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Investigation of prognostic factors in gliomas

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1. Introduction

The central nervous system (CNS) comprises two primary cell types: neurons (nerve cells) and neuroglial or supportive cells. Within the neuroglial category of cells, two main subtypes are distinguished. These are the ectodermal-origin macroglia (astrocytes, oligodendrocytes, and ependymal cells) and the mesodermal-origin microglia. Astrocytes are the most prevalent glial cells in the CNS. They play a crucial role in maintaining the blood-brain barrier and regulating extracellular homeostasis. Oligodendrocytes facilitate rapid signal transmission among neurons by producing the myelin sheath. Ependymal cells line the ventricular system, participating in cerebrospinal fluid (CSF) production and circulation, while microglia serve as the resident immune cells of the CNS. From a neuro-oncological perspective, gliomas are tumors emerging from the neoplastic transformation of these neuroglial cells. Gliomas are considered primary CNS tumors, originating within the CNS itself, as opposed to secondary tumors that metastasize from other organ systems to CNS structures.

This doctoral thesis aims to investigate the prognostic factors in two major glioma subtypes: low-grade diffuse infiltrative astrocytomas and glioblastomas. Their significance lies partly in their frequency, as these tumors constitute three-quarters of all malignant CNS tumors, and partly in the limited availability of therapeutic options. Surgically, the infiltrative nature of these tumors often precludes complete resection. Oncologically, treatment efficacy is constrained by pronounced intratumoral heterogeneity and aggressive growth patterns. Although malignant CNS tumors represent only 1–2% of all malignancies, challenges related to their treatment, high morbidity rates, and impact on the working-age population impose a disproportionately large socio-economic burden. Over the past decade, significant advancements have been made in the classification of these tumor groups and the integration of prognostic factors into clinical practice. This dissertation aims to summarize the clinically relevant prognostic factors currently used in daily practice, review the associated classification changes, and elucidate the

multifaceted relationship between the characteristic diffuse infiltrative growth patterns and the extracellular matrix (ECM).

1.1. Changes in Central Nervous System Tumor Classification

In the past century, the classification of glial tumors was primarily based on the lines of histogenesis, which differentiated tumors according to their microscopic characteristics, presumed different cells of origin, and states of differentiation. The histological distinction was based on the light microscopic features of the cells, the immunohistochemical (IHC) expression of proteins related to the cells' origin, and ultrastructural properties. However, studies from the last three decades have confirmed the role of several molecular genetic alterations in the tumorigenesis of brain tumors, which have gradually become diagnostic criteria as well.

A significant turning point occurred in 2014 with the Haarlem consensus, where the century-old diagnostic principle based entirely on microscopic examination was abandoned in the context of tumor classification. The concept of integrative diagnosis was introduced, in which phenotypic and genotypic features characteristic of the tumor type became the decisive factors, aiming to create more biologically homogeneous and precisely defined diagnostic entities.

The 2016 WHO (World Health Organisation) revision marked an important distinction by designating gliomas with diffuse infiltrative characteristics, as opposed to circumscribed gliomas that do not exhibit this pattern of spread. In the past, all astrocyte-origin tumors were grouped together; following the revision, diffuse infiltrative gliomas were grouped together based not only on their growth pattern and behavior but more importantly on the common presence of genetic driver mutations in the IDH1 and IDH2 genes. According to the 2016 WHO CNS classification, the group of diffuse gliomas includes WHO grade 2 and grade 3 astrocyte-origin tumors, grade 2 and grade 3 oligodendrogliomas, grade 4 glioblastomas, and related pediatric diffuse gliomas.

The advancement of molecular diagnostics has prompted a reinterpretation of the prognostic expectations associated with the newly defined entities. The previously relevant prognostic

difference between IDH-mutant WHO grade 2 (diffuse) astrocytomas and IDH-mutant WHO grade 3 (anaplastic) astrocytomas diminished in light of recent findings. At present, diffuse infiltrative gliomas lacking high-grade features are collectively referred to as lower-grade gliomas, reflecting the difficulty of exact separation based on mitotic activity and the observation that survival parameters in subgroups defined by certain molecular alterations correlated more with tumor aggressiveness and chemo- and radiosensitivity than with the grade.

Following the 2016 revision, the classification underwent further significant changes based on the work of the consortium to inform molecular and practical approaches to CNS tumor taxonomy (cIMPACT) working groups, culminating in the 5th edition of the WHO classification. The terms “primary glioblastoma” and “secondary glioblastoma” were definitively removed; hence, glioblastoma is currently only diagnosed in the case of IDH wild-type diffuse infiltrative tumors, while IDH-mutant cases are classified as Grade 4 astrocytomas.

In summary, the current taxonomy of gliomas is guided by the 5th edition of the WHO Classification of Central Nervous System Tumors published in 2021. CNS tumor types are divided into 13 main groups, with gliomas classified alongside glioneuronal and neuronal tumors. Within the glioma group, several entities are distinguished; however, this dissertation focuses on the most common group characterized by diffuse infiltrative growth patterns: astrocytomas, oligodendrogliomas, and glioblastomas.

1.2. Epidemiology

Approximately 100,000 people worldwide are diagnosed with diffuse glioma annually. Although this number accounts for only about 2% of all cancer diagnoses, the morbidity and mortality associated with these tumors have a significant societal impact. A noteworthy epidemiological observation is that, in countries where populations of Northern European descent predominate, the incidence rates are much higher (North America, Australia, and New Zealand; incidence: 7.8–9.6 per 100,000 people) compared to regions with predominantly Asian and African populations

(Southeast Asia and India; incidence: 1.9–3.3 per 100,000 people). Notably, the incidence in Western Europe is four times higher than that in affluent East Asian countries (Western Europe: 8.5 per 100,000; Japan and Singapore: 1.9 per 100,000), suggesting that ethnic, cultural, and environmental factors may play a significant role in incidence beyond commonly cited reporting biases.

Furthermore, 27.9% of CNS tumors are considered malignant. Glioblastoma is responsible for more than 50% of all malignant lesions; overall, glial tumors constitute over 80% of malignant CNS tumors.

Regarding age-adjusted incidence rates, diffuse astrocytic and oligodendroglial tumors occur at a rate of 4.45 per 100,000 people (95% CI: 4.42–4.48), while glioblastomas have an incidence of 3.27 per 100,000 people (95% CI: 3.24–3.29). These tumors exhibit a 1.5 to 2 times higher predominance in males and are more common in individuals over 40 years of age.

In terms of survival data, the one-year and five-year survival rates are the standard metrics used in oncology practice. The median overall survival (OS) is eight months (95% CI: 8–9) for glioblastoma, whereas the best prognosis is seen in WHO grade 2 oligodendroglioma, with a median survival of 205 months (95% CI: 196–209).

1.3. Treatment

From both a clinical and a prognostic perspective, the role of surgical intervention in tumor treatment is noteworthy. According to current guidelines, if the patient's general condition and comorbidities are favorable, surgical removal should be the primary procedure of choice. The extent of radicality in surgical removal is considered a positive prognostic factor for all types of glial tumors.

In surgical management, maintaining an onco-functional balance is the most prominent guiding principle. Although the extent of resection (EOR) correlates with OS, its determination must be

preceded by a thorough risk-benefit assessment to achieve the maximal EOR with the least-possible impact on the patient's quality of life. It is known as maximal function-preserving surgery.

With regard to postoperative treatment strategies, it is difficult for clinical trials to keep fully up to date with the continuously expanding body of knowledge. In general, for lower-grade tumors with favorable prognostic markers, the preferred approach is combination chemotherapy with procarbazine, lomustine (CCNU), and vincristine—PCV—while in less favorable cases, temozolomide (TMZ)—also classified as an alkylating chemotherapeutic agent—is preferred in combination with radiotherapy. For higher-grade tumors, including glioblastomas, the treatment protocol includes concurrent chemoradiotherapy, known as the Stupp protocol.

1.4. The Extracellular Matrix

The ECM is a dynamic, constantly remodeling, complex environment surrounding the cells, playing a crucial role in the formation of tissue structure as well as in tissue functionality. The extracellular matrix should be considered a functional unit that fills the space between cells. It contributes to cell mobility by serving both as an anchoring structure and a migratory barrier—and, in some cases, by facilitating cell migration. It actively participates in signal transduction processes, including the generation of biomechanical forces and the signaling conveyed by these forces.

The ECM additionally contributes to the composition of other tissue structures; however, in the brain, its qualitative and quantitative composition differs. Quantitatively, under physiological conditions, the ECM constitutes about 20% of brain tissue volume, whereas in primary brain tumors, this volume proportion can reach up to 50%.

In diffuse infiltrative gliomas, the limitation of surgical removal is the peritumoral infiltration of tumor cells, where tumor cells can migrate several centimeters away from the primary tumor mass, infiltrating the normal brain tissue.

The extracellular matrix is involved in multiple aspects of the peritumoral invasion process. On the one hand, it participates in the formation of the glioma stem cell niche, considered one of the starting points of tumorigenesis; on the other, it mediates every step of tumor cell migration.

The phenomenon can be exemplified by the secondary structures described by Scherer in 1938, which help understand how glioma cells infiltrate the brain parenchyma of the CNS and spread along its anatomical structures. These histological structures include perineuronal and perivascular satellitosis, as well as spread along the subpial surface and white matter fiber tracts (such as the corpus callosum).

Four characteristic steps can be distinguished in the invasion process.

1. Detachment of cells from the primarily growing tumor mass

Tumor cells maintain their intercellular communication through cadherin-mediated junctions. The first step is the destabilization of these junctions, followed by changes in the adhesive properties of glioma cells through the epithelial-mesenchymal transition (EMT) described above, resulting in cells' acquisition of a more motile character.

2. Tumor cell adhesion to the ECM

Glioma cells can interact with the ECM in several different ways. This receptor–ligand binding pattern occurs between receptors on the surface of tumor cells and various ligands in the ECM. In this process, a key role is played by the integrin receptor family, which mediates binding to laminins, tenascin molecules, and fibronectins. The CD44 receptor, which binds hyaluronan—present in excessive amounts in the brain ECM—also plays a crucial role in this process.

3. Degradation of the ECM

Remodeling of the ECM is an essential part of tumor cell invasion. This enzyme-mediated process involves matrix metalloproteinases (MMPs), hyaluronidases, as well as other cathepsin-type proteases.

4. Achievement of movement through cytoskeletal contraction

Cells are able to move due to cytoskeletal contractility, which is mediated by focal adhesion kinases in the tumor cell–ECM interaction. The cell becomes polarized, and membrane protrusions form, containing actin filaments and various structural and signaling molecules. The process of cytoskeletal contraction, involving actin filaments and isoforms of the myosin II molecule, is responsible for cell motility.

1.4.1. Composition of the Extracellular Matrix

In the CNS, the molecule that represents the ECM in the largest quantity is hyaluronic acid (HA), a non-protein-bound, space-filling carbohydrate macromolecule, classified as a glycosaminoglycan (GAG). Molecules involved in HA binding include CD44 and CD168/RHAMM (receptor for hyaluronate-mediated motility). The properties of GAGs are determined by their composition, associated sulfate groups, and saccharides. Key members of this group include chondroitin sulfate, keratan sulfate, dermatan sulfate, and heparan sulfate. Their most important function lies in the structure of proteoglycans.

Proteoglycans consist of a central protein core to which various numbers and types of GAG side chains are attached. An important subgroup is the hyaladherins, which can bind HA; within this group, lecticans—proteoglycans that also bind chondroitin sulfate GAG side chains—play a prominent role. Lecticans (chondroitin sulfate proteoglycans [CSPGs]) include brevican (brain-enriched hyaluronan binding [BEHAB]), versican, neurocan, and aggrecan.

Fibrillar glycoproteins—the components of the fibrous ECM—are found in greatest amounts in the basal membranes of blood vessels, subpial, and subependymal regions. This group includes collagens, fibronectin, and laminin molecules. Notably, in other parts of the body, these fibrous

ECM elements provide the structural framework for tissues. The brain is an exception in this regard; its framework and overall soft tissue characteristics are primarily determined by HA. In astrocytomas, increasing tumor grade is associated with the elevated production of collagen, fibronectin, and laminin. Fibrillar glycoproteins play a significant role in glioma cell invasion, as indicated by their localization. Laminins are composed of three long polypeptide chains (α, β, and γ) linked by disulfide bonds and form complexes with integrins, which serve as cell surface receptors. Beyond cell adhesion, laminins participate in invasion, proliferation, and differentiation processes.

Integrins are protein-based transmembrane receptors with a dimeric structure, consisting of one α and one β subunit. A total of 8 different α and 18 β chains can combine into 24 distinct heterodimers. The dimer composition determines the binding properties of each receptor. Integrins influence the direction of cell movement, as binding to their ligands enables the formation of focal adhesion clusters, typically in regions of protrusions that define the direction of invasion.

MMPs are zinc-dependent endopeptidases and the best-known ECM-degrading proteases. Their increased expression is observed at migratory edges, and their ligands include lecticans and fibrillar glycoproteins.

2. Aims

In our research, we aimed to address the question of whether, in the case of diffuse infiltrative astrocytic tumors and glioblastoma, the expression of the relevant ECM components involved in peritumoral infiltration, as well as their corresponding cell surface receptors, and the expression pattern of this group of molecules (referred to as the invasion panel)—the *invasion spectrum*—exhibit correlation with:

1. Groups of patients with grade 2 diffuse infiltrative astrocytomas with different prognoses,
2. Groups of patients with grade 2 and grade 3 diffuse infiltrative astrocytomas with different prognoses,
3. Groups of patients with grade 3 diffuse infiltrative astrocytomas prior to oncological radiotherapy and those with recurrent tumors who have already undergone such treatment, and
4. Groups of patients with glioblastoma exhibiting different prognoses.

The ECM molecules forming the invasion panel were examined in each group comparison. These molecules were individually compared at both the gene and protein expression levels to evaluate their specific contributions to peritumoral infiltration. Furthermore, a combined evaluation of the molecules' expression (the invasion spectrum) was performed to identify characteristic expression patterns specific to each study group, thereby exploring the relationship between invasive activity and prognosis.

3. Methods

3.1. Patients and Tumor Samples

The tumor samples used in our investigations were collected during neurosurgical procedures indicated as part of the patients' treatment plans. Following surgical removal, the samples were preserved on the surface of liquid nitrogen at -196 °C, then stored in an ultra-low temperature freezer at -80 °C until their use. Immunohistochemical analyses were conducted at the University of Debrecen, Department of Pathology and Department of Anatomy, Histology and Embryology on sections prepared from formalin-fixed, paraffin-embedded tissue blocks belonging to the patients. The tissue samples, which were handled completely independently from patient care, were analyzed by an experienced neuropathologist. The Brain Tumor Bank operates with the approval of the Scientific and Research Ethics Committee (TUKEB), and all research-related examinations were performed with the written consent of the respective patients. Approval number: 51450/2015/EKU (0411/15).

Our follow-up period ranged from January 2003 to February 2020, during which treatment and follow-up for all examined patients took place at the University of Debrecen, Department of Oncology. All samples processed originated from the Brain Tumor and Tissue Bank operating at the Department of Neurosurgery.

In the examined groups, we performed a comparison of astrocytic and glioblastoma-type tumors in the histopathological sense, with the classification carried out by a trained neuropathologist according to the protocols valid at the time of diagnosis.

3.1.1. Invasion panel

At the gene expression level, we examined our invasion panel, which is composed of ECM matrix molecules involved in invasion and contains 25 ECM molecules. Within this panel, evaluable identification was achieved for 22 molecules in the grade 2 astrocytomas, 23 molecules in the grade 3 group, and 18 molecules in the glioblastoma group. Although part of the invasion panel, GFAP and MKI67 are ECM molecules that are not strictly related to invasion. The former was determined to confirm glial origin, while the latter was used to verify the presence of tumor tissue. With regard to protein expression, determinations were performed for 10 molecules in the grade 2 tumors, whereas in glioblastomas, each molecule under investigation was determined. For the grade 3 tumor group and the prognostic groups of glioblastoma, immunohistochemical staining of ECM molecules with a prominent role was performed for illustrative purposes.

3.1.2. Grade 2 Astrocytomas with Different Prognostic Groups

In the case of low-grade grade 2 diffuse astrocytomas, patient groups with differing prognoses were compared. For low-grade tumors, classification into prognostic groups was based on survival data: patients with overall survival of at least 40 months were assigned to the favorable prognostic group (N = 11), while those who died earlier were placed in the unfavorable prognostic group (N = 8). The groups were compared regarding clinical parameters including age, tumor localization and laterality, as well as the extent of radicality of the primary surgical treatment. No significant differences were found between the two groups for any of the examined parameters.

Regarding survival times, comparisons were made of the mean overall survival and progression-free survival (PFS) times. For each patient, various progression-free survival intervals observed during the disease course were determined and labeled in chronological order. These survival times were always distinguished based on progression confirmed radiologically and/or clinically that necessitated further therapeutic intervention. Comparison of the first PFS intervals (PFS I) showed no significant difference.

It should be noted that in three cases within the better prognostic group, reoperation was performed primarily for reasons affecting quality of life (epileptic seizures) rather than due to significant tumor progression. In all three cases, reoperation occurred within four months following the initial surgery, which can be considered a confounding factor regarding PFS. Accordingly, for these patients, the combined PFS I + II intervals were used as the time to first progression in our calculations. By eliminating this confounding factor, a significant difference in progression-free survival time between the two groups was demonstrated ($p = 0.02^*$). Comparison of the second PFS intervals (PFS II) was possible in seven cases between the groups, and a significant difference was also found for this parameter ($p = 0.04^*$).

Among the 25 molecules comprising the invasion panel, we performed individual comparisons of mRNA expression of 22 ECM molecules involved in invasion between the two prognostic groups. For 10 ECM molecules included in our invasion panel, immunohistochemical analysis was conducted to assess their protein expression levels. Based on the biological functions of the molecules, for 4 molecules only the extracellular matrix was evaluated, while for the remaining 6 molecules both the ECM component and tumor cells were assessed immunohistochemically.

3.1.3. Grade 3 Astrocytomas Before and After Oncotherapy

In the case of grade 3 astrocytomas, Group “A” included those cases where the tumor sample used for molecular biological analysis was removed during the first neurosurgical intervention, and no prior radio-oncological treatment had been administered (“primary group,” n = 12). Group “B” consisted of cases in which the samples were obtained during reoperation indicated due to tumor progression, and the patients had already received prior radio-oncological treatment (“pre-treated group,” n = 9). Within the pre-treated group, radiotherapy alone was administered in five cases, oncotherapy alone in one case, and combined radio-oncotherapy in three cases.

Clinico-pathological comparisons were made between the two groups regarding clinical parameters such as age, tumor localization,laterality, extent of radicality of the primary surgical

treatment, and overall survival. No statistically significant differences were found between the two groups for any of the examined parameters.

Among the 25 molecules comprising the invasion panel, individual and combined comparisons of mRNA expression of 23 ECM molecules involved in invasion were performed between the two groups.

For grade 3 astrocytic tumors, statistically evaluable immunohistochemical staining was not possible; immunohistochemistry was performed solely for illustrative purposes.

3.1.4. Prognostic Groups of Grade 2 and Grade 3 Gliomas

Through inter-grade comparison, we analyzed patients belonging to the poorer prognostic subgroup of grade 2, as well as the grade 3 patient group. The significance of this comparison lies in the prognostic evaluation of the invasion spectrum in lower-grade gliomas, specifically in assessing the applicability of the invasion spectrum for risk group stratification.

Clinical parameters compared between the two groups included age, tumor location and laterality, the extent of radicality of the primary surgical treatment, and overall survival. A statistically significant difference was found only in patient age ($p = 0.003$).

From the 25 molecules comprising the invasion panel, we conducted both individual and combined comparisons of the mRNA expression of 22 ECM molecules involved in invasion between the two groups.

3.1.5. Glioblastoma Patient Groups with Different Prognoses

The largest comparative analysis, in terms of patient numbers, was carried out for glioblastoma, with a total of 132 patient data sets included in this subgroup. Glioblastoma patients were divided into two prognostic groups based on overall survival time. Patients with survival shorter than 24 months were classified as having a "worse prognosis" and formed Group A ($n = 74$), while those

who survived 24 months or longer were considered to have a "better prognosis" and formed Group B (n = 58). The rationale for choosing the 24-month threshold for overall survival is that patients receiving bevacizumab following treatment according to the Stupp protocol typically have an average overall survival of around 24 months. This is supported by both the literature and previously published patient data analyses from our institution.

Patients underwent tumor resection followed by radiotherapy and concurrent and maintenance temozolomide chemotherapy (according to the Stupp protocol). Upon tumor recurrence, patients received bevacizumab monotherapy until further progression occurred.

In Group A, the median age of patients was 61.0 years (CI: 57.97–63.28 years), while in Group B it was 58.5 years (CI: 55.53–61.47 years). Statistical analysis revealed a significant difference between the two groups ($p = 0.0293$). Although preoperative and postoperative Karnofsky Performance Status (KPS) scores were higher in Group B, the differences in KPS values were not statistically significant. Other clinical characteristics of the tumors did not differ between the groups.

Kaplan–Meier analyses confirmed the expected differences in both progression-free survival (PFS) and overall survival (OS) between Groups A and B (Figure 6). In Group A, the median OS was 9.0 months (CI: 8.14–9.86 months), whereas in the better prognosis group, the median OS was 27.0 months (CI: 22.17–31.83 months) ($p < 0.0001$). Median PFS differences were also statistically significant: 5.5 months in Group A (CI: 3.79–7.21 months) and 8.5 months in Group B (CI: 4.36–12.64 months) ($p = 0.024$).

From the 25 molecules comprising the invasion panel, we performed both individual and combined comparisons of the mRNA expression of 16 ECM molecules involved in invasion between the two groups. Protein expression levels of these 16 ECM molecules associated with GBM invasion were also assessed using immunohistochemical staining.

3.2. Determination of mRNA expression

The transcriptional levels of ECM components were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Frozen samples were first pulverized, then homogenized at 25 °C using TRI reagent (Invitrogen; Carlsbad, CA, USA). Total RNA was isolated from the TriReagent lysates according to the manufacturer's protocol. The quantity and purity of RNA were measured using a NanoDrop® ND 1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). Subsequently, reverse transcription was performed to convert total RNA into single-stranded complementary DNA (cDNA) using a High Capacity cDNA Archive Kit in the presence of RNasin (Applied Biosystems; Thermo Fisher Scientific, Inc.). The cDNA was then loaded onto a microfluidic card (corresponding to 100 ng total RNA per port). TaqMan® low-density array experiments were carried out using an Applied Biosystems 7900HT real-time PCR system with Micro Fluidic Card upgrade (Applied Biosystems; Thermo Fisher Scientific, Inc.). The microfluidic cards were analyzed with SDS 2.1 software (Applied Biosystems; Thermo Fisher Scientific, Inc.) for relative quantification, and cycle threshold (Cq) values were exported for further analysis. β2-microglobulin, β-actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping genes showed the least variation among samples. GAPDH was used as the reference gene for calculating ΔCq values for each gene. Expression levels were determined using the comparative Cq method (ΔΔCq) as previously described.

3.3. Determination of protein expression

Protein expression was measured by semi-quantitative immunohistochemistry (IHC). Fresh-frozen samples were fixed overnight at 4°C in 4% paraformaldehyde solution and then embedded in paraffin. IHC analyses were performed in the laboratories of the Department of Pathology and the Department of Anatomy, Histology and Embryology at the University of Debrecen. Sections were first deparaffinized with xylene and then rehydrated through graded ethanol solutions.

Subsequently, heat-induced antigen retrieval was performed in citrate buffer (pH 6.0). Primary antibodies were diluted according to the manufacturer's instructions and incubated for 48 hours. For glioblastoma samples, visualization was carried out using the MACH 4 Universal AP Polymer Kit® (Biocare Medical, CA, USA) and 3,3'-diaminobenzidine (DAB), followed by hematoxylin counterstaining. Positive and negative control sections were stained alongside each series of samples.

For illustrative purposes, visualization of grade 3 samples was performed using secondary fluorescent antibodies diluted 1:500 and incubated overnight. Sections were stained with DAPI at 1:2000 dilution and mounted with Hydromount. Confocal microscopy was used for evaluation of the staining.

Semi-quantitative evaluation of IHC staining in glioblastoma samples was performed as follows: protein expression was independently assessed by three investigators in 10 randomly selected high-power fields per section. The evaluation considered both the percentage of stained cells and/or extracellular space and the staining intensity. The percentage of staining was classified as follows: negative or <10% staining (0), 10–25% staining (1), 25–50% staining (2), 50–75% staining (3), >75% staining (4), according to the method described by Bondarenko et al. Staining intensity was scored on a scale from 0 to 3 (negative: -, weak positivity: + (1), moderate positivity: ++ (2), strong positivity: +++ (3)). A combined score for each section was calculated by multiplying the percentage score by the intensity score, and an average score was determined for each sample.

3.4. Statistical methods

Statistical analysis of the data was performed with the assistance of a biomathematician. To compare clinical data of the patients, two-sample t-tests, Mann-Whitney tests, and χ^2 tests were used. Kaplan-Meier survival analysis was conducted to examine survival differences between prognostic groups.

Comparison of the entire invasion panel between groups was carried out using various statistical classifiers, including linear discriminant analysis and the nearest neighbor method. The statistical classifiers were implemented using the Weka v3.6 software (University of Waikato, Hamilton, New Zealand), while other statistical tests were performed with GraphPad Prism v8.01 (GraphPad Software, La Jolla, CA, USA).

4. Results

4.1. Grade 2 Astrocytomas with Different Prognostic Groups

4.1.1. mRNA Expression Results

Based on the individual comparison of extracellular matrix molecules, a statistically significant difference in gene expression was confirmed for six molecules. These were HMMR / CD168 ($p = 0.02$*), IDH-1 ($p = 0.009$**), laminin α-5 ($p = 0.03$*), MKI-67 ($p = 0.03$*), PDGFA ($p = 0.04$*), and versican ($p = 0.03$*).

Using a linear discriminant analysis, the expression patterns of ECM molecules characteristic of specific prognostic groups were evaluated. This analysis determined the extent to which the expression of each individually assessed ECM molecule influenced the classification toward a given prognostic group, in relation to the other molecules examined.

In order of contribution, the molecules that had the greatest impact—and, thus, enhanced the ability to distinguish between prognostic groups—were integrin β1, integrin αV, integrin β5, CSPG-5, HMMR/CD168, CD44, and EGFR.

Based on the evaluation of ECM mRNA expression using the linear discriminant analysis statistical classifier, additional derived parameters were determined. According to this analysis, the sensitivity of our prognostic classification model based on the ECM expression pattern was 87.5%, and its negative predictive value was 88.9%.

4.1.2. Protein Expression Results

Based on our results, a statistically significant difference between the two prognostic groups was observed only in the staining intensity of integrin αV in tumor cells ($p = 0.04^*$). Our findings pertaining to protein expression demonstrated concordance with gene expression levels in the following molecules: integrin αV, brevican, CSPG-5, versican, integrin β5, and CD44—all of which exhibited consistent results across all evaluated parameters. Additionally, the staining intensities of MDM2, MMP2, and FLT-4 in tumor cells were also concordant with their corresponding mRNA expression levels.

4.2. Grade 3 Astrocytomas Before and After Oncotherapy

4.2.1. mRNA Expression Results

As a result of the comparative analysis of individual ECM molecules, a statistically significant difference in gene expression between the examined patient groups was observed only for the integrin α3 molecule ($p = 0.04^*$).

Based on the statistical classification carried out using linear discriminant analysis, the molecules that provided the greatest differentiation potential, in order, were GFAP, HMMR, CD44, integrin α3, IDH-1, and integrin αV.

The sensitivity of the group classification based on the linear discriminant analysis was 85.7%, and the negative predictive value was 88.9%.

4.2.2. Protein Expression Results

In the case of grade 3 astrocytic tumors, statistically evaluable IHC staining could not be performed; IHC staining was carried out solely for illustrative purposes.

4.3. Prognostic Groups of Lower-Grade Gliomas (Grade 2, 3)

4.3.1. mRNA Expression Results

In the comparison of mRNA expression levels, a statistically significant difference was observed for CSPG-5 ($p = 0.02^*$), IDH-1 ($p = 0.01^*$), and integrin α3 ($p = 0.0003^{***}$). In all three cases, higher expression levels were found for the grade 3 group.

According to the statistical classification based on the linear discriminant analysis, the molecules providing the greatest differentiation potential, in order, were MDM2, HMMR, integrin β5, and brevican.

The distinction between astrocytomas of different grades using the invasion spectrum was found to be highly accurate, with a sensitivity of 93.7% and a specificity of 100%.

4.4. Glioblastoma Patient Groups with Different Prognoses

4.4.1. mRNA Expression Results

The comparison of glioblastoma patient groups with different prognoses revealed significant differences in mRNA expression. Our results are illustrated in Figure 8, where the individual expression values are presented on a base-10 logarithmic scale.

In the comparison of individual molecules, the expression of three invasion-related molecules was significantly different between the two prognostic groups. The expression levels of FLT4/VEGF-3, MDM2, and MMP2 were significantly higher in the samples from the poorer prognosis group ($p = 0.0285$; $p = 0.02$; and $p = 0.0023$, respectively).

After analyzing individual ECM components, we used the nearest neighbor search statistical classification method to assess the invasion panel as a whole. The classification algorithm identified the following molecules as the most important influencing factors: CD44, EGFR, FLT4/VEGF-3, IDH1, MMP2, PDGFA, TNC, and VCAN.

The expression of these ECM components may play a key role in distinguishing tumors with different prognoses. Out of 132 patients, the classifier correctly identified in 94 cases whether the patient would survive for at least 24 months. Thus, the patient's prognosis could be identified with high accuracy using this algorithm. Sensitivity and positive predictive value were higher in tumors with poorer prognosis. The method had an ROC (Receiver operating characteristic) value of 0.706, and the Matthews correlation coefficient (MCC)—which ranges from –1 to +1—was 0.414.

4.4.2. Protein Expression Results

Among the analyzed invasion-related proteins, the expression levels of brevican, CD44, HMMR, integrin αV, integrin β1, and MDM2 exhibited significant differences between the two groups.

5. Discussion

5.1. General Overview

Diffuse infiltrative astrocytomas are the most common malignant CNS tumors. Depending on the mutation status of the IDH1/2 genes, they are classified into lower-grade astrocytomas (grade 2 and grade 3) and the so-called high-grade grade 4 astrocytoma, previously considered as secondary glioblastoma. Currently, glioblastoma refers exclusively to cases with wild-type IDH1/2. Peritumoral infiltration, which is characteristic of these tumors, serves not only as a diagnostic criterion but also plays a crucial role in therapeutic strategies. From a neurosurgical perspective, complete resection of the tumor is unattainable due to the migration of tumor cells several centimeters beyond the original tumor mass. Consequently, recurrence occurs in every case.

Infiltration is a dynamic, multi-step process that occurs with the active involvement of components of the ECM, which fills the intercellular space. Quantitative and qualitative analysis of ECM molecules, and the expression patterns they exhibit in patient groups with different prognoses or treatment protocols, may offer both prognostic and therapeutic advantages for neuro-oncologists.

Considering the presence of subgroups among lower-grade tumors with differing growth potential, as well as glioblastoma subgroups characterized by unusually long survival, the identification of prognostic markers that define risk groups provides significant clinical value.

Glioblastoma is the most aggressive known human malignancy. Due to its privileged intracranial localization protected by the blood-brain barrier, both local and systemic treatment options have limited efficacy. Furthermore, pronounced intratumoral heterogeneity and clonal evolution result in the selection of therapy-resistant cell populations, posing a serious challenge.

Currently used chemotherapeutic agents primarily exert anti-proliferative effects. To date, drugs targeting tumor invasion have not demonstrated any meaningful improvement in either progression-free survival (PFS) or OS.

In light of these challenges, our research group investigated the role of ECM molecules involved in peritumoral infiltration in both lower-grade astrocytomas and glioblastoma across patient groups with different prognoses and treatment algorithms. Our aim was to assess the individual contributions and expression patterns of ECM molecules for potential prognostic use.

5.2. Distinction of Prognostic Subgroups of Grade 2 Astrocytomas Based on the Invasion Spectrum

Recently, gliomas classified as grade 2 and grade 3, including astrocytic tumors, have been collectively referred to as lower-grade gliomas. The patient group with lower-grade gliomas clearly demonstrates the limitations of classification based solely on classical histopathological features and mitotic activity, as treatment protocols may differ significantly and gliomas previously categorized as grade 2 diffuse astrocytomas can exhibit markedly different prognoses.

The importance of molecular markers is highlighted by the fact that even among histologically similar lower-grade gliomas, patients can exhibit very different PFS and OS outcomes despite receiving the same therapy. Consequently, the previously considered homogeneous group of lower-grade gliomas is currently subdivided into low- and high-risk subgroups to better inform treatment decisions.

In the case of low-grade astrocytic tumors, the determination of risk groups influences postoperative oncological management. Currently, there is no scientific consensus on the exact criteria that should guide the immediate postoperative use of radiotherapy and/or chemotherapy for low-grade glial tumors. Conversely, which low-risk patients with low proliferative potential require only close monitoring—the so-called watchful waiting strategy—is not clearly defined.

In our current study, the comparison of prognostic subgroups within grade 2 astrocytomas, based on expression pattern-based classification, yielded a sensitivity of 87.5% and a negative predictive value of 88.9%, as determined by the linear discriminant analysis. Overall, this result suggests that the invasion spectrum can serve as a suitable tool for identifying grade 2 astrocytomas associated with poor prognosis, and its routine use may serve as a complementary method for stratifying risk groups.

Additionally, several molecules demonstrated statistically significant differences between the prognostic subgroups at both the transcriptional (HMMR, versican, PDGF-A, and laminin α5) and translational levels (integrin αV). A strong concordance among our results emphasizes the individual and significant role of these molecules in the process of peritumoral infiltration (see the subchapter: *ECM Molecules with a Key Role in Infiltration*).

5.3. Limited Anti-Invasive Potential of the Treatment Strategy Applied to Diffuse Infiltrative Gliomas

In consideration of the therapeutic strategies employed for treating diffuse gliomas, the nature of current molecular targets, and their influence on survival outcomes and quality of life, it is evident that no substantial advancements in glioma treatment have been achieved since the introduction of temozolomide and bevacizumab. This stagnation is primarily attributable to the immune-privileged and anatomically protected status of the CNS.

In the case of radiotherapy, defining an appropriate target volume is particularly challenging, as infiltrating cell populations can only be visualized to a limited extent—or not at all—using standard imaging techniques. While determining the radiation dose and distribution, especially in the case of lower-grade gliomas associated with more favorable survival outcomes, the long-term effects of radiation on cognition and other higher brain functions must also be considered.

In oncotherapy, the greatest challenges are the limited CNS penetration of drugs and the selection of therapy-resistant glioma cell subpopulations through mutational mechanisms.

From our study of grade 3 astrocytomas, it is clear that while the separation of patient groups based on ECM expression profiles is reasonably effective, a closer evaluation reveals that integrin α3 is the only molecule that exhibited a statistically significant difference between the two groups. Furthermore, even the fold change differences among non-significant expression values do not indicate meaningful expression variation. In most cases, higher expression levels were observed in the treatment-exposed group.

These results support the hypothesis that radiotherapy and antiproliferative chemotherapy have little to no significant impact on the invasive potential of gliomas.

5.4. Overlap of Grade 2 and Grade 3 Diffuse Infiltrative Astrocytomas—the Inability of Current Grading Alone to Provide Complete Prognostic Information

The significance of lower-grade gliomas has already been discussed. The term was introduced to group together tumors that were previously classified separately as grade 2 and grade 3, as these tumors, while different in their histological appearance, display similar biological and clinical behavior. Therefore, they are often treated using the same strategies. Our study groups were defined based on this idea, comparing tumors of different grades.

However, based on the invasion spectrum, the two groups do not appear to be clearly distinct. Nevertheless, while considering individual molecules, the ones showing the most noticeable differences in expression were CSPG-5 and integrin α3, in addition to the MKI67 marker, which indicates mitotic activity. These molecules exhibited higher expression in the higher-grade tumor samples.

Other than these findings, our comparisons did not show any significant difference between the two groups. This result suggests that tumor grade alone may not provide sufficient information to fully predict the behavior or outcome of diffuse astrocytomas.

5.5. The invasion spectrum as an Effective Prognostic Marker in Patients with Glioblastoma

It has been established that the components of the ECM show differential expressions at both the transcriptional and translational levels in the case of glioblastoma. The mRNA expression of the FLT4, MDM2, and MMP-2 genes differed significantly between the two groups; furthermore, for MDM2, a significant difference was confirmed at the protein expression level. FLT4 and MMP-2 were selected as key molecules by the statistical classifier for distinguishing between different prognostic groups.

A statistical classification algorithm based on the nearest neighbor method was able to identify the prognostic groups of individual samples based on the invasion spectrum. The method accurately determined whether a patient belongs to the poorer or better prognosis group. Clinically, this process is of great importance, as it enables access to individual patient survival information practically simultaneously with the histological diagnosis. The higher positive predictive value observed in patients with poorer prognosis helps identify those whose treatment needs to be intensified using the available methods. Our results also provide supplementary information on the molecular composition of glioblastomas, which may enable the selection of appropriate anti-invasive therapeutic agents in the future.

5.6. ECM Molecules of Highlighted Importance in Infiltration, Potential Therapeutic Targets, and Their Characteristics

The issue of invasiveness and the available information on specific ECM molecules mostly pertain to glioblastoma. Through our results, we aimed to expand the amount of available information on certain characteristic molecules within the context of lower-grade gliomas, while also identifying their potential use as therapeutic targets.
Below, we aim to elaborate on the role of ECM molecules that were consistently identified as relevant within the examined groups:

The role of the integrin molecule family in peritumoral invasion has long been a focal point of scientific interest. Integrins are transmembrane cell surface receptors belonging to the glycoprotein family, with a heterodimeric structure composed of alpha and beta subunits. These subunits determine the receptor's specificity toward its ligands. Based on ligand-binding characteristics, integrins can be categorized as collagen-, laminin-, or RGD (arginine-glycine-aspartate) sequence-binding integrins, as well as leukocyte receptors. Although integrins do not possess intrinsic enzymatic activity, they can activate downstream signaling pathways through focal adhesion kinases (FAK).

A key aspect of invasion is the interaction between the tumor cell, the tumor microenvironment, and the stromal components of the extracellular matrix—an interaction that integrins support in multiple ways. By activating signaling pathways, they transmit angiogenic and proliferative signals and serve as physical anchoring points.

Among the eight known beta subunits, the beta-1 subunit is considered one of the most significant. It can form heterodimers with several alpha subunits in the perivascular space. In vitro experiments have shown that neutralizing antibodies targeting this subunit alone can reduce invasive potential and may also enhance the effect of anti-angiogenic therapy, such as

bevacizumab, used in clinical practice. Our study clearly confirmed the prognostic role of this molecule, as its expression primarily aided in identifying patient groups with poorer prognoses.

Integrin αV, which can dimerize by recognizing the RGD sequence, is another important subunit. Its ligands include fibronectin, fibrinogen, and tenascin, among others. The role of the alpha-V subunit has been detailed mainly in high-grade gliomas. This is evidenced by the development of cilengitide, a specific neutralizing antibody targeting αvβ3 and αvβ5 subunits, which has been the subject of multiple studies. While it has not yet been adopted into everyday clinical practice, neutralizing this subunit continues to attract scientific interest. In our current study, the alpha-V subunit played a significant role in distinguishing between the three analyzed groups. Our results support the importance of integrin αV in influencing peritumoral infiltration in lower-grade gliomas as well, positioning it as a promising prognostic and anti-invasive molecular target even in this less-studied patient group.

Another notable member of the integrin family is integrin α-3 (ITGA3), whose role in invasion is supported by its elevated expression in glioma stem-like cells (GSCs). A study conducted on glioblastoma cell lines revealed increased ITGA3 expression in areas around blood vessels, which are considered stem cell niches. It was also overrepresented in infiltrative tumor cells, and ITGA3 expression correlated with the invasive potential of these cells.

In glial-origin tumors, hyaluronic acid—a member of the glycosaminoglycan family—plays a significant role in the invasion process. In neoplastic cases, its quantity is elevated compared to that in the normal brain extracellular matrix, and its expression correlates with tumor grade. Hyaluronic acid binds to the HMMR (CD168) and CD44 molecules. The majority of available studies have focused on higher-grade glial tumors; however, analyses utilizing large datasets that include cases across all tumor grades (e.g., The Cancer Genome Atlas – TCGA) have confirmed the prognostic roles of CD168 and CD44. Our own findings have reinforced the prominent role of these molecules in infiltration processes even in lower-grade glial tumors.

Based on our results, FLT4, MDM2, and MMP2 also emerge as noteworthy molecules with prominent relevance to peritumoral infiltration.

FLT4 is a receptor for vascular endothelial growth factor (VEGF) types C and D. It is not normally expressed in the endothelium of the human brain, but has been shown to be expressed in the endothelium of blood vessels within glioblastomas. This may contribute to the “escape” phenomenon observed in patients treated with bevacizumab, whereby secondary neovascularization occurs despite anti-angiogenic therapy.

MDM2 is an inhibitor of the p53 protein. Elevated levels of MDM2 represent one potential mechanism by which the gatekeeping function of p53 is bypassed in TP53 wild-type glioblastomas. Additionally, MDM2 has p53-independent roles. It induces genomic instability, likely by inhibiting DNA damage repair and suppressing cell cycle arrest. It also has a proven role in promoting epithelial-mesenchymal transition (EMT), thereby increasing cell motility and tumor invasiveness.

MMP-2 plays a well-established role in the dynamic remodeling of the tumor extracellular matrix and contributes to other oncogenic functions that facilitate the invasion of glioma cells into the brain parenchyma.

6. Summary

Astrocytic tumors are the most common malignant tumors of the CNS. Curative treatment is currently unachievable due to peritumoral infiltration, which hinders complete surgical removal, and due to the limited effectiveness—by several measures—of current radiotherapy and chemotherapy treatments. Recently introduced molecular genetic diagnostic approaches offer prognostic and predictive advantages for neuro-oncologists. However, identifying risk groups associated with different treatment protocols remains challenging.

Our research group aimed to support the identification of such risk groups by evaluating the expression patterns of ECM molecules involved in the phenomenon of peritumoral infiltration. It was done by examining the molecular profiles of astrocytic tumor subgroups with varying levels of aggressiveness, prognosis, and prior therapeutic protocols.

Based on our findings, it can be concluded that, by considering the expression levels of specific ECM molecules, prognostic subgroups within both glioblastomas and grade 2 diffuse infiltrative astrocytomas can be effectively distinguished. Furthermore, it is evident that the current grading system does not sufficiently reflect the differences between grade 2 and grade 3 astrocytic tumors within the lower-grade glioma group. Additionally, current treatment protocols lack substantial anti-invasive potential.

7. Novel Scientific Findings

1. Subgroups of grade 2 astrocytomas with differing prognoses can be distinguished based on the invasive spectrum of ECM molecules. Evaluating the invasive spectrum aids in identifying intra-grade risk groups.
2. The treatment strategy currently applied to diffuse infiltrative gliomas has no significant effect on the expression of ECM molecules, indicating that it possesses limited anti-invasive potential.
3. Grade 2 and grade 3 diffuse infiltrative gliomas demonstrate considerable overlap. The currently used grading system alone does not provide comprehensive prognostic information; introducing the concept of “lower-grade glioma” into everyday clinical practice is recommended.
4. The invasive spectrum serves as an effective prognostic marker in glioblastoma patients and may support the identification of glioblastoma subgroups associated with longer survival outcomes.
5. Extracellular matrix molecules belonging to the invasion panel play a crucial role in the process of peritumoral infiltration not only in higher-grade gliomas but also in lower-grade gliomas (e.g., integrin α3, αV, β1, MMP2).

8. Keywords

Lower grade glioma, astrocytoma, glioblastoma, prognosis, risk groups, peritumoral infiltration, extracellular matrix, invasion spectrum

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List of publications related to the dissertation

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