy blue).

4. Discussion

The characterization of U87MG (ECACC 89081402) and its response to ATRA were undertaken in this study. Our findings revealed the extensive stem cell attributes of this cell line as shown by the widespread expression of prominin-1 (CD133) which becomes significantly decreased in a dose dependent manner in response to elevated ATRA levels. An intercellular adhesion molecule (ICAM-1, CD54) was also found to be highly expressed in U87MG when exposed to ATRA, offering a potential target against chemotherapy resistance. Focusing on this cell lines response to ATRA enabled the deciphering of alternative proliferation rates between the established cellular monolayer and leading-edge zones. The expression profile of long non-coding RNAs *PARTICLE* and *GAS5* were characterized in this cell line, whereby both responded inversely in a dose dependent manner to serial concentration increases in ATRA, but only at the leading edge. Prolonged U87MG passaging significantly decreased expression of *PARTICLE* in contrast to elevated levels determined for *GAS5* in the presence of ATRA. Karyotyping U87MG confirmed the female origins of this cell line due to the absence of the Y chromosome and presence of the X chromosomal pair. The frequency of chromosomal aberrations increased with passaging from passage 10 to 16 with extensive polyploidy evident at the late stage. This report reveals the dynamic nature of this cell line that provides challenges for stable data acquisition for glioblastoma research. U87MG (ECACC 89081402) and U87MG (HTB-14; ATCC) share identical STR profiles except for sex chromosome profiles according to supplier websites. These sources indicate that U87MG (ECACC 89081402) reaches 100% confluency between seventy to ninety hours cultivation, in contrast to U87MG (HTB-14; ATCC) which tends to become non-adherent during proliferation.

Prominent features of glioblastoma which hamper effective treatment include rapid growth and clonal spatial and temporal heterogeneity with stem-like features [34]. Prominin-1 (CD133) is regarded as a tool for hematopoietic stem cell isolation [35] as well as an identification marker for targeting populations of malignant transformation in certain cancers such as leukemia [35]. The extensive and high levels of prominin-1 in U87MG reported here is supported by others utilizing novel adherent culture methods [36]. ICAM-1 was also prevalent in U87MG, which is associated with invasion and metastases in several types of cancer [25]. It has been shown that inhibition of ICAM-1 reduced glioma invasion in vitro and in vivo [37]. Targeting ICAM-1 offers potential for overcoming glioblastoma's resistance to antiangiogenic therapy and improve prognosis [25].

Compromised retinoid signaling in carcinogenesis proposed a role for retinoic acid deficiency in tumor development [38]. ATRA was shown to inhibit proliferation of some glioma cell lines [39]. However, the therapeutic role of retinoic acid in cancer remains uncertain due to increased risks of mortality associated with its supplementation [7]. It has been proposed that gliomas may be initiated by an increase in endogenous production of retinoic acid in glia with alternative effects on receptor expression levels [7]. The differential effects of ATRA on the leading edge versus the established cellular monolayer within an in vitro culture dish shown in this report, highlights the ambiguities of its proliferative instigation properties.

Evidence has shown the important role of lncRNAs in tumor suppressor regulation [7,13–17,40] yet their involvement in GBM is very much under explored to date [41]. The lncRNA *PARTICLE* controls the expression of tumor suppressors *MAT2A* and *WWOX* in breast cancer and osteosarcoma respectively [13–17]. This foremost study represents the initial demonstration that *PARTICLE* is expressed in U87MG with significantly higher levels of expression at P5 compared to P15 (0.4-fold decrease, $p < 0.05$). These results support the role of *PARTICLE* as a negative regulator of tumor suppressors [13–17]. Nevertheless, the potential for the role of *PARTICLE* in epigenetic modulation associated with tumorigenicity loss can also be possible [13]. Findings reveal its significant dose dependent reduction to elevated ATRA concentrations in leading edge U87MG in vitro at the P5 stage. Surprisingly, this effect was not found in the established cellular monolayer zone, highlighting the differential response of lncRNA to this retinoid within the in vitro microenvironment. The implications for this are so far unknown and may reflect the dysregulated expression of lncRNAs associated within brain tumors [42,43].

LncRNA profiling of GBMs using The Cancer Genome Atlas database, identified *GAS5* among others closely associated with patient overall survival [44]. A functional role as a tumor suppressor has been previously reported for *GAS5* in U87 (obtained from the Shanghai Institutes for Biological Sciences Cell Resource Center) [40]. Our findings showed a significant elevation in *GAS5* with passaging as reflected in the 4.8-fold increase in expression levels in P15 compared to P5 in response to similar concentrations of ATRA. Likewise, findings showed an inverse expression level of *GAS5* with increased ATRA levels only in leading-edge cells and not in the established cellular monolayer zone similar to the *PARTICLE* profile in this regard.

Continuous serial in vitro cellular passaging is routinely undertaken in biological research laboratories. It has been reported that late passage U87MG show decreased tumorigenicity with lower invasion capabilities than early passaged cells without significant differences in proliferation and migration [41]. Given the suggestion that passage-induced changes may lead to notable changes in biological characteristics [41], this study examined the potential for chromosomal alterations post ATRA exposure between passage 10 and passage 16 of U87MG. While other studies have investigated the effects of serial passaging up to 100 times, our study noted the extensive polyploidy by passage 16 followed by cellular deterioration and apoptosis. This work supports the necessity to avoid lengthy serial passaging and encourages the use of identical or similar passage number for experimental data acquisition. Human U87MG is one of the most commonly studied grade IV glioblastoma cell lines. It has come into focus recently given the probable misidentification

of U87MG procured from ATCC [12]. Genomic sequencing of U87MG unveiled an exceptionally dense mutational landscape that supports a model where cancer mutations occur via structural instability rather than novel point mutations [45].

In conclusion, U87MG (ECACC 89081402) offers a glioblastoma cell line model for exploitation of stem cell differentiation and chemotherapy resistance. Caution is advised when assessing proliferation outputs due to molecular differences within the in vitro microenvironment. An avoidance of continuous passaging is recommended due to the development of karyotype aberrations. While more representative GBM models are keenly sought, the U87MG may still offer a platform for glioblastoma research on condition that the challenges associated with this cell line do not go unheeded by the scientific community.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/brainsci11060812/s1, Figure S1: Representative bright field images of the glioblastoma cell line U87MG (ECACC 89081402) treated for 7 days with ATRA. Figure S2: The stem cell marker prominin-1 shows low levels of expression in U87MG (ECACC 89081402) after 7 days treatment with ATRA (10^{-4} M). Figure S3: ICAM-1 shows low levels of expression in control U87MG (ECACC 89081402) not treated with ATRA.

Author Contributions: M.P. and M.V. undertook laboratory experimentation and data analysis; M.H. and I.J. contributed flow cytometry data; M.P. and Z.P. provided karyotyping analysis; J.P. and V.K. provided confocal microscopy data; S.V.O. undertook graphic design and statistical analysis; M.Č. provided facilities and project support; V.B.O. conceived the project, wrote the manuscript and supervised the research. All authors have read and agreed to the published version of the manuscript.

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Biomolecules to Biomarkers? U87MG Marker Evaluation on the Path towards Glioblastoma Multiforme Pathogenesis

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Abstract: The heterogeneity of the glioma subtype glioblastoma multiforme (GBM) challenges effective neuropathological treatment. The reliance on in vitro studies and xenografted animal models to simulate human GBM has proven ineffective. Currently, a dearth of knowledge exists regarding the applicability of cell line biomolecules to the realm of GBM pathogenesis. Our study's objectives were to address this preclinical issue and assess prominin-1, ICAM-1, *PARTICLE* and *GAS5* as potential GBM diagnostic targets. The methodologies included haemoxylin and eosin staining, immunofluorescence, in situ hybridization and quantitative PCR. The findings identified that morphology correlates with malignancy in GBM patient pathology. Immunofluorescence confocal microscopy revealed prominin-1 in pseudo-palisades adjacent to necrotic foci in both animal and human GBM. Evidence is presented for an ICAM-1 association with degenerating vasculature. Significantly elevated nuclear *PARTICLE* expression from in situ hybridization and quantitative PCR reflected its role as a tumor activator. *GAS5* identified within necrotic GBM validated this potential prognostic biomolecule with extended survival. Here we present evidence for the stem cell marker prominin-1 and the chemotherapeutic target ICAM-1 in a glioma animal model and GBM pathology sections from patients that elicited alternative responses to adjuvant chemotherapy. This foremost study introduces the long non-coding RNA *PARTICLE* into the context of human GBM pathogenesis while substantiating the role of *GAS5* as a tumor suppressor. The validation of GBM biomarkers from cellular models contributes to the advancement towards superior detection, therapeutic responders and the ultimate attainment of promising prognoses for this currently incurable brain cancer.

Keywords: prominin-1; ICAM-1; lncRNA; Glioma; Eker rats; U87MG; *PARTICLE*; *GAS5*; GBM; Biomarker

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1. Introduction

Specialized glial cells, known as astrocytes, outnumber neurons in the central nervous system (CNS) by over fivefold [1,2]. Furthermore, astrocytes support the detoxification of blood capillary networks and guidance during migration [3,4]. Deregulation of astrocytic pathways along with the build-up of somatic mutations in glial progenitor cells empower malignant glioma formation, a subset of which is glioblastoma multiforme (GBM) [5,6]. Despite its relatively low occurrence rate in comparison to all cancer types, GBM (WHO grade IV) is an aggressive high-grade oligo—astrocytoma with mixed cellular features

and, on average, a maximum fifteen-month median survival rate [7,8]. The heterogeneous morphological features of GBM make classification a challenge for neuropathologists due to the presence of diverse tumor and non-tumor cell types along with microenvironmental components [9,10]. Notwithstanding advances in conventional treatments involving surgical resection followed by radiotherapy and adjuvant chemotherapy interventions as well as electric tumor-treating fields (TTFields) [11], GBM remains incurable due to therapeutic resistance and its structural infiltrative characteristics [12,13]. The incorporation of molecular data, such as Ki-67, isocitrate dehydrogenase (IDH) and *BRAF* V600E mutations, have been recognized as potential prognostic and predictive biomarkers of GBM [14,15]. Recently, the fifth edition of the WHO Classification of Tumors of the Central Nervous System has greatly emphasized the importance of molecular parameters to grade gliomas and estimate prognosis more precisely [16]. Current genetic markers of glioma also include codeletions of chromosomal arms 1p and 19q, histone H3F3A alterations, nuclear alpha-thalassemia/mental retardation X-linked syndrome (ATRX) gene mutations, O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation status, loss of cyclin-dependent kinase inhibitor 2A (CDKN2A), epidermal growth factor receptor (EGFR) amplification, combined gain of chromosome 7 and loss of chromosome 10 and telomerase reverse transcriptase (TERT) promoter mutations [17]. Further research is required to fully assess their prognostic value in the context of glioblastoma and their influence on treatment selection. Immunotherapy is at an early stage with tyrosine kinase inhibitors (e.g., gefitinib and erlotinib), proving unsuccessful in GBM phase II clinical trials [18]. New GBM molecular markers are required from recently recognized biological avenues, such as tissue-specific long non-coding RNAs and their epigenetic influence [17]. Advanced statistical methodology are warranted to assist in prognostic accuracy [19]. The current issue is that an imperfect understanding of GBM potentially accounts for such failures and has hastened the need to identify nuances in associated biocompatible nano-molecules to combat the challenge in visualization and grading GBM tumors towards durable effective therapeutic responses [20,21]. Microscopic features of GBM include microvascular proliferation, hypercellularity, high mitotic count, nuclei atypica and pseudo-palisades adjacent to hypoxic and necrotic foci [22,23]. Simulating human GBM has relied heavily on in vitro cell line platforms and xenografted animal models, yet the data from such sources has generally proven untranslatable in clinical trials focused on treatment pathways [24,25].

Recently, we validated and deciphered the phenotypic nuances of the GBM cell line U87MG (ECACC 89081402; p53 wild-type; methylated MGMT promoter) [2,26,27]. Findings revealed that exposure to all-trans retinoic acid (ATRA) attenuated the expression of prominin-1 (CD133), a stem cell marker. Histochemical evidence highlighted the presence of prominin-1 in the cytoplasm but its absence in perinuclear space [26]. Conversely, the intercellular adhesion molecule-1 (ICAM-1; CD54), a chemotherapeutic targeting molecule, showed increased expression in response to ATRA [26]. ICAM-1 is regarded as an important mediator in tumor migration and invasion [28,29]. The long non-coding RNA (lncRNA) *PARTICLE*, a negative regulator of tumor suppressors, demonstrated significantly high expressivity in early passaged U87MG exposed to ATRA with transcripts confined to the leading edge of the microenvironment [26]. Nevertheless, low transcript levels of the lncRNA *GAS5* were noted in this location under similar conditions [26], reflective of its tumor suppressor status and association with overall GBM patient survival [30].

The objective of this study was to evaluate previously obtained in vitro U87MG derived data within rodent and human ex vivo GBM platforms. A germline insertion of the tuberous sclerosis (*Tsc2*) gene gave rise to the established Eker rat model [31]. Our previous investigations in aged Tsc2 +/− rats determined hallmarks of gliomas [32]. This study focused on the Eker rat model and four GBM patients representing responders or non-responders to temozolomide chemotherapy. Evidence of prominin-1 and ICAM-1 is presented and their pertinence toward GBM patient histopathology is considered. This foremost examination highlights the expression profile of lncRNA *PARTICLE* in GBM

derived from patients and validates *GAS5* in this context, providing insights into potentially valuable targeting biomolecules for GBM therapeutics.

2. Materials and Methods

This research focused on the examination of histological sections obtained from patients displaying the classical pattern of GBM and elicited various responses to chemotherapy. Where possible, analysis was performed in parallel with sections from an Eker rat glioma model (inclusion criteria: 18-month-old males, subcortical tumors in the cerebrum, tsc2 +/− genotype [32,33]). All experimental procedures were approved by the local Committee for Animal Protection and were conducted in accordance with the Animal Protection Code of the Czech Republic and the directive of the European Community Council on the use of laboratory animals (2010/63/EC).

2.1. Patient Diagnosis and Pathology

Patients diagnosed with GBM (Table 1), who underwent surgical resection with/without temozolomide chemotherapy, were part of a standard therapeutic intervention at the University Hospital Královské, Vinohrady, Prague, Czech Republic. Tissue sections (5 µm) were cut with a microtome from paraffin embedded GBM and stained for morphological and molecular biomarker readouts as indicated below.

Table 1. Clinicopathological characteristics of patients involved in this study who were diagnosed with glioblastoma multiforme (WHO Grade IV) and treated with surgical resection with/without temozolomide (TMZ). Abbreviations: NT: temozolomide not tolerated; NK: temozolomide treatment not known; Met.: metastasis; IDH: isocitrate dehydrogenase 1; WT: wild-type; +: presence of the *IDH* R132H mutation.

Patient Number	Identification Number	Gender	Birth Year	Diagnosis Year	Death Year	Primary Tumour	IDH	TMZ	Met.
P1	5488/19	Female	1962	2019	2019	Yes	WT	NK	No
P2	11724/19	Male	1949	2019	2020	Yes	+	NT	No
P3	47/19	Male	1952	2019	2020	Yes	WT	Yes	No
P4	286/19	Male	1943	2019	2020	Yes	WT	Yes	No

2.2. Haematoxylin and Eosin Staining of Human GBM Pathogenesis

Slides were deparaffinized in xylene (3 × 10 min), submerged in absolute ethanol (3 × 5 min) and rehydrated in a 95–70% ethanol series (5 min). Slides were washed in running water (2 min) and then stained in Mayer's haematoxylin solution (3 min) according to standard protocols [34]. After washing in running water (15 min), slides were immersed in eosin solution (2 min) and subsequently rinsed in 70% ethanol and 95% ethanol. Slides were dehydrated in ethanol (100%, 5 × 3 min), cleared in xylene (10 × 3 min), cover-slipped and viewed under an Olympus BX43F light microscope using the 10× and 40× objectives.

2.3. Immunohistochemistry for Prominin-1, ICAM-1 and/or GFAP Astrocyte Marker in Cortical Sections

Following deparaffinization and fixation in absolute ethanol (outlined above), pathological tumor sections were permeabilized in PBST (PBS, 0.4% Triton X-100) for 30 min and washed in PBS (10 × 3 min). Sections were exposed to a blocking solution (5% bovine serum albumin in PBS). The presence of prominin-1 or ICAM-1 was determined with incubation (O/N 4 °C; 1: 200 dilution in blocking solution) in rabbit monoclonal anti-prominin-1/CD133 (cat. # ab216323; Abcam, Cambridge, UK) or rabbit monoclonal anti-ICAM-1/CD54 (cat. # 4915, Cell Signaling Technology, Danvers, MA, USA), respectively. Following washes in PBST (15 × 3 min), sections were incubated in the dark in Alexa fluor®488 goat anti-rabbit IgG (H + L) (cat. # A-11008; Thermo Scientific (Waltham, MA USA); 1:500; 1 h RT in blocking

solution). The protocol continued as required for double immunostaining with the astrocyte marker glial fibrillary acidic protein (GFAP). After washing in PBS (15 min × 3), the presence of GFAP was determined with incubation (O/N 4 °C; 1:200 dilution in blocking solution) in mouse monoclonal anti-GFAP (cat. # 53-9892-82; Thermo Scientific). After washing in PBS (15 min × 3), sections were mounted in ProLong® Gold Antifade reagent (cat. #8961S, Cell Signaling Technology, New York, NY, USA) containing DAPI and cover-slipped. Images were acquired with a Leica TCS SP8X confocal system (Leica Microsystems, Mannheim, Germany) using an HCX PL APO 40_/1.30 Oil objective or for low magnification HCX PLS-APO 5× and appropriate excitation with 405 and 488 nm lasers, which were then analyzed with LAS AF software (Version 2.6 Light, Leica Microsystems, Mannheim, Germany) and ImageJ 1.47 software (NIH, Bethesda, MD, USA). The brightness and contrast of images were adjusted in a standardized manner for all images as previously described [32,33]. All images and graphs were generated and assembled in figures using SPSS (version 6) and Microsoft PowerPoint (Microsoft Office 10). Colocalization between prominin-1 or ICAM-1 and GFAP was determined using the ImageJ plugins (Just Another Colocalization Finder (JACoP) and Colocalization Finder (NIH, Bethesda, MD, USA)). Pearson's (r) and Mander's (M) correlation coefficients were used as determinants of red and green fluorescence signal alignment. Complete colocalization was considered equal to 1.

2.4. Fluorescence In Situ Hybridization and Confocal Microscopic Analysis of lncRNAs PARTICLE and GAS5

Procedures were carried out as previously reported [35,36] and in accordance with Stellaris fluorescence in situ hybridization (FISH) instructions (www.biocat.com (accessed on 20 July 2022)). Through an online probe design tool (www.biosearchtech.com/stellarisdesigner/ (accessed on 10 August 2022), specific probes were chosen from input sequences (*PARTICLE* NR_038942.1; *GAS5* NR_152521.1) for optimal binding properties to the target RNA. Probe fluorophores 5′carboxyfluorescein FAM (excitation (Ex): 495 nm; emission (Em): 520 nm) were chosen for the detection of these lncRNAs. Confocal fluorescence microscopic imaging was acquired with a Leica TCS SP8X confocal system (Leica Microsystems, Mannheim, Germany) using an HCX PL APO 40_/1.30 Oil objective and appropriate excitation using 488 nm and emission using 509 nm lasers. Emitted fluorescence signals were sampled at a resolution of 30 nm/pixel with a dwell time of 1.5 µs. Image analysis was carried out as indicated above.

2.5. RNA Isolation and Real-Time Quantitative PCR from GBM Pathology Sections

A modified protocol for long non-coding transcript isolation from paraffin embedded sections was utilized [37]. In brief, this involved GBM removal from microscope slides with a blade cleaned with xylene followed by deparaffinization. Protein digestion was carried out with lysis buffer (20 mM Tris HCl pH 8.0, 1mM $CaCl_2$, 0.5% sodium dodecyl sulphate, 5 mg proteinase K (cat. # 19131, Qiagen, Venlo, The Netherlands)) and with incubation at 56 °C for 1 h and immediate transfer to ice. Trizol reagent (cat. # T9424, Sigma Aldrich, St. Louis, MO, USA) with chloroform (cat. # 17110, Penta Chemicals, Prague, Czech Republic) extraction was added, followed by centrifugation for phase separation. Isopropanol (cat. # 17500-11000, Penta Chemicals) was added to the upper layer with RNA, followed by O/N precipitation. After centrifugation, RNA pellets were air dried and resuspended in nuclease free water with purity assessment using O.D. 260/280 ratio determination (NanoDrop 1000, Thermo Scientific). RNA was treated with DNase I for 20 min at 37 °C to remove genomic DNA. Total RNA (1 µg) was converted into first strand cDNA using standard protocol procedures (with the inclusion of random hexamers) and reagents from Life Technologies, Darmstadt, Germany. Quantitative PCR for the determination of lncRNA *PARTICLE* utilizing the following reaction conditions: cDNA (50–100 ng), 1 × Taqman universal PCR master mix (no AmpErase UNG; Life Technologies, cat. # 4324018) and 1 × Taqman *PARTICLE* expression assay (cat. # Hs03847241_s1, Thermo Scientific).

2.6. Statistical Analysis

A two tailed Student's *t*-test was employed for comparative purposes between samples (Excel, Microsoft Office, Version 10). Data are presented as mean ± standard error with alpha significance determined at $p = 0.05$. Interquartile range represented the median and data range distribution (SPSS version 28 software, IBM). Experiments were replicated three times for patients ($n = 4$, total R.O.I = 10 per sample) and Eker rats (cortical sections obtained from a previous study involving $n = 6$ [32], R.O.I = 10 per sample).

3. Results

3.1. Morphological Correlates of Malignant Glioma/GBM in Patient Histopathology

Individuals represented responders or non-responders to temozolomide chemotherapy (Table 1), with the hallmarks of GBM (according to WHO diagnostic criteria [16]) evident in all patients. While the tumor margins were poorly defined, heterogeneous-shaped clusters of tumor cells could be seen intercalating with necrotic regions (Figure 1A) and (Supplementary Materials). The tumors were highly vascularized (Figure 1B), with proliferative cells preferentially adjacent to newly formed microvessels (Figure 1C). Tumor cells, with heterochromic polymorphic nuclei, were associated with regions showing extensive necrosis (Figure 1D). Complex anatomical features included zones of proliferating cells coinciding with areas of complete cellular depletion, which were caused presumably by apoptotic events (Figure 1E). The tumors were of high cellularity, with an apparent extensive mitotic activity that resulted in pleomorphic nuclei (Figure 1F). This tended to cause a high nuclear-to-cytoplasmic ratio in tumor cells (Figure 1F). Such features of malignant GBM confirmed the clinicopathological characteristics in these classical GBM patients.

3.2. Prominin-1 Independent from Astrocytes Elicits Elevated Expression in Pseudo-Palisades Adjacent to Necrotic Foci

The astrocytoma basis of GBM prompted the examination of prominin-1 expression in astrocytes in patient samples. Analysis revealed a lack of colocalization of prominin-1 in this cell type in pseudo-palisades or surrounding tissue (Pearson's correlation coefficient r = 0.386 or r = 0.482, respectively; Figure 2(A1–A6)). Equal intensity threshold values for green or red fluorescence micrographs showed divergent Mander's colocalization coefficient values in pseudo-palisades versus the surrounding tissue (Figure 2(A6)). It has been proposed that pseudo-palisades are hypoxic tumor cells actively migrating away from a central vascular lumen which has either degenerated or thrombosed [38]. This unique feature of malignant glioma was evident in all four GBM patient pathology sections, as well as in the Eker rat cortical brain regions examined in this report. All human GBM slides contained expansive necrotic regions (Figure 2B) within which were ovoid elongated structures predominated by prominin-1 expression along their peripheral palisades (Figure 2(B1–B5)). A similar pattern was evident in the Eker rat cortex, whereby prominin-1 showed a specific association with ring-like structures within degraded tissue (Figure 2(C1–C3)). Previously shown to be widely distributed within U87MG [26], dispersion data indicated that the median prominin-1 signal was higher in the Eker rat cortex (median = 110.6 arbitrary units (a.u.)) than in the GBM patients (46.5–92.33 a.u.) (Figure 2D). The prominin-1 signal intensity range for patient 3 (P3) and patient 4 (P4) were identical at 50.5 a.u. for both cases, though P3 had a lower interquartile range (IQR: 27.15 a. u.) compared to P4 (IQR: 45.05). Similar mean prominin-1 intensity values were also determined for these individuals (P3: 52.17 ± 18 a.u.; P4: 58.85 ± 21 a.u.; Student's *t*-test $p = 0.784$). Both patients had undergone similar chemotherapeutic adjuvant therapy (Table 1), potentially contributing to the alignment of prominin-1 signal intensity ranges within their respective pathological specimens. The IQR for patient 1 (P1) was lower and was positively skewed, while the IQR for patient 2 (P2) was wider and had a negative skew. The dispersion range for prominin-1 in the Eker rat cortex was short (29.66 a.u.; IQR = 19.65 a.u.) and almost symmetrical. When the focus is placed solely on the ovoid pseudo-palisade areas, significant differences emerged with prominin-1 expression between humans and rodents. The percentage of prominin-1 signal per pseudo-palisade

area (R.O.I = 10) was significantly higher in the Eker rat cortexes compared to all GBM patients (P1, p = 0.0028; P3, p = 0.0005; P4, p = 0.0028; Student t-test), except for one individual (P2, p = 0.55) who had been intolerant to chemotherapeutic adjuvant intervention (Figure 2E). While prominin-1 appears confined to similar areas in rodent and human GBM sections, variable expression was noted between species. Given the varied prominin-1 expression between GBM cases, future larger studies are envisioned to determine whether this is reflective of previous therapeutic interventions. Future perspectives should consider proteomic investigations of prominin-1 containing membrane particles (not exosomes) in human body fluids (e.g., human urine, seminal fluid and saliva) to diagnose diseases involving the down-regulation of stem-cell properties or differentiation [39].

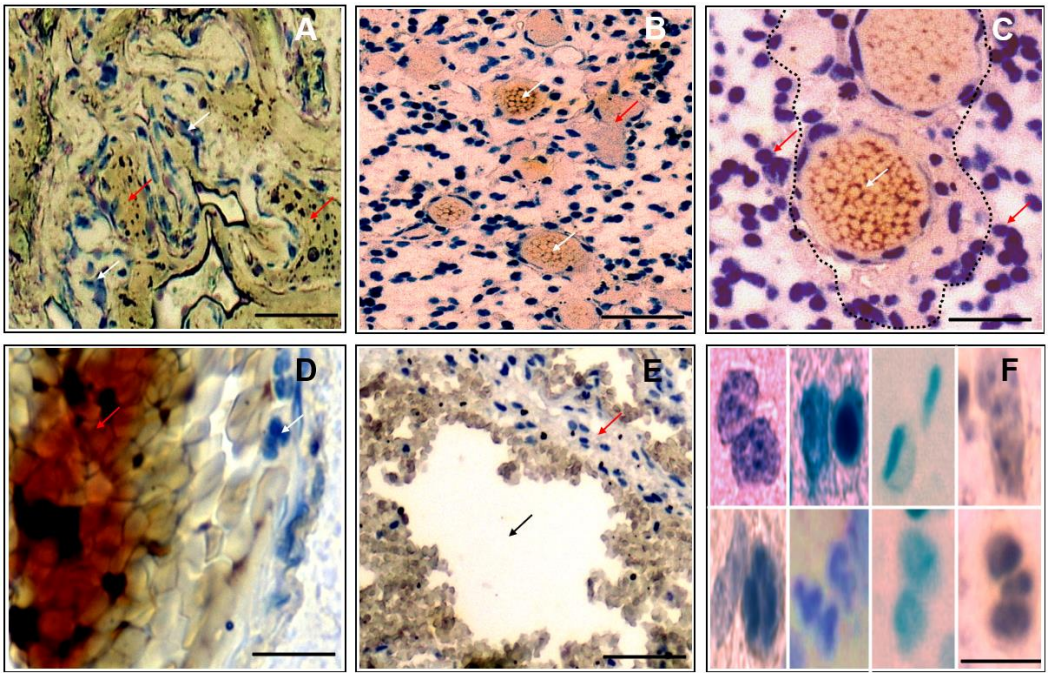

Figure 1. Histopathological features of human glioblastoma multiforme (GBM) following surgical resection and hematoxylin and eosin staining. Representative light micrographs showing (**A**) heterogeneous shaped clusters of tumor cells (white arrows) intercalating within necrotic regions (red arrows). Scale bar 200 μm. (**B**,**C**) Highly vascularised tumor regions with proliferative cells (red arrows) adjacent to microvessels (white arrows). Scale bar 200 μm (**B**) and 100 μm (**C**), respectively. (**D**) Tumor with heterochromic polymorphic nuclei (white arrow) associated with extensive necrosis (red arrow). Scale bar 100 μm. (**E**) Proliferating cells (red arrow) coinciding with apoptotic zones (black arrow). Scale bar 200 μm (**F**) Representative examples of tumor cells with pleomorphic nuclei. Scale bar 50 μm.

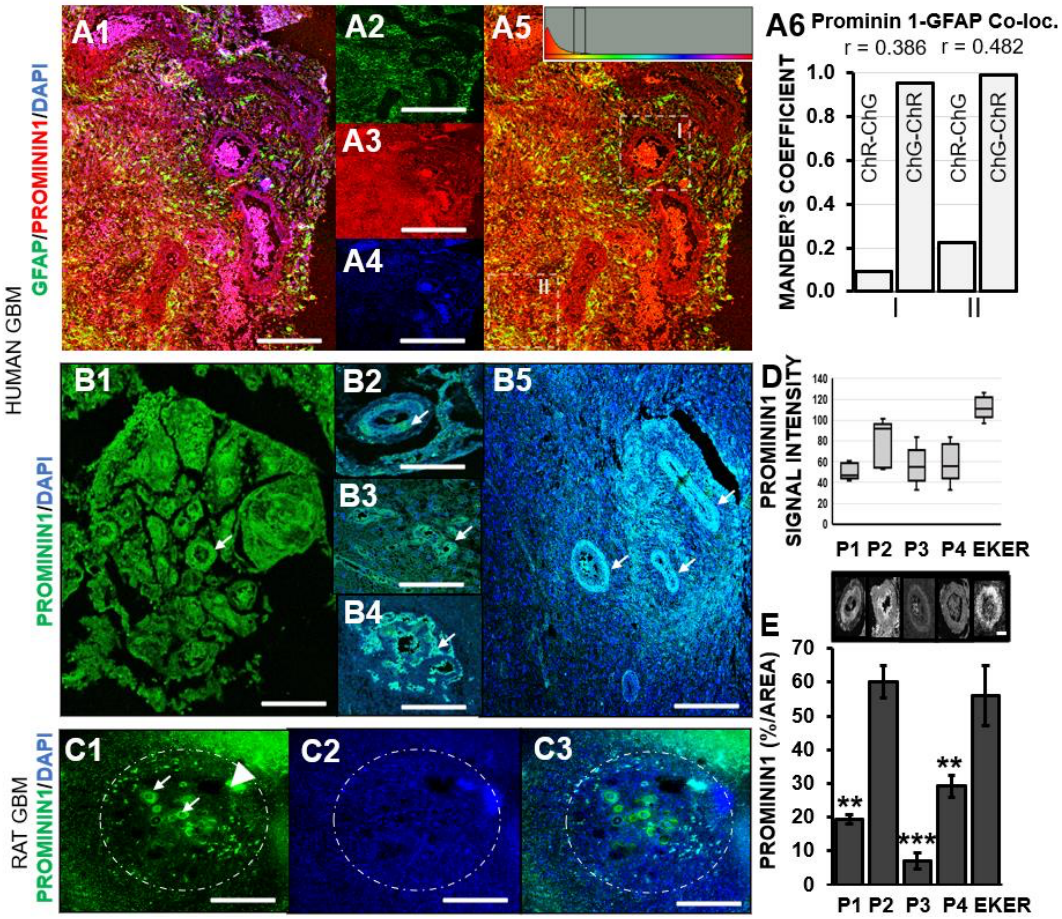

Figure 2. Prominin-1 does not colocalize with the astrocyte marker GFAP but is expressed in circular zones of palisading endothelial cells in GBM. (**A1**) Representative confocal micrograph of merged images for GFAP (astrocyte marker) and prominin-1 in human GBM. Scale bar 250 µm. (**A2**–**A4**) Separated fluorescence images representing GFAP (green; **A2**), prominin-1 (red; **A3**) and nuclei stained with DAPI (blue; (**A4**)). (**A5**) Cytofluorogram with threshold indicator in yellow zone. (**A6**) Histograms of Mander's coefficient of colocalization for zones I and II (as shown in (**A5**)). (**B**,**C**) Representative confocal micrographs showing prominin-1 expression in human GBM and Eker rat cortical brain sections. (**B**) Immunohistochemical detection revealed elevated prominin-1 expression in palisading endothelial cells located in circular zones surrounding necrotic regions in human GBM pathological sections (white arrows). Scale bar 2 mm (**B1**), 1 mm (**B2**–**B5**). (**C**) Prominin-l expression in rodent GBM model ie. Eker rats in similar circular zones surrounding necrosis in the brain. Scale bar 2 mm. **C1**—Prominin; **C2**—Dapi, **C3**—merged image) (**D**) Interquartile range of prominin-1 signal intensity in pathological GBM sections (ROI = 10 per patient (P) or EKER rat). (**E**) Representative grey scale micrographs showing prominin-1 in circular cellular zones within GBM above histogram quantitation of percentage expression per area. Asterisks represent significant differences in the levels of prominin-1 in Eker rats compared to patients (P1, P3 and P4), ** $p \leq 0.005$; *** $p \leq 0.0005$.

3.3. Chemotherapeutic Target ICAM-1 Associates with Degenerating GBM Vasculature

Our previous study noted incremental ICAM-1 in response to increasing ATRA dosage [26]. It has been suggested that targeting this molecule may provide a strategy for enhanced efficacy of anti-angiogenic GBM therapy [26]. While ICAM-1 appeared widely distributed in human GBM and Eker rat cortical sections, patterns of its specific expression emerged especially in human pathological samples. Of note, ICAM-1 did not predominate in astrocytes (Pearson's correlation coefficient r = 0.327, Figure 3A,E). High ICAM-1 intensity was noted in small ovoid structures, perhaps representing zones of microvascular hyperplasia (Figure 3B,F; zone = 1). Whether such regions signified the development of new blood vessels could be speculated given the proximity to necrotic pseudo-palisading tissue within which tumor cells potentially migrated towards the emerging vasculature (Figure 3B,F; zone = 2). Of note, low levels of ICAM-1 expression were demonstrated throughout human GBM (Figure 3B,F; zone = 3). ICAM-1 was also intensely expressed within centralized tumor cells, as well as in the surrounding pseudo-palisade circular boundary (Figure 3C). Eker rat cortical tissue revealed significantly higher ICAM-1 signal per area compared to all human GBM patients examined (Figure 3D,G), along with a generalized distribution pattern that tended to align with blood vessels (Figure 3D asterisk). This was reflected in upper-end values within the IQR for ICAM-1 in the Eker rats (median = 107 a.u., min. − max. = 96–129 a. u.) compared to GBM patients (Figure 3H). Significant differences were noted within GBM patients when comparing the percentage of ICAM-1 expression per area (R.O.I = 10). While similar levels were noted for P3 and P4 (p = 0.06), differences in ICAM-1 were found when P2 was compared to P3 (p = 0.0005) or P4 (p = 0.0037). This was supported by overlapping dispersion ranges for P3 (median = 50.87 a.u., min. − max. = 47–60 a. u.) and P4 (median = 63.94 a.u., min. − max. = 50–71 a. u.) (Figure 3H). The IQR of P1 also overlaps with these two patients, while P2 showed a negative skew towards the higher intensity ICAM-1 range (Figure 3H). As the chemotherapeutic intervention for P1 was unknown, conclusions cannot be drawn on its influence on ICAM-1 expression. Elevated ICAM-1 was found in the patient (P2) with chemotherapeutic non-tolerance. Nevertheless, ICAM-1 levels in P1 align with both responders to adjuvant therapy, which tends to suggest a potential therapeutic influence on this biomolecule. This will be deciphered in a future larger experimental cohort.

3.4. Long Non-Coding RNA PARTICLE Expression in Tumor Cells within Necrotic GBM Degenerative Zones

PARTICLE has been recognized as a silencer of tumor suppressor genes and a maintainer of cancer viability [35,36,40]. Our previous study demonstrated the significant curtailment of *PARTICLE* by ATRA at the leading edge of U87MG [26]. This contrasted with its sustained elevated expression with resistance to ATRA treatment within the established cellular monolayer microenvironment [26]. Likewise, this study reveals the high nuclear expression of *PARTICLE* in tumor cells still surviving in necrotic zones in human GBM. In situ hybridization confocal micrographs of *PARTICLE* in P1–P4 GBM pathological sections were converted to optical density threshold images. Maximum intensity levels for *PARTICLE* were associated with the upper end threshold levels for tumor clearance zones (Figure 4A). Despite being expressed in both nucleus and cytoplasm in other cancer types (e.g., breast and osteosarcoma; [35,36]), *PARTICLE* expression is confined to the nucleus in human GBM (Figure 4B,C). Intriguingly, *PARTICLE* tended to predominate along one side of a necrotic clearance zone (Figure 4D) and to be also expressed in atypically shaped nuclei (Figure 4E). A highly significant 1.93-fold increased difference (65.9%, p = 0.008) in *PARTICLE* was noted between such peripheral regions (Figure 4D,F, demarcated zones I and II). Quantitative real-time PCR detection successfully amplified *PARTICLE* from deparaffinized pathology sections, highlighting the robust elevated expression of this non-coding transcript in GBM. Given the role of *PARTICLE* as a tumor activator in several cancers examined to date [35,36], it can be speculated that this lncRNA assists in GBM survival in proximity to zones of degeneration.

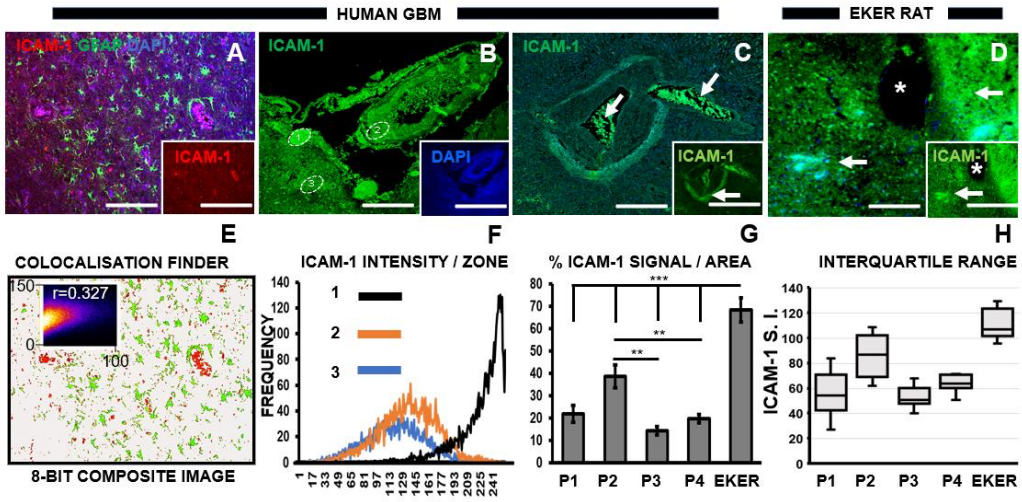

Figure 3. ICAM-1 does not colocalize with the astrocyte marker GFAP but reveals predominance adjacent to zones of tumor decay. (**A**) Representative confocal micrograph of merged images for GFAP (astrocyte marker) and ICAM-1 (red; inset) in human GBM. Scale bars 250 µm. (**B**–**D**) Representative confocal micrographs showing ICAM-1 expression in human GBM and Eker rat cortical brain sections. (**B**,**C**) ICAM-1 (green) signal detected in human GBM. Numbered ROI were analyzed for ICAM-1 signal intensity as shown in panel F, showing the high signals (zone 1) close to regions devoid of cellular content. Inset indicates dual immunofluorescence staining for both ICAM-1 (green) and nuclei (DAPI, blue). (**C**) High levels of ICAM-1 are located within (arrow) and surrounding circular zones (arrow, inset) in GBM sections. (**D**) ICAM-1 (green) reveals a highly distributed expression pattern (arrow) in EKER rat model compared to human GBM adjacent to blood vessel (asterisk). Scale bars in main micrographs and insets 2 µm and 1 µm, respectively. (**E**) Composite image (8-bit) showing the lack of colocalization between GFAP and ICAM-1 with scatterplot (inset). (**F**) Frequency polygons of ICAM-1 intensity per zone (1–3 indicated in panel B). (**G**) Histogram showing interpatient comparison of percentage ICAM-1 signal/area and between EKER rats and human GBM patients (P). Asterisks indicate significant differences, ** $p \leq 0.005$; *** $p \leq 0.0005$. (**H**) Interquartile range of ICAM-1 signal intensity between GBM patients (P1–P4) and an EKER rat glioma model.

3.5. GBM Necrosis Associated with Elevated Long Non-Coding RNA GAS5 in Chemotherapeutic Responsive Patients

It has been reported that elevated *GAS5* is closely associated with cancer patients overall survival [30,41–43] and acts as a functional tumor suppressor in the U87MG cell line [44]. This investigation found *GAS5* in necrotic regions of human GBM pathological sections. Lower levels of *GAS5* were detected in proliferative regions in GBM compared to centralized degenerative zones (Figure 5A). In situ hybridization confocal micrographs of *GAS5* expression in P2–P4 GBM pathological sections were transformed to optical density threshold images. Maximum intensity levels for *GAS5* were associated with upper-end threshold levels for deep tumor necrotic zones (Figure 5B). GBM sections derived from the patient who was non-tolerant to adjuvant therapy (P2) showed preferential *GAS5* expression in these hypoxic regions compared to its expression in more structurally intact tumor cells (39% increase; P2; Figure 5A,B). Further examination of patients tolerant to chemotherapy (P3, P4; Figure 5C), demonstrated a similar *GAS5* distribution pattern to GBM sections from P2 (Figure 5A,B). Nevertheless, a significantly greater mean *GAS5* intensity was revealed in necrotic regions compared to adjacent proliferative zones (Figure 5C,E, 82% increase; $p = 0.003$). Of note, *GAS5* expression was perinuclear-cytoplasmic in the GBM intracellular distribution (Figure 5D). While *GAS5* could be detected with in situ hybridiza-

tion, amplification of *GAS5* from RNA extracted from deparaffinized GBM sections proved unsuccessful. This investigation has examined GBM sections from patients with alternative responses to adjuvant therapy. While *GAS5* predominated in degenerating tumor cells in all patients, it can be speculated that patients who responded to chemotherapeutic intervention demonstrated even further elevated levels of this tumor suppressor than non-responders, perhaps influencing an extension in overall survival.

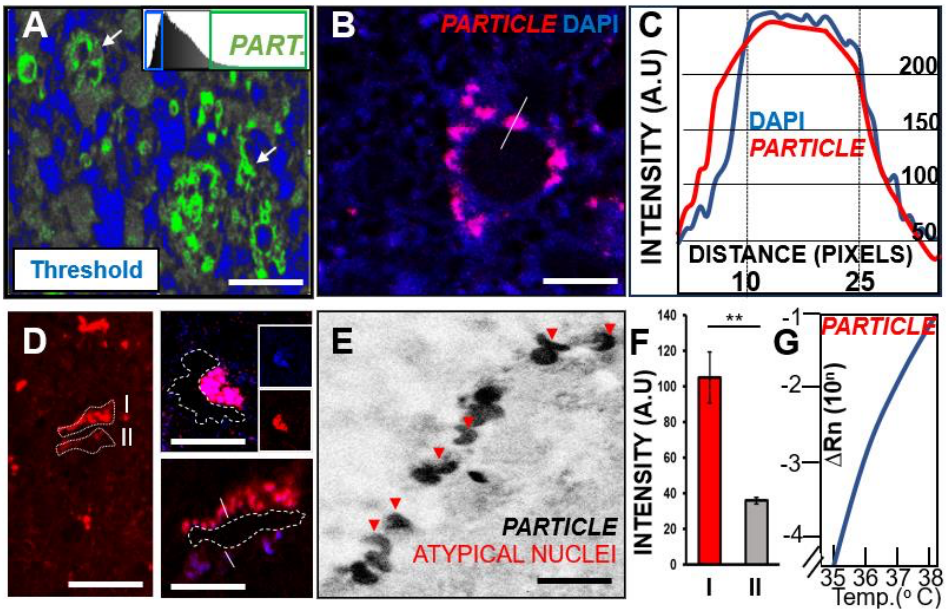

Figure 4. Tumor activator long non-coding RNA *PARTICLE* reveals nuclear expression pattern predominantly in tumor cells adjacent to zones of decay in human GBM. (**A**) Pseudo-colored high threshold image highlighting elevated *PARTICLE* (green) expression in regions adjacent to cellular free zones (blue) within a human GBM pathological section (arrows). Threshold demarcation schematic (upper right). Scale bar 100 µm. (**B**) Representative confocal micrograph of *PARTICLE* expression next to cell free regions of the tumor. Nuclear presence shown with DAPI staining (Blue). Line indicates transection point analyzed in panel C. Scale bar 200 µm. (**C**) *PARTICLE* (red) reveals nuclear (DAPI, blue) expression pattern as shown by representative line graphs of signal intensity. (**D**) Representative confocal low and high magnification micrographs show increased *PARTICLE* (red) expression predominantly along one side adjacent to tumor free zones. Analyzed regions (I, II) demarcated in dashed lines in main micrograph. Scale bar 1 mm. Insets show tumor free zones (dashed lines). Nuclei stained with DAPI (blue, insets). Scale bar 200 µm. (**E**) Grey scale micrograph showing *PARTICLE* expression in atypical nuclei in human GBM (red arrowheads). Scale bar 100 µm. (**F**) Histogram of significant *PARTICLE* intensity signal differences between demarcated zones (shown in panel D) with the GBM tumor. (**G**) Real-time quantitative PCR amplification curve of *PARTICLE* expression in human GBM. ** $p < 0.005$.

In summary, prominin-1, ICAM-1 and two long non-coding RNAs *PARTICLE* and *GAS5* elicited specific territorial patterns of expression within the GBM pathological sections. Significant signal intensity differences between GBM patients open further avenues worth exploring in the quest for effective targeted treatment strategies.

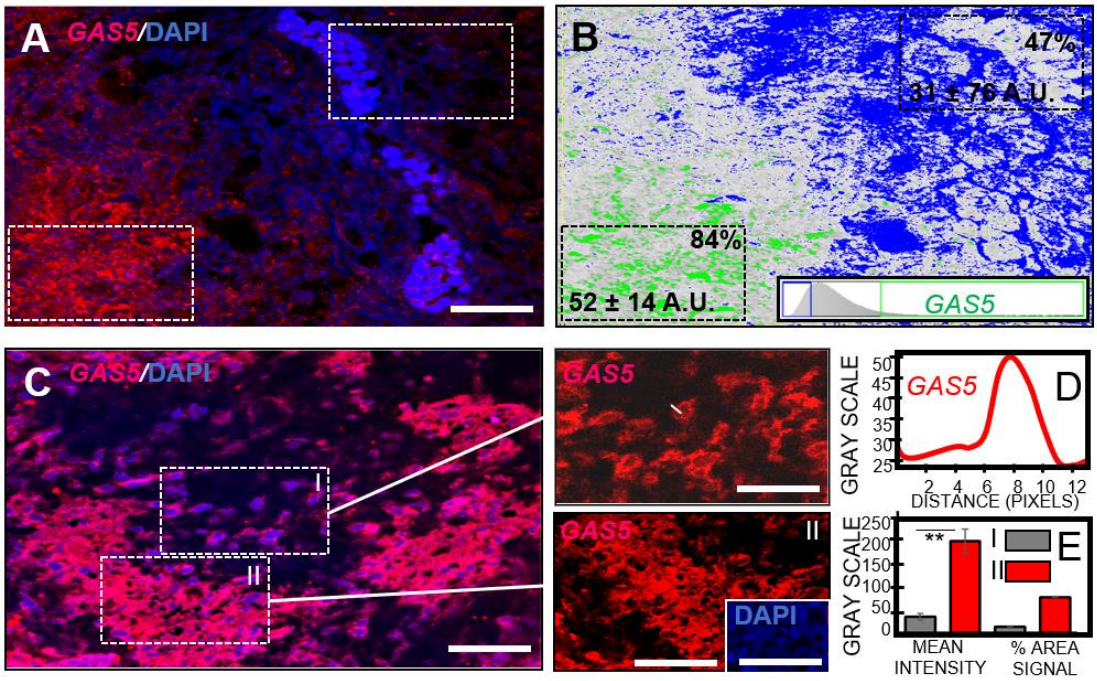

Figure 5. Tumor suppressor long non-coding RNA *GAS5* enhanced in necrotic human GBM. (**A**) Representative confocal micrograph showing *GAS5* (red) and DAPI stained nuclei (blue). Dashed lines indicate regions quantified in panel B. Scale bar 1 mm. (**B**) Pseudo-colored high threshold image highlighting elevated *GAS5* (green) expression in necrotic regions compared to cellular zones (blue-white) within a human GBM pathological section. Threshold demarcation schematic (lower right). *GAS5* intensity and percentage signal per demarcated area (dashed line) indicated. (**C**) Representative confocal micrographs of *GAS5* (red) and DAPI stained nuclei (blue). Rectangle boxes (I, II) further highlighting increased levels of *GAS5* in region II with amorphic nuclei (inset) indicative of tissue necrosis and tumor suppression. Scale bar 50 µm. (**D**) Grey scale line graph profiling *GAS5* in perinuclear/cytoplasmic signal (line shown in panel C upper right). (**E**) Histograms of *GAS5* mean signal intensity and percentage area of expression in regions I and II (panel C). Asterisk represent significant differences, ** $p \leq 0.005$.

4. Discussion

The nuances associated with evaluating U87MG proliferation have been previously conveyed, enabling this glioblastoma cell line model to be suitable for stem cell differentiation and chemotherapeutic resistance studies [26,45–47]. This conclusion was reached from cell-based experimentation focused on the response of the stem cell marker prominin-1, the chemotherapeutic target ICAM-1 and the alternative expression profiles of the lncRNAs *PARTICLE* and *GAS5* within this in vitro GBM microenvironment [26]. Herein, we present the pre-clinical applicability of these important biomolecules through analytical expansion to a representative glioma animal model and GBM patients that elicited alternative responses to adjuvant chemotherapy.

An in-depth microscopic evaluation, identifying necrotic foci, enhancement of cellular proliferation and vascular remodeling, concluded that the Eker rat was a suitable model for primary brain tumor studies [32,48]. This investigation recognized such morphological correlations of malignancy in GBM patient pathological sections from haemoxylin and eosin staining. Immunofluorescence imaging highlighted an elevated prominin-1 expression in

pseudo-palisades adjacent to necrotic foci in both animal and human GBM. By extending our previous work [26], we revealed ICAM-1 to be associated with degenerating vasculature within patient GBM and rodent subcortical tumors. In situ hybridization experiments demonstrated that the lncRNA *PARTICLE* elicited a predominantly nuclear intracellular expression profile potentially activating tumor survival within necrotic degenerative GBM zones. Such regions of tumor necrosis were associated with elevated lncRNA *GAS5* expression in chemotherapeutic responsive patients, supporting its role as a tumor suppressor and prognostic biomolecule of extended overall survival.

This investigation centered on the evaluation of GBM pathological sections obtained from patients who succumbed within twelve months of diagnosis. Ranging from 57 to 77 years, this group included female and males of unknown and mixed responsiveness, respectively, to temozolomide treatment. Histopathological analysis of brain tumor sections from these patients identified typical GBM characteristics [49,50]. These features included atypical cells with hyperchromic polymorphic nuclei [49] and the presence of necrotic regions coinciding with high cellular zones, potentially representative of tumor cell migration from degrading vasculature destined for further microvessel formation [51]. The relevance of such pseudo-palisades around necrotic vessels is believed to be an underappreciated feature that contributes to GBM clinical progression [52]. Reports have recognized the perivascular 'railway-like' expression profile of the stem cell marker prominin-1 (CD133+) within GBM [53]. Such striped patterns of prominin-1 expression were noted in our samples on occasion and strongly support the predominant presence of prominin-1 within the peripheral zones of ovoid structures, representing blood vessels within human GBM and Eker rat cortical sections. Importantly, our findings align with the first identification of prominin-1 expression within tumor cells of pseudo-palisade formations that delineate necrosis within GBM [53]. This study noted higher prominin-1 expression in the rodent samples compared to GBM patients who responded to TMZ treatment. Given similar elevated prominin-1 signals between Eker rats and a TMZ non-tolerant patient compared to treatment-responsive patients, it can be speculated that prominin-1 may be worth further investigation in this regard using a larger cohort. This is supported by previous studies calling for further investigation of intercellular communication between prominin-1 ($CD133^+$) niches and adjacent blood vessels within GBM, with the view to design specific targeted treatments directed at brain tumor stem cells [53]. Of note, $CD133^+$ cells elicited greater tumorigenic potential than $CD133^-$ cells in response to ionizing radiation [54]. It has been suggested that CD133+ tumor cells confer glioma radio-resistance and act as a potential source of tumor recurrence after radiation [55]. The basis for therapeutic resistance has yet to be fully deciphered, yet evidence of the involvement of microvesicle-mediated transfer of the metabolic enzyme nicotinamide phosphoribosyltransferase (NAMPT) to radiosensitive cells has been reported [56].

GBM is considered one of the most angiogenic tumors characterized by microvascular proliferation involving tumor cell transformation in a process of tubular vasculogenic mimicry [57,58]. In contrast with reports of the association of strong vascular ICAM-1 staining in GBM zones with fewer blood vessels [58], this study identified elevated ICAM-1 expression in ovoid structures most likely representing blood microvessels. ICAM-1 has been reported to be rapidly induced after the onset of severe hypoxia [59], supporting the findings from this study whereby ICAM-1 elicited intensive expression within centralized tumor cells bounded by circular pseudo-palisades. While the implication of increased ICAM-1 in GBM has not been fully elucidated, the results presented herein point to significant differences in its expression between patients with varying adjuvant therapy tolerance. Nevertheless, in larger studies staining intensity of vascular ICAM-1 was not associated with GBM patient survival [58].

Recognition of the functional importance of lncRNA in the context of GBM has recently emerged following their identification as master regulators of tumorigenesis [60–62]. Nevertheless, limited mechanistic studies of lncRNAs exist in relation to their role in GBM etiology, with most studies executed using traditional serum-growth cell lines, such as

U87MG [26,61]. The lncRNA *PARTICLE* is a key epigenetic mediator involved in the intercellular communication and recruitment triplex platform for down regulators of tumor suppressor genes in response to irradiation [35,36]. The in vitro investigation of *PARTICLE* demonstrated its robust expression in non-irradiated U87MG and its curtailment in response to ATRA [26]. The view that lncRNA expression is dynamic across multiple single cells in GBM tumors and cell lines [60] was supported by *PARTICLE* being responsive to ATRA only within the U87MG leading edge cells, yet elicited resistance to this active metabolite of vitamin A in the established monolayer [26]. This investigation extended the assessment of *PARTICLE* to human GBM pathology and demonstrated its significantly elevated expression in tumor cells adjacent to necrotic GBM degenerative zones. The role of *PARTICLE* as an enhancer of cancer viability has been demonstrated [36]. The predominance of *PARTICLE* expression along one side of a necrotic tumor niche is perhaps indicative of its activation potential for tumor cell survival and proliferation as a regulator of the dominant growth direction in GBM.

In contrast, the tumor suppressor *GAS5* has been reported to be decreased in GBM and associated with the overall and disease-free survival relative to patients with high expression of this lncRNA [63,64]. This study demonstrated that *GAS5* predominated in degenerating GBM extracted from patients with alternative responses to adjuvant therapy. While a similar *GAS5* distribution pattern was apparent in all GBM patients, those that responded to temozolomide revealed significantly higher levels of this tumor suppressor.

The limitations of this investigation center on the patient cohort size and findings are likely to be confirmed in a larger-scale study. Emphasis was placed solely on biomolecules previously studied in vitro using a representative GBM cell line. Eker rat data were confined to prominin-1 and ICAM-1 investigations due to potential conservation issues with lncRNA assessment. The merits of this study center on encouraging in vitro research to be extended into the assessment of patient-derived tissue. Our findings for prominin-1, ICAM-1 and *GAS5* in GBM support the data of other research groups [53,59,61,65]. In addition, this investigation is the first to show the predominant expression of *PARTICLE* in GBM pathology, facilitating future research into this important tumor activator. The technical aspects of clinical implementation involve challenges associated with simultaneous examination of prominin-1, ICAM-1 immunohistochemistry combined with *GAS-5* and *PARTICLE* in-situ hybridization. In addition, overcoming the blood-brain barrier needs to be considered for effective GBM patient treatment.

5. Conclusions

This study highlighted the specific expression pattern of prominin-1, ICAM-1, *PARTICLE* and *GAS5* within GBM histological sections. While cell lines provide a platform for biomolecule response to controlled media, extending investigations into the complex environment of GBM sections makes us a step closer to future promising biomarkers for GBM detection, therapeutic response and prognosis prediction.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pharmaceutics16010123/s1, Figure S1. Low magnification confocal micrographs of prominin-1 or ICAM-1 expression in GBM. (A) Representative confocal microscopic 5× image of prominin-1 (green, upper and lower) and nuclear staining (DAPI, blue, middle) in GBM. (B) Representative confocal microscopic 5× image of ICAM-1 (green, upper and lower) and nuclear staining (DAPI, blue, middle) in GBM. Scale bar 250 mm.

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Informed Consent Statement: The Ethics Committee waived the requirement for informed consent in accordance to Czech Law (Act. no. 373/11, and its amendment Act no. 202/17).

Data Availability Statement: The data presented in this study are available in this article and Supplementary Materials).

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biomedicines

MDPI

Review

LncRNAs as Biomarkers of Glioblastoma Multiforme

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Abstract: Long noncoding RNAs (lncRNAs) are RNA molecules of 200 nucleotides or more in length that are not translated into proteins. Their expression is highly specific, with the vast majority involved in the regulation of cellular processes and functions. Many human diseases, including cancer, have been shown to be associated to deregulated lncRNAs, rendering them potential therapeutic targets and biomarkers for harder-to-diagnose diseases. LncRNAs are expression is highly specific to different cell types, with the majority represented in brain tissue. These are involved in the regulatory system of neurons and glial and assist in the development and coordinated functioning of brain cells. Reports have shown a link between changes in lncRNA molecules and multiple processes involved in the etiopathogenesis of brain neoplasia, including glioblastoma multiforme (GBM). GBM is an aggressive variant of brain cancer with an unfavorable prognosis and a median survival of 14-16 months. GBM is considered a complete brain disease because the malignant cells involved are highly invasive and spread throughout the brain, impeding the complete resection of the cancerous tissue, leading to post-surgery recurrences, which are the leading cause of mortality. Early detection of this disease could increase the success rate of interventions and extend the survival, with profiling biological fluids for specific markers providing promising avenues for early diagnosis and interventions. A rapid and minimally invasive diagnostic method is blood analysis, with changes in lncRNAs providing critical information about normal and disease processes, including in the brain. This review provides a systematic overview of deregulated lncRNAs in association with GBM and the dynamics of their profile in the blood of GBM patients.

Keywords: lncRNA; noncoding RNA; glioblastoma multiforme; glioma; plasma; serum; blood; biomarker; liquid biopsy; AC016405.3; ADAMTs9-AS2; AGAP2-AS1; AHIF; ANRIL; lncRNA-ATB; CASC2; CASC7; CASC9; CCND2-AS1; CRNDE; DCST1-AS1; DGCR5; DLEU1-AS1; ECONEXIN; LINC00461; FAM66C; GAS5; H19; HMMR-AS1; HOTAIR; HOTAIRM1; HOXA-AS2; HOXB13-1; HOTTIP; HULC; KTN1-AS1; LINC00467; LINC00565; LINC00641; LINC01393; LINC01426; LINC01446; LINC01494; LINC01503; LINC01711; LINC02283; LINC-ROR; lnc-TALC; MAFG-DT; MALAT1; MATN1-AS1; MDC1-AS; MEG3; MIAT; MIR210HG; MNX1-AS1; NCK1-AS1; NEAT1; PART1; PARTICLE; PCAT1; PCA1; PVT1; RBPMS-AS1; RPSAP52; RUNX1-IT1; SAMMSON; SOX2-OT; TALNEC2; TP73-AS1; TSLC1-AS1; TUSC7; TUG1; TUNAR; UCA1; XIST; ZEB1-AS1; ZBED3-AS1

1. Introduction

To date, more than 100,000 transcripts that are not translated and do not encode proteins have been identified [1] (see Figure 1). These noncoding molecules are very heterogeneous and vary in length, function, location in the genome, and localisation in cells or tissues. Noncoding RNAs (ncRNAs) form a large heterogeneous set of functional RNA molecules that are transcribed from different locations throughout the genome. Although ncRNAs are not translated into proteins, they play an important role in the physiological cellular processes and in the regulation of gene expression. The importance of the noncoding transcriptome is supported by the direct correlation between the proportion of ncRNAs in the genome of organisms and their developmental complexity [2]. There is no such correlation in the number of protein-coding genes [3]. Reports also show that the number of lncRNA types corresponds to the complexity of the nervous system. The human brain has been found to have the highest number of lncRNA types in all organisms studied to date [4][5].

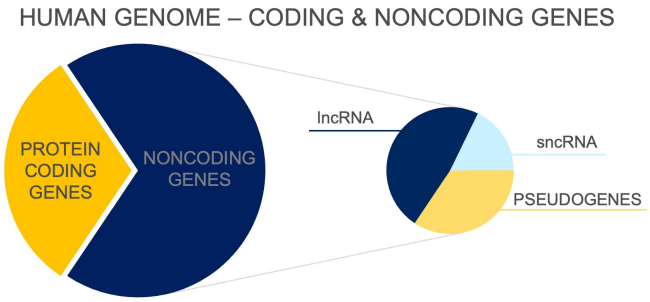

Figure 1. Distribution of coding and noncoding genes in the human genome according to ENCODE Release version 45 [6]. LncRNA - long noncoding RNA; sncRNA - small noncoding RNA.

Transcripts longer than 200 nucleotides belong to a large group of long noncoding RNAs (lncRNAs) [2][7]. Their total number is increasing due to more sensitive detection methods and is greater than the sum of all protein-coding genes [5]. LncRNAs are mainly transcribed by polymerase II and may subsequently undergo post-transcriptional modifications. Through interaction with proteins and regulatory segments of genome, lncRNAs of neurons are involved in control of many cellular processes including differentiation, proliferation, migration, signalling, as well as in an array of epigenetic mechanisms [1][7]. LncRNAs have been detected in the nucleus, nucleolus, cytoplasm, and mitochondria [1]. There is rising evidence suggesting a mechanistic link of many human diseases, including cancer, to lncRNA dysregulations, making lncRNA molecules potential therapeutic targets and biomarkers for diseases, which may facilitate the detection and diagnosis of various disorders and diseases [1].

The exact function for most lncRNAs remains unknown, with substantial evidence suggesting that the localisation of the lncRNA can predict its likely role in the cell. Indeed, the transcripts that prevail in the nucleus are involved in the regulation of gene expression, chromatin modification, and imprinting [8]. LncRNAs prevalent in the cytoplasm, on the other hand, are involved in mRNA splicing and regulation of protein translation and may also be precursors for small noncoding RNAs (sncRNAs), e.g. microRNAs (miRNAs) [1][8].

Several lncRNA specific databases have been created that contain information on their origin, functions, and action mechanisms (e.g., LNCipedia 5.2; lncRNAfunc), along with their alternative names and various identifiers, e.g. gene ID, Hugo nomenclature, and Ensembl tags for both genes and transcripts. Most databases do not list all the data

and all the names for a given lncRNA, and some use their own, specific lncRNA identification system. This makes it difficult to find information about a particular lncRNA molecule not only in databases, but also in peer-reviewed publications. Hence, there is pressing need in the use of uniform and standardized lncRNA nomenclature to improve communication and avoid confusion or duplication of individual molecules. Due to the constant new discoveries related to lncRNAs, in many cases, once the function of a molecule has been discovered, it is renamed [9][10]. There is lack of consensus and standardized nomenclature for labeling lncRNA, which lead to duplications and confusion in the field, warranting an urgent attention. Some lncRNAs, for instance, can appear in results of search under two or more names, and can be easily confused for multiple distinct lncRNAs.

The response of lncRNAs to glioblastoma multiforme (GBM) with emerging recognition of their diagnostic and prognostic relevance makes their profiling and analysis of prime relevance to diagnosis and therapy of this malignant brain condition. GBM, refer to the most common and aggressive malignant brain tumour in adults that resists conventional complex therapy, which includes maximal surgical resection followed by radiation therapy and chemotherapy [10]. GBM is considered a whole brain disease because the neoplastic cells are highly invasive, infiltrating in surrounding tissue and spreading beyond the lesion area. This characteristic makes complete tumour resection highly challenging, and leads to frequent post-surgery recurrences, which are the main cause of mortality [11]. Despite the relatively low incidence (3-4 cases per 100,000 people), GMB remains one of the greatest challenges and priorities for research and clinical translation, owing to its severity and very high mortality. On average, treated patients live 14-16 months from the first diagnosis, with only 5-10% of patients surviving 5 years from the manifestation of the disease [11]. The effectiveness of treatment and the progress are largely hampered by the high infiltration of the malignant tissue in the surrounding areas and the high heterogeneity of neoplastic cells. In addition to highly malignant neoplastic cells, the lesion of GBM typically contains endothelial cells, neurones, astrocytes, oligodendrocytes, microglia, and non-cellular components such as apocrine and paracrine signalling factors, exosomes, and others cell types and tissue debris [12]. These components are typically segregated into several distinct compartments known as tumour niches, which may differ morphologically and functionally even within a single tumour. Numerous studies confirm the involvement of lncRNAs in many molecular processes in GBM tissue [13][14][15][16]. Revealing their precise function could serve to discover new therapeutic approaches. These molecules may also serve well as biomarkers - directly in tumour tissue for more accurate diagnosis and setting up of more effective therapy after tumour resection.

In this article, we provided a systematic review of lncRNAs associated with GBM, with their response in the disease and diagnostic relevance as biomarkers. Like in several other cancer types, the classification of brain cancer faces challenges, with the term glioma often used for experiments with glioblastoma multiforme (GBM, subtype of glioma) cell lines and for tissues from patients with a confirmed diagnosis of GBM. We refer to the commonly used names of lncRNA, provide the description to their chromosomal localizations as well as to the identifiers presented by the Ensembl gene (ENSGs) database.

2. Genome Localisation and Expression of lncRNA

Sequences from which lncRNAs are transcribed can occur almost anywhere in the human genome. The lncRNA molecules can be divided into several groups based on their location in the genome. Sequences of intron lncRNAs are found in the introns of protein-coding genes. Intergenic lncRNAs (lincRNAs) are in the region between the two coding genes. Enhancer lncRNAs (elncRNAs) are localized in the enhancer regions of protein-coding genes. The lncRNA sequences may also be positioned in the genome regions where protein-coding genes are found. Sequences for lncRNAs, thus, may overlap with the exon, intron, or both parts of a gene, or they may overlap the entire sequence of a protein-coding

gene. Importantly, unlike protein coding genome, the genome encoding lncRNA can be localised on both strands of DNA and be transcribed in both directions. Genomic sequences within these transcription units can be shared not only with coding regions, but also with each other in both sense and antisense directions [1][17]. In most cases, the lncRNA sequences are transcribed by RNA Polymerase II and rarely by RNA Polymerase I or III [18]. The resulting transcripts can be post-transcriptionally modified in manner shared with protein transcripts, involving binding of 7-methylguanosine at the 5' end, polyadenylation at the 3' end, or splicing [19][20]. Reverse editing can produce circular RNAs (circRNAs), which are another subtype of lncRNA. CircRNAs form covalently closed loops, which makes them resistant to nucleases. Like lncRNA, circRNAs have been detected in biological fluids such as blood, cerebrospinal fluid and urine, making them potential biomarkers for various disease conditions. Some of these molecules have been associated with different forms of cancers, including gliomas [17].

The expression of lncRNAs is highly specific to various tissue types, with their profiles responding to pathophysiological conditions affecting cells and tissue, as well as developmental stage, circadian rhythms and others [20][21]. Quantitative studies suggest that the specificity index of lncRNAs is significantly higher than that transcriptome of protein-coding genes. This fact contributes to the theory of lncRNAs as regulators of gene expression in specific cell types [4]. Most types of lncRNA have been found in the nervous tissue, which is composed of many cell types that require a highly complex regulatory mechanisms. These mechanisms are influenced by lncRNA molecules, which play an important role in the development, maintenance, and influence of neural functionality, contributing to brain mechanisms. The set of lncRNAs in human brain tissue differs from primate brain by a greater degree than the transcriptome of encoding genes, with the extent of the differences correlating with the developmental stage, functionality and disease state [4].

3. Molecular Mechanisms Underlying lncRNA Functions

To date, studies have implicated lncRNAs in almost all processes of gene expression regulation, including chromosome inactivation, imprinting, chromatin dynamics, protein modification and nucleic acid stability [22][23]. The expression of lncRNAs can be influenced by a variety of factors, including environment, stress and the pathophysiological state of the cell. The genes for lncRNAs may be subject to epigenetic modifications, such as promoter methylation [24].

There are four basic molecular mechanisms by which lncRNAs can interact with surrounding biomolecules and influence their activity [17]:

1. Signals - These lncRNAs are transcribed at a specific site at a specific time. Their transcription is cell type-specific, inducing an active signalling event.

2. Decoys (Figure 2) - Transcripts of lncRNAs serve as decoys for target proteins. The lncRNA molecules occupy the binding site, and the proteins cannot interact with DNA. Thus, they can interact with transcription factors, repressors, chromatin modifiers and other proteins. Within this regulatory mode, lncRNAs can also interact with miRNAs (Figure 3). Specific lncRNAs act as sponge for some miRNAs, i.e. the lncRNA binds to the miRNA, which then cannot perform its function.

3. Guides (Figure 4) - These lncRNA molecules control the placement of ribonucleoprotein complexes at specific target sites.

4. Scaffolds (Figure 5) - Transcripts act as scaffolds for other molecules that can bind to a given lncRNA to form a ribonucleoprotein complex.

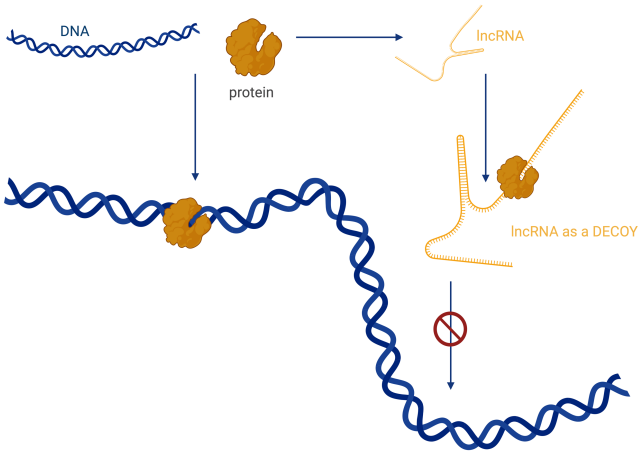

Figure 2. Decoy - transcripts of lncRNAs serve as decoys for target proteins. Created with BioRender.com.

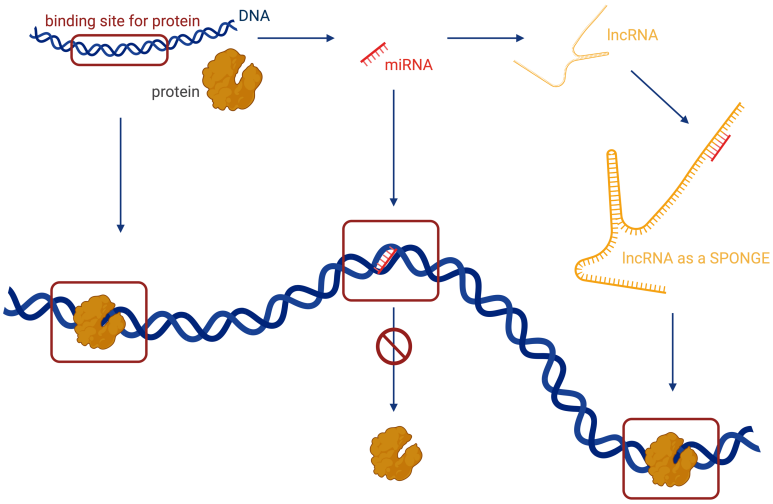

Figure 3. Sponge - specific lncRNAs act as sponge for some miRNAs. Created with BioRender.com.

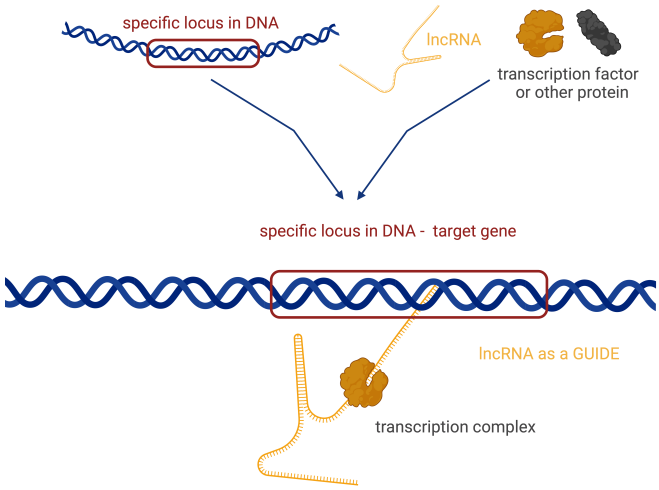

Figure 4. Guides - lncRNA molecules control the placement of ribonucleoprotein complexes at specific target sites. Created with BioRender.com.

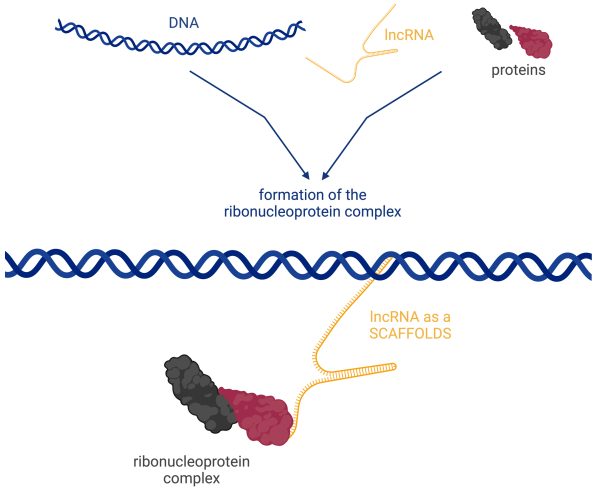

Figure 5. Scaffolds – lncRNA is a scaffolds for other molecules that can bind to a given lncRNA to form a ribonucleoprotein complex. Created with BioRender.com.

A large proportion of lncRNAs use more than one of the mechanisms described above to regulate cellular processes and thus can perform multiple functions. Therefore, lncRNAs we cannot be strictly divided into these 4 groups [17][25].

4. LncRNAs and Cancer

The lncRNA molecules are involved in almost all cellular processes, including growth, development, and differentiation. They also participate in many signalling pathways and mechanisms including p53, growth hormone production and signalling, glucose metabolism, cytokine expression, V(D)J recombination of immune cells and inflammation [18]. Mutations or altered expression of lncRNAs can cause pathophysiological changes, contributing to wide variety of cancers [18][26], neurological and

neurodegenerative diseases [27][28] and genetic conditions (e.g., phenylketonuria) [29]. Furthermore, guide lncRNAs form complexes with regulatory or enzymatically active proteins, targeting them towards specific gene promoters or genomic loci, thus regulating downstream signaling events and gene expressions [22]. Examples of guide lncRNAs include AIR, CCND1 (cyclin D promoter associated lncRNA), lincRNA-p21, and others. Using genome-wide RNA-Seq analyses, numerous lncRNAs have been identified, exhibiting either upregulation or downregulation in various form forms of malignancies, including renal, breast, and brain cancer [30]. Among these lncRNAs, MALAT1, RCAT1, DUXAP9, TCL6, LINC00342, AGAP2 Antisense1, DLEU2, NNT-AS1, LINC00460 and Lnc-LSG1 are notably highly specific to renal cancer, while HOTAIR, ANRIL, ZFAS1, HOTAIRM1, PVT1, MALAT1, LNP1, and others are associated with breast and brain cancer [31].

Determining the exact function of a given lncRNA molecule is difficult as in most cases, changes in their expression does not cause phenotypic alterations. Based on previous studies, some lncRNAs have been assigned as oncogenic (MALAT1, PCA3, HOTAIR, H19, PARTICLE etc.) or as tumour suppressor (GAS5, MEG3, TERRA, etc.) [17][18][32]. Some lncRNAs may exhibit variability in their properties and effects depending on the type of cancer. For instance, lncRNA AC016405.3 has tumour suppressor function in GBM, while in the higher level breast cancer it is considered an oncogenic molecule [33][34].

LncRNA molecules are specifically expressed in certain types of cancer, and some can be detected in blood or urine. Given the relatively large number of different types of lncRNA and their high tissue specificity, lncRNAs are explored as potential biomarkers for various diseases. A good example is the lncRNA PCA3, whose increased expression signals a prostate cancer and can be detected in urine together with other lncRNAs MALAT1 and LincRNA-p21. The expression levels of some cancer-specific lncRNAs have been shown to correlate with the degree of malignancy, stage of the disease, metastasis, or prognosis [35]. They have also been associated with resistance to therapy and subsequent tumour recurrence [36]. For instance, lncARSR, which showed high expression levels in sunitinib-resistant renal cancer cells has been found to be essential for the resistant phenotype, through competition with endogenous RNA for miR-34 and miR-449, leading to the upregulation of AXL/c-MET and activation of STAT3, AKT, and ERK pathways. Remarkably, lncARSR was identified as a predictive marker for poor response in patients with renal cancer, with emerging data suggesting its exosomal release from therapy-resistant cells, thereby conferring treatment resistance [30].

5. LncRNAs and GBM

Given the large number of lncRNA types in healthy brain tissue and the extensive heterogeneity of GBM tissue, it is necessary to consider these transcripts when looking into grading the condition, making prognosis, or exploring their response to experimental therapies. Studies have shown a link between lncRNAs and many processes involved in the formation and growth of GBM. LncRNA transcripts are involved in cell proliferation (MIAT) [37], cell apoptosis (MALAT1) [38], cell invasion (lncRNA ATB) [39][40], angiogenesis (HULC) [41], DNA damage response (PCAT1) [42] and cell cycle regulation (CASC7) [43], regulation of tumour microenvironment (FAM66C) [44], hypoxia (MIR210HG) [45], BBB permeability (TUG1) [46], tumour progression (TUNAR) [47], recurrence (lnc-TALC) [48], resistance to temozolomide (TMZ; ADAMTs9-AS2) [49], radiation resistance and response (HMMR-AS1) [50] and others (see Table 1). LncRNAs can interact directly with various molecular processes and mechanisms, or indirectly, through regulation of miRNAs, using methylation or affecting chromatin modification [36]. LncRNAs also play a regulatory role in the microenvironment of GBM, where they can act on cytokines and growth factors [51]. Finally, lncRNA molecules affect cancer stem cells and thus participate in tumorigenesis, recurrence, and resistance to therapy [36]. Accordingly, deregulated levels of lncRNA were detected in resected GBM tissue, and their analysis can provide more accurate differential diagnoses. Expression profiles of different lncRNAs can be also used to determine the grade of glioma and its subtype [13].

Importantly, analysis of deregulated lncRNAs circulating in the blood can be also used for determining prognosis and monitoring response to treatment.

Table 1. Systematic list of long noncoding RNAs (lncRNAs) associated with glioblastoma multiforme (GBM). This list includes lncRNAs whose expression is deregulated in association with GBM both *in vitro*, *in vivo*, and *ex vivo* - in GBM cell lines, in GBM primary tissue, in xenografts, in GBM tissue from resected tumours, in blood from GBM patients, and data from clinical associations. In addition to their most common names, lncRNAs are also identified by an Ensembl tag and by specifying their position on chromosomes.; lncRNA - long noncoding RNA, GBM - glioblastoma multiforme, TMZ - temozolomide, CSC – cancer stem cells, TCGA – The Cancer Genome Atlas Program.

lncRNA name / alternative transcript name /	Gene Location Class Ensembl Gene ID	Role in GBM	Expression	Function in GBM	Data	Ref.
AC016405.3 / RP11-44N11.2 / / lnc-DERL1-3 /	8q24.13 bidirectional ENSG00000272384	suppressor	↓	suppressing of proliferation and invasion	clinical association; GBM primary tissue; GBM cell line	[33]
ADAMTs9-AS2 /NONHSAT090261/	03p14.1 antisense ENSG00000241684	oncogenic	↑	TMZ resistance	clinical association; GBM cell line	[49]
AGAP2-AS1 / HSALNG0091650 /	02q14.1 antisense ENSG00000255737	oncogenic	↑	proliferation, viability	GBM primary tissue; GBM cell line	[52]
AHIF / lnc-TMEM30B-9 / / HIFiA-AS2 /	14q23.2 antisense ENSG00000258777	protumour	↑	invasion, viability, GSC, radiation resistance	GBM cell line; GSC mesenchymal line	[53]
ANRIL / CDKN2B-AS1 /	09p21.3 antisense ENSG00000240498	oncogenic	↑	cell proliferation	GBM cell line GBM tissue GBM patients serum	[13][54]
lncRNA-ATB	14q11.2 intronic -	protumour	↑	invasion of cell	GBM cell line	[40]
CASC2	10q26.11 antisense ENSG00000177640	suppressor	↓	inhibitor of proliferation	GBM cell line; GBM tissue; xenograft	[55]
CASC7 / lnc-AGO2-1 /	8q24.3 intronic ENSG00000259758	suppressor	↓	inhibitor proliferation, regulation of cell cycle	GBM primary tissue; GBM cell line	[43]
CASC9 / LINC00981 / / RP11-697M17.1-003 /	8q21.13 intronic ENSG00000249395	oncogenic	↑	tumourigenesis	GBM cell line	[56]
CCND2-AS1	12p13.32 antisense ENSG00000255920	protumour	↑	proliferation and growth	GBM cell line; GBM patient tissue	[57]
CRNDE / lnc-IRX3-80 /	16q12.2 antisense ENSG00000245694	oncogenic	↑	proliferation, invasion, migration, inhibition of apoptosis	GBM cell line; GBM patient tissue	[58]
DCST1-AS1	01q21.3 antisense ENSG00000232093	protumour	↑	proliferation	clinical related; GBM primary tissue;	[59]
DGCR5	22q11.21 antisense ENSG00000237517	suppressor	↓	proliferation, migration, invasion, apoptosis	GBM cell line; GBM tissue	[60]
DLEU1-AS1	13q14.3 intronic ENSG00000186047	biomarker	↑	proliferation, cell cycle, autophagy; correlation with prognosis	GBM cell line; GBM tissue	[61]
ECONEXIN / LINC00461 /	05q14.3 intronic ENSG00000245526	protumour	↑	proliferation	GBM cell line; GBM tissue; TCGA data	[62]

FAM66C	12p13.31 antisense ENSG00000226711	-	↑↓	tumour microenvironment	GBM cell line; GBM tissue; TCGA data	[44]
GAS5	01q25.1 antisense ENSG00000234741	suppressor	↓	inhibition of proliferation, invasion and viability	GBM cell line; GBM tissue; GBM patients serum	[63]
H19 / D11S813E / / ASM1 /	11p15.5 intronic ENSG00000130600	protumour	↑	proliferation, invasion, angiogenesis	GBM cell line	[64]
HMMR-AS1	05q34 antisense ENSG00000251018	protumour	↑	tumourigenesis, proliferation, invasion, radiation resistance	GBM cell line	[50]
HOTAIR	12q13.13 antisense ENSG00000228630	protumour	↑	proliferation, invasion, therapy resistance, chromatin remodeling	clinical association; GBM patient tissue/serum; cell line; xenoimplants	[65] [66]
HOTAIRM1 / HOXA-AS1 /	07p15.2 antisense ENSG00000233429	oncogenic	↑	proliferation, invasion, viability	clinical association TCGA; GBM primary tissue; GBM cell line	[67]
HOXA-AS2	07p15.2 antisense ENSG00000253552	protumour	↑	migration, invasion, viability	GBM tissue; GBM cell line	[68]
HOXB13-AS1 /lncHOXB13-1/	17q21.2 intronic ENSG00000159184	protumour	↑	proliferation, progress	GBM tissue; GBM cell line	[69]
HOTTIP / HOXA-AS6 /	07p15.2 antisense ENSG00000243766	antitumour	↓	inhibition of cell cycle, induction of apoptosis	GBM tissue; GBM cell line	[70]
HULC / lnc-BMP6-106 /	06p24.3 intronic ENSG00000285219	protumour	↑	proliferation, angiogenesis, activity of MGMT	GBM cell line; GBM patient tissue	[29]
KTN1-AS1	14q22.3 antisense ENSG00000186615	tumour suppres-sor	↑	viability and invasion of cell; correlation with prognosis	GBM tissue; GBM cell line; TCGA data	[71]
LINC00467 / NR_026761 /	01q32.3 intronic ENSG00000153363	protumour	↑	proliferation and invasion	GBM cell line	[72]
LINC00565	13q34 intronic ENSG00000260910	unknown; biomarker	↑	correlation with prognosis	GBM patient serum	[73]
LINC00641	14q11.2 intronic ENSG00000258441	unknown; biomarker	↑	correlation with prognosis	GBM patient serum	[73]
LINC01393	07q31.2 intronic ENSG00000225535	unknown; biomarker	↑	tumour progress; correlation with prognosis	GBM tissue; GBM cell line; TCGA data	[74]
LINC01426	21q22.12 intronic ENSG00000234380	oncogenic	↑	proliferation, invasion, viability	clinical association TCGA; GBM primary tissue; GBM cell line	[75]
LINC01446	07p12.1 intronic ENSG00000205628	protumour	↑	tumourigenesis, progress	clinical association; GBM cell line; xenografts	[76]
LINC01494	02q35 intronic ENSG00000228135	oncogenic	↑	proliferation, invasion	clinical association; GBM tissue; GBM cell line	[77]

LINC01503	9q34.11 intronic ENSG00000233901	oncogenic; biomarker	↑	migration, invasion, apoptosis; correlation with malignancy grade and prognosis	GBM tissue; GBM cell line; TCGA data	[78]
LINC01711	20q13.32 intronic ENSG00000268941	protumour	↑	proliferation, migration, invasion correlation with prognosis	GBM tissue; GBM cell line	[79]
LINC02283	04q12 intronic ENSG00000248184	oncogenic	↑	correlation with expression of PDGFRA, malignancy	patient GSC lines; xenoimplants; GBM tissue	[80]
LINC-ROR / ROR /	18q21.31 intronic ENSG00000258609	unknown	↑↓	GSC	GBM tissue; GBM cell line	[81]
lnc-TALC / LNCARSR / / linc-GNAQ-7 /	09q21.31 intronic ENSG00000233086	protumour	↑	TMZ resistance, tumour relapse	TMZ-selected GBM cell lines	[48]
MAFG-DT /MAFG-AS1-001/	17q25.3 intronic ENSG00000265688	protumour	↑	proliferation	GBM tissue; GBM cell lines	[82]
MALAT1	11q13.1 intronic ENSG00000251562	unknown	↑↓	invasion, proliferation, migration, apoptosis, permeability of BBB, chemosensitivity	clinical association; GBM patient tissue and serum; GBM cell lines; xenografts	[38]
MATN1-AS1	01p35.2 intronic ENSG00000186056	suppressor	↓	inhibition of proliferation and invasion	GBM primary tissue lines; GBM cell lines	[38]
MDC1-AS	06p21.33 antisense ENSG00000224328	suppressor	↓	inhibition of proliferation	GBM cell lines	[83]
MEG3 / lnc-DLK1-3 /	14q32.2 intronic ENSG00000214548	suppressor	↓	inhibition of proliferation	GBM tissue; GBM cell lines	[84]
MIAT	22q12.1 intronic ENSG00000225783	oncogenic	↑	proliferation, migration, metastasis	GBM tissue; GBM cell lines	[37]
MIR210HG	11p15.5 intronic ENSG00000247095	unknown; biomarker	↑	hypoxia, invasion, TMZ resistance; correlation with prognosis	GBM cell line; xenografts; TCGA data GBM patient plasma	[45]
MNX1-AS1 / CCAT5 / / LOC645249 /	07q36.3 intronic ENSG00000243479	oncogenic	↑	proliferation, migration, invasion	GBM tissue; GBM cell lines	[85]
NCK1-AS1 / SLC35G2-AS1 / / NCK1-DT /	03q22.3 antisense ENSG00000239213	protumour	↑	TMZ resistance	primary tissue; GBM cell lines	[86]
NEAT1 / LINC00084 /	11q13.1 intronic ENSG00000245532	protumour	↑	proliferation, glycolysis	GBM primary tissue; cell lines; xenografts	[87]
PART1	05q12.1 antisense ENSG00000152931	tumour suppres- sor	↓	inhibition of progression and tumour growth	clinical association TCGA; GBM tissue; GBM cell lines	[88]
PARTICL / PARTICLE /	2p11.2 circulating ENSG00000286532	regulation of tumour suppressors	-	tumour microenvironment, chromatin dynamics	GBM cell lines; GBM tissue	[32] [89]

PCAT1 / PCA1 /	08q24.21 intronic ENSG00000253438	unknown	↑↓	viability, DNA repair	GBM cell lines	[42]
PVT1 / lncRNA1331 /	08q24.2 intronic ENSG00000249859	oncogenic	↑	tumourigenesis, progress	GBM tissue; GBM cell lines; xenoimplants	[90]
RBPMS-AS1	08p12 antisense ENSG00000254109	antitumour	↓	radiosensitivity, apoptosis	GBM tissue; GBM cell line; xenoimplants	[91]
RPSAP52	12q14.3 antisense ENSG00000241749	unknown; biomarker	↑	correlation with prognosis	clinical association; GBM primary tissue; GBM cell line	[92]
RUNX1-IT1	21q22.12 intronic ENSG00000159216	protumour	↑	cell cycles, proliferation	GBM tissue; GBM cell line	[93]
SAMMSON / LINC01212 /	03p13 intronic ENSG00000240405	oncogenic; potential biomarker	↑	proliferation, viability, invasion, apoptosis	GBM tissue; GBM cell line; GBM patient serum	[94]
SOX2-OT	3q26.3 overlapping ENSG00000242808	unknown; biomarker	↑	migration and invasion; correlation with prognosis	GBM tissue; GBM cell lines	[87]
TALNEC2 / LINC01116 /	02q31.1 intronic ENSG00000163364	protumour	↑	tumourigenesis, radiation resistance	clinical association TCGA; GBM primary tissue; GBM cell lines	[95]
TP73-AS1 / lnc-LRRC47-78 / / KIAA0495 /	01p36.32 antisense ENSG00000227372	unknown; biomarker	↑	correlation with prognosis; resistance and metabolism TMZ in GSC	clinical association TCGA; GSC lines	[96]
TSLC1-AS1 / lnc-NXPE2-1 / / RP11-713B9 /	11q23.2 antisense ENST00000546273	tumour suppressor	↓	inhibition of cell proliferation, migration and invasion	GBM tissue; GBM cell lines	[97]
TUSC7 / LINC00902 /	03q13.31 antisense ENSG00000243197	tumour suppressor	↓	inhibition, resistance to TMZ, tumour malignancy	GBM cell line; GBM patients resistant to TMZ tissue	[98]
TUG1	22q12.2 antisense ENSG00000253352	unknown	↑↓	permeability of BBB	GBM tissue; GBM cell line	[46]
TUNAR	14q32.2 intronic ENSG00000250366	unknown	↑	regulation of tumour progress, cell cycles	GBM cell lines	[99]
UCA1 / UCAT1 / /oncolncRNA-36/	19p13.12 intronic ENSG00000214049	protumour	↑	proliferation, invasion, migration; glycolysis	GBM tissue; GBM cell lines	[100] [101]
XIST	Xq13.2 intronic ENSG00000229807	protumour	↑	permeability of BBB, angiogenesis, proliferation of CSC, migration, invasion	GBM tissue; GBM cell line	[102]
ZEB1-AS1	10p11.22 antisense ENSG00000237036	protumour	↑	cell proliferation, migration, invasion	GBM cell line	[103]
ZBED3-AS1	05q13.3 antisense ENSG00000250802	unknown; biomarker	↓	TMZ resistance	TMZ-resistant GBM cell line a tissue	[104]

6. LncRNAs as Biomarker in Clinical Use

LncRNAs can regulate gene expression by binding to transcription factors, can compete for binding sequences for miRNAs, and thus inhibit their action. They can also bind to regulatory proteins and participate in the formation of ribonucleoprotein complexes

and induce modification of chromatin. Finally, lncRNAs can regulate mRNAs at several levels, from translational inhibition and splicing to degradation, thus, effecting protein synthesis and functions [105]. Alterations in the expression of various lncRNAs have been detected in association with many diseases, including cancer [17], depression [28], cardiovascular disease and others [106][28]. These characteristics of lncRNAs renders them potential therapeutic targets and instructive biomarkers for difficult-to-diagnose diseases [1][21]. A good example is the clinical application of lncRNA deregulation of PCA3 (*Prostate Cancer Antigen 3*). In 2012, the FDA approved a diagnostic test for prostate cancer based on the detection of elevated expression of lncRNA PCA3 in urine [17].

Reports suggest that some lncRNAs have better diagnostic and prognostic properties than standardised biomarkers [105]. LncRNA molecules meet all major requirements for biomarkers used clinical diagnostics. They are produced continuously in cells, respond to homeostatic and environmental challenges, are secreted into the biological fluids and can be readily detected by analytical methods. Changes in the level of these molecules in cells and in biological fluids, therefore, provide valuable information about the alterations of health and disease states [107]. The fact that most lncRNAs are relatively stable, can be released in body fluids including plasma, serum, urine and cerebrospinal fluid and can be readily detectable, makes them highly suitable as biomarkers [26]. LncRNAs have also been detected in exosomes of biological fluids, including blood (see Table 2), which makes them better protected from ribonucleases and more stable over time. In this form, lncRNA molecules are also protected from the effects of repeated thawing, assisting their detection and research [108][109]. Expression levels lncRNA, like that of other proteins, can be quantified using sensitive laboratory methods such as real-time PCR, NGS, RNA microarrays, RNA-seq, which are becoming increasingly available. Importantly, changes in lncRNA levels in tissues and body fluids may also reflect alterations in the response of the body to therapeutic intervention [106][110].

Table 2. Systematic list of potential GBM lncRNA biomarkers and their deregulation in blood in other diseases. LncRNAs proposed in available studies as GBM biomarker (Table 1) are also deregulated in the blood of patients with other diseases.

lncRNA	Expression	Disease	Expression level correlates with	Fluids	Ref.
ADAMTs9-AS2	↓	ischemic stroke	severity of disability	plasma	[111]
	↓	non-small cell lung cancer	aggressive tumour behavior	serum	[112]
ANRIL	↑	breast cancer	metastasis	serum	[113]
	↑	coronary artery disease	prognosis, degree of inflammation, severity of disability	plasma	[114]
	↑	COVID-19	severity of disability	blood	[115]
	↑	Crohn's disease	diagnosis	serum	[116]
	↑	diabetes mellitus	diagnosis	serum	[117]
	↑	glioma	tumour grade and prognosis	serum	[54]
	↑	intraductal papillary mucinous neoplasms of the pancreas	malignant prediction	plasma	[118]
	↑	ischemic stroke	severity of disability	serum	[119] [120]
	↑	multiple myeloma	prognosis	plasma	[121]
	↑	neonatal sepsis	higher risk of mortality	plasma	[122]
	↑	non-small cell lung cancer	prognosis	serum, plasma	[123] [124]
	↑	pituitary adenomas	prognosis	plasma	[125]
	↑	sepsis	severity of disability and prognosis	plasma	[126]
	↑	stable angina	level of troponin 1	plasma	[127]
	↑	ulcerative colitis	diagnosis	serum	[116]

	↓	acute exacerbation of chronic obstructive pulmonary disease	levels of inflammatory cytokines	plasma	[128]
	↓	acute ischemic stroke	clinico-pathological symptoms	plasma	[129]
	↓	glaucoma	clinico-pathological symptoms	serum	[130]
	↓	multiple sclerosis	diagnosis	blood	[131]
	↓	pediatric inflammatory bowel disease	diagnosis	serum	[132]
	↓	preeclampsia	diagnosis	serum	[133]
CASC2	↑	osteoarthritis	level of IL-17	plasma	[134]
	↑	aphthous stomatitis	level of IL-6 and IL-18plasma	plasma	[135]
	↓	diabetic nephropathy	diagnosis	serum	[136]
	↓	hepatocellular carcinoma	tumour grade	serum	[137]
	↓	childhood asthma	diagnosis	serum	[138]
	↓	chronic renal failure	diagnosis	serum	[139]
	↓	oral squamos	prognosis	plasma	[140]
	↓	rheumatoid arthritis	diagnosis	serum, plasma	[141] [142]
	↓	sepsis	clinico-pathological symptoms	serum, blood	[143] [144]
CRNDE	↑	acute myeloid leukemia	clinico-pathological symptoms	blood	[145]
	↑	colorectal carcinoma	aggressive tumour and liver metastais	serum, plasma	[146] [147]
	↑	hepatocellular carcinoma	tumour size and differentiation	serum	[148]
	↑	nasopharyngeal carcinoma	lymph node metastasis	serum	[149]
	↑	non-small cell lung cancer	diagnosis	plasma	[150]
	↑	sepsis	severity of disability	serum	[151]
	↑	severe pneumonia	prognosis	serum	[152]
	↓	chronic lymphocytic leukemia	prognosis	serum	[153]
	↓	sepsis	increasing levels after treatment	plasma	[154]
DGCR5	↓	gastric cancer	clinico-pathological symptoms, metastasis	plasma	[155]
	↓	hepatocellular carcinoma	diagnosis	serum	[156]
DLEU-AS1	↑	diabetic foot ulcer	diagnosis	serum	[157]
	↑	endometrial cancer	clinico-pathological symptoms	serum	[158]
GAS5	↑	atherosclerosis	diagnosis	serum	[159]
	↑	malignant mesothelioma	diagnosis	plasma	[160]
	↑	multiple sclerosis	clinico-pathological symptoms	serum	[161]
	↑	myelofibrosis	clinico-pathological symptoms	plasma	[162]
	↑	osteporosis	diagnosis	plasma	[163]
	↑	osteporosis with fractures	upregulated in the presence of a fracture	serum	[164]
	↑	polycystic ovary syndrome	diagnosis	plasma	[165]
	↓	glioblastoma multiforme	prognosis	serum	[166]
	↓	breast cancer	diagnosis	serum	[167]
	↓	cerebrovascular stroke	diagnosis	serum	[168]
	↓	coronary artery disease	diagnosis	plasma	[169]
	↓	COVID-19	severity of disability	serum	[170]
	↓	diabetes mellitus 2	diagnosis	serum	[171]
	↓	hepatocellular carcinoma	diagnosis	plasma	[172]
	↓	chronic hepatitis B virus infection	liver fibrosis	serum	[173]

	↓	mycobacterium tuberculosis	diagnosis	serum	[174]
	↓	non-small cell lung cancer	tumour size and metastasis	serum, plasma	[175] [176]
	↓	osteporosis	diagnosis	serum	[177]
	↓	polycystic ovary syndrome	biomarker of insulin resistance	serum	[178]
	↓	rheumatoid arthritis	diagnosis	serum, plasma	[179] [180]
	↓	sepsis	diagnosis	serum, plasma	[181] [182]
	↓	systemic lupus erythematosus	diagnosis	plasma	[183]
HOTAIR	↑	Alzheimer's disease	clinico-pathological symptoms, decreasing levels after treatment (exercises)	serum	[184]
	↑	breast cancer	lymph node metastasis	plasma, serum	[185] [186]
	↑	colorectal carcinoma	diagnosis	plasma	[187]
	↑	congenital heart diseases	diagnosis	plasma	[188]
	↑	coronary artery disease	diagnosis	blood	[189]
	↑	diabetes mellitus 2	complications of diabetes	serum	[190]
	↑	esophageal squamous cell carcinoma	tumour grade, decreasing levels after treatment	serum	[191]
	↑	gastric cancer	tumour grade and metastasis	plasma, serum	[192] [193]
	↑	gestational diabetes	body mass index, fasting plasma glucose	plasma	[194]
	↑	glioblastoma multiforme	level of tissue expresion, progression	serum	[195] [66] [166]
	↑	hepatocellular carcinoma	diagnosis	serum	[196]
	↑	laryngeal squamous cell carcinoma	lymph node metastasis	serum	[197]
	↑	multiple myeloma	disease stage	plasma	[198]
	↑	non-small cell lung cancer	histology subtype and tumour-node-metastasis stage	plasma	[199]
	↑	osteoarthritis	diagnosis	plasma	[200]
	↑	papillary thyroid carcinoma	tumour grade	serum	[201]
	↑	rheumatoid arthritis	decreasing levels after treatment	serum	[202]
	↑	systemic lupus erythematosus	level of IL-6	serum	[203]
	↓	acute myocardial infarction	diagnosis	plasma	[204]
	↓	atherosclerosis	diagnosis	plasma	[204]
LINC00467	↓	acute myeloid leukemia	increasing levels after treatment	serum	[101]
LINC00565	↑	glioblastoma multiforme	survival pattern patients	serum	[73]
LINC00641	↑	Crohn's disease	diagnosis	serum	[205]
	↑	glioblastoma multiforme	survival pattern patients	serum	[73]
	↑	ulcerative colitis	diagnosis	serum	[205]
MALAT1	↑	acute kidney injury	diagnosis	serum	[206]
	↑	acute pancreatitis	diagnosis	serum	[207]
	↑	angina pectoris	severity of disability	serum	[208]
	↑	breast cancer	diagnosis	serum	[209]
	↑	gastric cancer	metastasis	plasma	[210]
	↑	gestational diabetes	diagnosis	serum	[211]
	↑	glioblastoma multiforme	TMZ chemoresitence	serum	[38]
	↑	hypertensis	diagnosis	plasma	[212]
	↑	multiple sclerosis	diagnosis	serum	[213]

	↑	nasopharyngeal carcinoma	tumour stage, decreasing levels after treatment	serum	[214]
	↑	non-Hodgkin lymphoma	tumour stage	plasma	[215]
	↑	non-small cell lung cancer	tumour grade and metastasis	serum	[216]
	↑	osteosarcoma	survival pattern patients	serum	[217]
	↑	ovarian cancer	metastasis	serum	[218]
	↑	parkinson disease	degree of inflammation	serum	[219]
	↑	prostate cancer	diagnosis	serum	[220]
	↑	rheumatoid arthritis	diagnosis	plasma	[221]
	↑	sespsis	clinico-pathological symptoms	plasma	[222]
	↑	severe pneumonia	prediction of survival of patients	serum	[223]
	↑	tongue squamous cell carcinoma	diagnosis	serum, plasma	[224]
	↑	ulcerative colitis	diagnosis	plasma	[225]
	↓	diabetes mellitus 2	diagnosis	serum	[226]
	↓	retinoblastoma	diagnosis	serum	[227]
MIR210HG	↑	glioma	diagnosis	serum	[228]
RPSAP52	↑	renal failure	diagnosis	plasma	[229]
	↓	diabetic retinopathy	diagnosis	plasma	[230]
SAMMSON	↑	glioblastoma multiforme	diagnosis	plasma	[231]
	↑	oral squamous cell carcinoma	levels of tissue expresion, decreasing levels after treatment	serum	[232]
	↑	papillary thyroid carcinoma	diagnosis	plasma	[233]
SOX2-OT	↑	head and neck squamous cell carcinoma	diagnosis	plasma	[234]
	↑	lung squamous cell carcinoma	tumour size and lymph node metastasis, decreasing levels after treatment	plasma	[235]
	↑	ovarian cancer	diagnosis	plasma	[236]
	↑	pulmonary arterial hypertension	diagnosis	serum	[237]
TP73-AS1	↑	non-small cell lung cancer	prognosis	serum	[150]
	↑	NK/T-cell lymphoma	diagnosis	blood	[96]
ZEB1-AS1	↑	esophaegal carcinom	diagnosis	serum	[238]
	↑	prostate cancer	diagnosis	serum	[239]
	↓	amyotrophic lateral sclerosis	diagnosis	blood	[240]
	↓	diabetes mellitus	complications of diabetes (diabetic lung)	plasma	[241]

For some diseases, it appears that the detection of changes in a single lncRNA is not specific enough to qualify as biomarkers. Indeed, a large percentage of lncRNAs are abnormally expressed in multiple diseases (See Table 2). Rising data show that stress and pathological changes in anatomically related structures, types of tissue, or embryologically interrelated organs are characterised by abnormal expression of a similar set of lncRNAs [109]. Thus, it is necessary to identify a set of lncRNAs, called a signature or fingerprints, that corresponds to a given disease [109] (See Table 3). An example is the combination of three lncRNAs SPRY4-IT1, ANRIL and NEAT1. These lncRNAs are abnormally regulated in the blood plasma of patients with non-small cell lung cancer. More than 90% specificity and 80% sensitivity have been achieved in the diagnosis of this disease using the detection of this set of lncRNAs [242]. Analysis of lncRNAs in blood plasma can also be used to determine the prognosis - changes in the regulation of lncRNAs XLOC_014172 and LOC149086, which can distinguish metastatic hepatocellular

carcinoma from nonmetastatic carcinoma, with a specificity and sensitivity of more than 90% and an AUC of 0.934 [109].

Table 3. Suggested lncRNA signatures for the selected diseases according to Table 2.

Disease	Deregulated levels of lncRNA biomarkers in peripheral blood
Breast cancer	ANRIL (↑), HOTAIR (↑), MALAT1 (↑), GAS5 (↓)
Coronary artery disease	ANRIL (↑), HOTAIR (↑), GAS5 (↓)
Diabetes mellitus 2	ANRIL (↑), HOTAIR (↑), GAS5 (↓), MALAT1 (↓), ZEB1-AS1 (↓)
Gastric cancer	HOTAIR (↑), MALAT1 (↑), DGCR5 (↓),
Glioblastoma multiforme	ANRIL (↑), HOTAIR (↑), LINC00641 (↑), LINC00565 (↑), MALAT1 (↑), SAMMSON (↑), GAS5 (↓)
Hepatocellular carcinoma	CRNDE (↑), HOTAIR (↑), CASC2 (↓), DGCR5 (↓), GAS5 (↓)
Multiple sclerosis	GAS5 (↑), MALAT1 (↑), ANRIL (↓)
Non-small cell lung cancer	ANRIL (↑), CRNDE (↑), GAS5 (↑), HOTAIR (↑), MALAT1 (↑), TP73-AS1 (↑), ADAMTs9-AS2 (↓)
Rheumatoid arthritis	HOTAIR (↑), MALAT1 (↑), CASC2 (↓), GAS5 (↓)
Sepsis	ANRIL (↑), CRNDE (↑↓), CASC2 (↓), GAS5 (↓)
Ulcerative colitis	ANRIL (↑), LINC00641 (↑), MALAT (↑)

Detection of changes in the expression of one or more lncRNAs (also as part of liquid biopsy), thus, might be an effective approach for the early diagnosis of various diseases, to ensure more targeted and personalized interventions with better therapeutic outcomes. With the use of biological fluids as a source of lncRNA biomarkers, sample collection ranges from non-invasive (urine, saliva) to minimally invasive (plasma, serum) and invasive (organ biopsy). Due to the properties of lncRNAs, there is pressing need in standardization of sample collection and stringent preparation of biomaterials for analysis across different settings, to ensure the specificity and reproducibility of the data [107]. More research on the correlation between various lncRNAs in the same condition and cross-correlation in different diseases is warranted, using extensive data collection and analysis with advanced computational methods and artificial intelligence approaches (AI).

Emerging lncRNA Biomarkers of GBM

ADAMTS9-AS2 (*ADAM metallopeptidase with thrombospondin type 1 motif 9 antisense RNA 2*) is considered a proto-oncogenic GBM lncRNA in most studies [49][243]. This lncRNA is also abnormally up-regulated in other malignancies, to significantly increased levels in ovarian cancer tissue [218] while in lung adenocarcinoma its level is reduced [244]. ADAMTS9-AS2 is involved in several major signalling pathways, including PI3K/AKT and MEK/Erk, and interacts with many miRNAs (in most cases as a sponge) [245]. ADAMTS9-AS2 has both tumour suppressor and proto-oncogenic functions depending on the type of cancer and can be used as a biomarker for cancer. Abnormal expression levels of this lncRNA measured in plasma or tissue have diagnostic value, with changes reported in patients with malignancy of lung, oesophageal and prostate cancer [245], and lung adenocarcinoma [112][246][247]. Decreased expression of ADAMTS9-AS2 in tumour tissue correlates with poor prognosis and shorter survival in patients with esophageal cancer [248], lung adenocarcinoma [244], breast cancer and bladder urothelial carcinoma [245]. The expression of ADAMTS9-AS2 was reduced and negatively correlated with the extent of tissue and organ damage, which make this lncRNA a potential qualitative biomarker [249]. ADAMTS9-AS2 has repeatedly demonstrated GBM oncogenic effects [49][243]. Its expression was measured in resected GBM tissue and cell lines, with levels correlating with glioma grade [243]. Increased ADAMTS-AS2 levels are also prognostic, as higher expression levels were found in GBM patients resistant to TMZ treatment compared to those responding to TMZ treatment [49]. Considering that elevated

levels of this lncRNA are also found in the blood of patients with several diseases, the most effective use of this lncRNA profile in patients with GBM would be in combination with other indicators of disease.

ANRIL (*Antisense Noncoding RNA in INK4 Locus*) is considered an oncogenic lncRNA linked to GBM. Dysregulation of ANRIL in blood has been associated with cancers in general, cardiovascular diseases [250] and type 2 diabetes mellitus [251] (see Table 2). This lncRNA can modulate gene expression at the posttranscriptional level by interacting with miRNAs and proteins [252]. Furthermore, ANRIL negatively and positively influences gene expression at the chromatin level [250]. ANRIL functions as a scaffold for PRC2 and therefore participates in epigenetic gene silencing [253] and is involved in alternative splicing in HEK293 and HUVEC cells [254]. Through these mechanisms, ANRIL contributes to tumorigenesis processes, increasing cell proliferation, migration, invasion, and metastasis, and suppressing apoptosis and senescence [251]. Up-regulated ANRIL expression levels have been found to be linked with cancers such as lung, stomach, breast, ovarian, cervical, colorectal, bladder, thyroid, brain, osteosarcoma, myeloma, prostate, endometrial, renal, and leukemia, melanoma, retinoblastoma, and hepatocellular carcinoma [251]. In addition to an increased risk of cancer, polymorphisms in the ANRIL gene are also associated with the risk of atherosclerosis, obesity, and type 2 diabetes. ANRIL expression is also affected by inflammation, with pro-inflammatory factor IFN-γ activating the transcription factor STAT1, thereby inducing ANRIL expression in endothelial cells [252]. Elevated ANRIL can affect the expression of NF-κB-dependent inflammatory molecules, such as IL-6 and IL-8 [253]. In GBM, the oncogenic lncRNA ANRIL is upregulated in cell lines, resected GBM tissue and serum of patients diagnosed with glioma [16][54]. High expression of this lncRNA in patient serum correlates with adverse prognosis, grade, size, and metastasis [54]. This lncRNA should be included in the GBM signature when testing for GBM disease in blood.

In addition to GBM [55], **CASC2** (*Cancer Susceptibility Candidate*) lncRNA is downregulated in various cancers such as endometrial, lung, gastric, colorectal and bladder cancer. In clinical practise, low levels of CASC2 are associated with a more aggressive cancer phenotype and shorter survival time [255]. CASC2 is involved in the MAPK and Wnt/B-catenin signaling pathways. This lncRNA functions as a sponge for some oncogenic miRNAs, such as miR-21 and miR-18a [255]. The lncRNA CASC2 was monitored in the blood of patients with type 2 diabetes. Low serum levels of CASC2 predict the appearance of chronic renal failure in these patients [256] and rheumatoid arthritis [257]. Different plasma levels of CASC2, along with IL-6 and IL-8, were found in patients treated for aphthous stomatitis compared to healthy controls. Higher levels of CASC2 after treatment predicted a higher rate of recurrence [135]. CASC2 expression levels measured in whole blood negatively correlate with liver cancer stage [258]. Deregulation of CASC2 expression was also investigated in serum of patients hospitalized with sepsis. Levels were negatively correlated with the II Assessment of Acute Physiology and Chronic Health (APACHE II) and the Sequential Organ Failure Assessment (SOFA). With lower CASC2 levels, the risk of death increases in these patients. CASC2 insufficiency may be a good biomarker, as it correlates with the reduced cytokine release, severity of multiorgan injury and prognosis in these patients [257]. On the other hand, up-regulation of CASC2 was observed in pancreatic tissues of patients with acute pancreatitis [259]. The expression of CASC2 was examined in GBM cell lines, xenografts and tissues resected from patients diagnosed with glioma [55][260][261]. The level of this lncRNA is upregulated compared to healthy controls. This fact leads to changes in the expression of some miRNAs, e.g. miR-193a-5p, and a decrease mTOR expression [260]. The expressional changes negatively correlate with the tumour grade and survival time in patients [261] and with its role in the efficacy of chemotherapy also reported [262]. Unfortunately, data reporting changes in CASC2 expression in the blood of GBM patients are not available. Given the diagnostic and prognostic value of this lncRNA in GBM patients and the diagnostic significance of

blood levels of CASC2 in other diseases, it is desirable to focus on the levels of this tumour suppressor lncRNA in the blood of GBM patients.

Oncogenic lncRNA **CRNDE** (*Colorectal Neoplasia Differentially Expressed*) has been detected in tissue from GBM patients and is associated with resistance to TMZ therapy [58]. This lncRNA is also abnormally expressed in other cancers. Alterations in CRNDE expression correlate with tumour clinicopathological characteristics and prognosis of patients diagnosed with colorectal cancer, breast cancer, cervical cancer, lung adenocarcinoma, multiple myeloma, chronic lymphocytic leukemia and ovarian cancer [263]. The physiological expression of CRNDE is tissue-specific; low levels are detected e.g. in the colorectal mucosa; on the other hand CRNDE is found in breast tissue and testes in higher amounts [263]. CRNDE interacts with a wide variety of targets involved in the activation of the Wnt/β-catenin signalling pathway, as well as some miRNAs (e.g. miR335-3p) and proteins [264]. CRNDE may serve as a scaffold for some tumour-associated proteins (e.g., DMBT1) [265][266]. Through the molecular mechanisms described above, CRNDE regulates the tumour microenvironment, contributing to tumorigenesis - proliferation, cell invasion, apoptosis, metastasis, and resistance [151]. Elevated levels of this lncRNA are a indicator of the prognosis of cancer patients [151] e.g. in a patient with osteosarcoma [264]. The deregulated expression levels found in the blood of patients hospitalized with sepsis are correlated with APACHE II and SOFA, as well as inflammation, and are a prognostic biomarker for sepsis [151]. Finally, CRNDE appears to be a good biomarker for the clinical course of hepatocellular carcinoma. The available analyzes suggest that serum measured exosomal lncRNA CRNDE is an independent marker of survival time in patients with hepatocellular carcinoma [148]. CRNDE is one of the best characterized lncRNA in association with gliomas and GBM, with increased expression observed in GBM cell lines (including CSCs [267]) and in resected GBM tissues [268]. Tissue expression levels of this lncRNA correlate with prognosis, tumour size, recidivity [267], GBM subtype [269], and predict patients' chemosensitivity to TMZ treatment [58]. *In vitro* experiments suggest that CRNDE knockdown enhances TMZ chemosensitivity in GBM cells [58]. This makes CRNDE a potential therapeutic target for further GBM treatment research. The level of CRNDE in the blood of GBM patients has been investigated [166]. Because it was detected in only 20 % of patients, its biomarker potential has not been further investigated [166]. In consideration of the facts described above, it would be a good idea to focus on a larger sample of patients with different subtypes of GBM to see if increased CRNDE expression in the blood of patients indicates only that subtype of GBM or decreased chemosensitivity to TMZ.

DGCR5 (*DiGeorge syndrome critical region gene 5*) is one of the GBM suppressor lncRNAs [60]. The oncogenic and suppressor functions of DGCR5 have been described depending on the type of malignancy (e.g. gallbladder cancer, lung cancer) [270]. Dysregulation of DGCR5 expression has also been documented in patients with Huntington's disease [271]. At the molecular level, this lncRNA is involved in various mechanisms of tumorigenesis, including cell proliferation, invasion, migration, apoptosis and response to therapy. It interacts with many miRNAs, including miR-21, and functions as competing endogenous RNA (ceRNA) [272]. Reduced expression compared to healthy controls has been observed in the following malignancies: cervical [273], laryngeal, bladder [274], pancreatic, thyroid, prostate, ovarian cancer, hepatocellular carcinoma [275][276], colorectal cancer [272] and gliomas [60]. For these types of malignancies, DGCR5 could be used as a biomarker, as reduced expression levels correspond to clinical stage, tumour size, survival time and amount of metastasis [270][272][277][278]. On the other hand, increased expression was detected in gallbladder cancer and triple negative breast cancer [270][279]. This lncRNA also correlates with the number of immune cells and the strength of the immune response in the tumour microenvironment [270]. DGCR5 is downregulated in glioma tissue and cell lines [60]. Analysis of data from xenograft experiments confirmed that this lncRNA acts as a tumour suppressor by inhibiting glioma growth [60]. The level of lncRNA in resected tissue negatively correlates with glioma grade and prognosis [280].

This lncRNA could be included in the tissue signature of GBM to refine diagnosis and prognosis. DGCR5 expression level correlates with the amount of immune and stromal cells and is thus associated with immune response and immune infiltration [280]. Further studies indicate that this lncRNA is involved in the process of angiogenesis and could be a promising therapeutic target [281]. Given its diagnostic and prognostic character in GBM and deregulation in the blood of gastric cancer patients [155], this lncRNA is an interesting target for analysis in the blood of GBM patients.

GAS5 (*Growth Arrest Specific 5*) is a GBM tumour suppressor lncRNA [63]. Decreased expression of this lncRNA is also detected in other cancers including breast, prostate, ovarian and cervical cancers, colorectal, gastric, kidney, bladder, lung, pancreatic, endometrial and renal cancers, as well as melanoma, osteosarcoma, neuroblastoma and gliomas [282]. GAS5 naturally accumulates in cells after growth arrest induced by, for example, nutrient deficiency. GAS5 affects cell cycle progression, and it is necessary for normal cell growth arrest. High levels of GAS5 expression inhibit cell cycle progression, while decreased GAS5 expression reduces apoptosis and promotes accelerated cell division [282]. GAS5 is considered a tumour suppressive lncRNA in association with many malignancies, in which reduced expression of this transcript has been detected. Clinicopathological characteristics, which include survival time, relapse-free survival, presence of distant metastases, presence of lymph node metastases, tumour size, and progression, are inversely correlated with expression levels in different types of cancer, suggesting that GAS5 could become a diagnostic and prognostic biomarker. Furthermore, it also has potental for a biomarker allowing monitoring therapeutic responses [283]. GAS5 tumour suppression has been associated with gliomas, and the expression level of this lncRNA is correlated with the degree of tumour malignancy and patient survival time. Differential expression levels of GAS5 are detected not only in tissues, but also in body fluids, including blood and urine [284]. Decreased plasma and serum GAS5 levels have been detected in patients with multiple sclerosis and in patients with myelofibrosis. Measured values were correlated with the clinicopatological status of the patient [161][162]. Reduced expression was detected in serum from patients with various diseases including breast cancer [167], stroke [168], COVID-19 [170], liver cancer, sepsis [285], rheumatoid arthritis, and osteoporosis [177]. Tumour suppression of GAS5 has been associated with gliomas, and the expression level of this lncRNA correlates with the degree of tumour malignancy and patient survival time. GAS5 transcription is higher in lower-grade gliomas compared to higher-grade gliomas, including GBM [286]. Low levels of GAS5 expression observed in GBM compared to healthy controls correlate with poor prognosis [275]. Serum levels of GAS5 may become a good prognostic biomarker as part of the lncRNA signature because deregulated levels of this lncRNA are associated with 2-year overall survival of GBM patients after surgery [166]. Deregulation of GAS5 in a large number of diseases shows the importance of this lncRNA. For the clinical use of GAS5 as a biomarker, specific sets of lncRNAs are needed to facilitate higher diagnostic specificity.

LINC00467 (*Long intergenic non-protein coding RNA 467*) is an oncogenic GBM lncRNA and its expression correlating with the grade of glioma [72]. This lncRNA has been shown to be pro-inflammatory in association with some other malignancies such as gastric cancer, with its increase reported in lung cancer, breast cancer, colorectal cancer, hepatocellular carcinoma, osteosarcoma, head squamous cell carcinoma and others [287]. LINC00467 is part of several signalling pathways including Akt, STAT and EGFR, and its deregulation may contribute to pro-inflammatory mechanisms [288][289]. Tumorigenesis can also occur through sponging of e.g. miR-4779 and miR-7978 [290]. LINC00467 can also act as a ceRNA and thus participate in the regulation of signalling pathways (e.g. EGFR) and tumorigenesis [291]. It correlates with clinical stage of the various cancer types, with their poor prognosis and survival time [288][292][293][294][295]. Interestingly, this lncRNA can encode a short ASAP peptide. Research shows that this micropeptide is involved in mitochondrial metabolism and high levels correlate with a poor prognosis in patients with colorectal cancer [296]. Another argument for considering this lncRNA

among diagnostic and prognostic biomarkers, including GBM, is the detection of LINC00467 deregulation in the plasma of patients with acute myeloid leukemia [101]. Increased expression levels of LINC00467 have also been detected in prostate cancer tissue. The level of expression varied between cells and specifically between two macrophage phenotypes, pro-inflammatory and anti-inflammatory. Studies show that LINC00467 is involved in the polarization of macrophages towards the pro-inflammatory type. These facts make LINC00467 a promising therapeutic target for patients with early-stage prostate cancer [297]. LINC00467 was analyzed in glioma tissues and also in cell lines [298]. The expression level is upregulated [298] and its knockdown inhibited proliferation of cell [298] and induced apoptosis [298]. These observations make LINC00467 a potential therapeutic target. More experiments, including analysis of this lncRNA in patients' blood, are required to designate LINC00467 as a GBM biomarker.

LINC00641 is a potential biomarker for GBM and is differentially expressed in other types of cancer [73][299]. LINC00641 can be classified as both a tumour suppressor and an oncogenic lncRNA depending on the type of cancer. The tissue expression of this biomolecule is up-regulated in association with gastric, renal, prostate, rectal cancers and acute myeloid leukemia [299][300]. On the other hand, reduced expression levels are linked with cervical, bladder, breast, non-small cell lung and thyroid cancer [299]. Differences in tissue expression are associated with prognosis and survival in patients with cancers that include prostate cancer, thyroid cancer, bladder cancer [301], gastric cancer, renal cell carcinoma and rectal cancer [299][302]. In patients with breast cancer, expression levels correlate with tumour size and clinical stage, including lymph node metastasis [302]. LINC00641 interacts with many miRNAs as a sponge for, e.g., miR-197-3p or as competing endogenous RNAs (ceRNAs) in cervical, bladder, rectal cancers, and acute myeloid leukemia [299]. LINC00641 is also involved in the regulation of several signaling pathways including PTEN/PI3K/AKT and Notch-1 [303]. Therefore, targeting the LINC00641/miR-197-3p/KLF10/PTEN/PI3K/AKT cascade could hold promise as a therapeutic strategy. LINC00641 has been shown to be involved in the regulation of proliferation and apoptosis, as well as invasion and metastasis in several cancer types. Many studies in cell lines demonstrated the therapeutic potential of LINC00641. Targeting this lncRNA has been reported to alleviate features of tumorigenesis in almost all cancer types mentioned above [299]. In some cases, for example, in gastric cancer, targeting LINC00641 also affects drug resistance [304]. The above facts nominate LINC00641 as a high-quality potential biomarker and therapeutic target in the context of cancer and other diseases. LINC00641 has also been detected at higher levels in the serum of patients diagnosed with inflammatory bowel diseases such as ulcerative colitis and Crohn's disease and may thus be part of a non-invasive diagnostic pathway [205]. Furthermore, the results of some studies suggest the involvement of this lncRNA in the autophagy process and its indirect effect on the expression of brain-derived neurotrophic factor (BDNF) [305][306]. It is reportedly downregulated in GBM cell lines and tissues and is proposed to be a tumour suppressor lncRNA acting as an inhibitor of GBM cell proliferation [307][308]. Based on bioinformatics analyses, LINC00641 is recommended as part of the lncRNA signature for more accurate diagnosis from resected tissue [307][309]. In the serum of GBM patients, LINC00641 was analyzed together with LINC00565, and both lncRNAs showed increased expression compared to healthy controls [73].

MIR210HG is an oncogenic GBM lncRNA [45] and is deregulated in other cancers (liver, lung, pancreatic, breast, gastric, cervical, ovarian, colorectal) and non-cancerous diseases such as preeclampsia, acute renal injury, and others [310]. MIR210HG is involved in cell proliferation, migration and invasion, energy metabolism, autophagy, hypoxia, radiosensitivity and chemoresistance. MIR210HG has been confirmed to interact with many miRNAs, e.g. by sponging (by sponging miR-520a-3p suppresses trophoblast migration and invasion *in vitro*) or as a ceRNA [311]. Cell culture studies and clinical data show that there is an association between drug resistance and abnormal expression of MIR210HG in certain cancers, including GBM, pancreatic cancer, non-small cell lung cancer,

ERPR/Her2-type breast carcinoma and colorectal cancer. These data make MIR210HG a novel therapeutic target that could improve tumour sensitivity to radiotherapy and chemotherapy and inhibit neoplastic process [45][310][312]. Elevated levels of MIR210HG expression have been found in other cancers, with its higher levels reported in hepatocellular carcinoma tissue, which correlates with the clinical stage of the disease and tumour characteristics, including size, vascular invasion, and histological differentiation. It is also negatively correlated with overall cancer survival and could therefore be a good prognostic marker [313]. Abnormal expression of MIR210HG was found in pancreatic tumour tissue and its level is also associated with the survival time of patients [314]. Expressional changes are also associated with the clinical presentation of patients with osteosarcoma, as well as breast cancer, colorectal cancer, gastric cancer, cervical cancer, and ovarian cancer [310]. Interestingly, this lncRNA is overexpressed in the sperm of infertile men with varicocele and negatively correlates not only with the quantity of sperm, but also with the motility of the sperm [315]. MIR210HG was detected in the placenta of patients with preeclampsia compared to healthy controls [311]. These data suggest that this lncRNA may be included in the list of potential prognostic markers related to various diseases. Detection in blood or urine would be a suitable method for rapid and early diagnosis of some difficult-to-diagnose diseases, e.g. gliomas. Upregulated expression levels of MIR210HG were detected in the serum of glioma patients compared to healthy controls [228]. In addition to blood, MIR210HG levels were also elevated in GBM tissue and cell lines [45]. As mentioned above, MIR210HG is involved in the mechanism of hypoxia, which affects tumour aggressiveness. Elevated levels of this lncRNA predict poor prognosis associated with cell invasion, CSC and TMZ resistance [45]. Based on bioinformatics analyses, MIR210HG was found to be part of a set of lncRNAs that can be used to distinguish GBM from other gliomas [316].

ZEB1-AS1 (*Zinc finger E-box-binding homeobox 1 Antisense 1*) is an oncogenic GBM lncRNA [79]. Pro-tumour ZEB1-AS1 is associated with several other malignancies, including colorectal cancer, breast cancer, gastric cancer, prostate cancer, hepatocellular carcinoma, non-small cell lung cancer, osteosarcoma and others [317]. ZEB1-AS1 may potentially boost the proliferation, invasion, and migration capabilities of melanoma cells by directly suppressing miR-1224-5p. A study showed that elevated levels of ZEB1-AS1 were correlated with a decrease in the overall survival rate among melanoma patients, suggesting that ZEB1-AS1 and miR-1224-5p play crucial roles in melanoma pathogenesis, and could serve as predictive biomarkers and potential therapeutic targets [318]. ZEB1-AS1 lncRNA also plays a role in non-malignant conditions such as atherosclerosis, pulmonary fibrosis, ischemic vascular disease, and complications accompanying diabetes, including diabetic nephropathy [103]. ZEB1-AS1 is involved in the regulation of gene expression and thus contributes to cancer cell proliferation and migration [317]. It is an important modulator of ZEB1 gene expression, which is one of the main regulators of the epithelial-mesenchymal transition. ZEB1-AS1 acts as a sponge for many miRNAs and can therefore influence other genes [103]. Based on the results of the studies, ZEB1-AS1 appears to be a good biomarker not only in the context of cancer. Measured serum and plasma levels correlate with prognosis, response to treatment and stage in the following diseases [103]. In the context of colorectal cancer, ZEB1-AS1 has a diagnostic function, its expression level correlates with clinical stage and histological grade, metastasis, microvascular invasion, and its overexpression is associated with a poor prognosis [317]. Serum expression levels of ZEB1-AS1 were measured in patients with oral squamous cell carcinoma before and after tumour resection, with highly detectable differences. Data from this study suggest that ZEB1-AS1 could be a good marker for measuring treatment success [318]. In patients treated for esophageal cancer, ZEB1-AS1 was also detected in serum. The measured levels were correlated with a poor prognosis and ZEB1-AS1 levels in tumour tissues. Clinical studies show that ZEB1-AS1 expression levels also correlate with complications of diabetes [319]. Different expression levels of this lncRNA were measured in plasma from patients treated for diabetes-related complications (e.g. lung damage, nephropathy)

compared to diabetics without complications and a healthy group, where the expression was higher [320]. The opposite trend was observed in the serum of patients with atherosclerosis, where increased expression of ZEB1-AS1 was detected compared to healthy controls [321]. Changes in ZEB1-AS1 expression were detected in glioma tissue (including GBM) and GBM cell lines [322]. In both types of material, ZEB1-AS1 is detected at high levels, and in resected tissue it correlates with tumour size and malignancy grade (I-IV) [322][324]. *In vitro* experiments suggest that knockdown of this lncRNA induces G0/G1 phase arrest and correspondingly reduces the percentage of cells in S phase, thus affecting GBM cell proliferation, invasion, and migration [324]. Given the diagnostic relevance of ZEB1-AS1 to GBM and its changes on tissue, and its deregulation in the blood of patients with other diseases, the analysis of the ZEB1-AS1 profile and dynamics in the blood of GBM patients in well warranted.

7. Conclusions

The lncRNAs, which are the largest group of noncoding transcripts, have recently received a lot of research and translational attention. Their expression in various tissue types is highly specific, and differences have been detected between different tissues and cells in different physiological and pathophysiological conditions. Some human diseases, including cancer, are linked to deregulated lncRNAs, making these molecules potential therapeutic targets and biomarkers. The lncRNA research field is rapidly advancing and numerous new transcripts are identified every year. Several databases specialised in lncRNAs have been created to sort and organize the growing information, some of which were used in the writing of this article (e.g., LNCipedia 5.2; lncRNAfunc). GBM belongs to the most aggressive malignant brain tumour in adults that resists conventional complex therapy, which includes maximal surgical resection followed by radiation therapy and chemotherapy. Despite the relatively low incidence (3-4 cases per 100,000 people), GMB remains one of the greatest challenges and priorities for research, owing to its severity and very high mortality. Treated patients live an average of 14-16 months from first diagnosis, with 90% of patients not surviving 5 years from disease manifestation. The effectiveness of treatment and its course are influenced by the heterogeneity of tumour tissue, whose regulation involves lncRNAs, as shown in many studies reviewed throughout this study.

Throughout this review, a systematic analysis of lncRNA molecules has been carried out with reference to GBM. In-depth analysis of the emerging potential GBM biomarkers and their changes in the blood of patients with GBM and other diseases has been carried out. Based on a literature search, a lncRNA fingerprints of GBM is proposed, which involves ANRIL (↑), HOTAIR (↑), LINC00641 (↑), LINC00565 (↑), MALAT1 (↑), SAMMSON (↑), GAS5 (↓). Detection of these lncRNAs in the blood of GBM patients could serve for early diagnosis, and potentially help to improve the diagnosis and personalized interventions. Furthermore, given deregulated serum and plasma levels in other diseases, we suggest other lncRNAs to be investigated in the blood of GBM patients, e.g. ADAMTS9-AS2 or CASC2, to improve the use of lncRNA as GBM biomarker. Given that the detection of lncRNAs in body fluids involves relatively simple sample collection and measurement procedures, their in-depth profiling could help with early and accurate diagnosis of GBM to facilitate therapeutic interventions.

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