

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Analysis of the Extracellular Matrix Composition in Various Grades of Astrocytoma

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The Examination takes place at the library of Department of Neurosurgery, Faculty of Medicine, University of Debrecen , at 11:00 AM, 01 June 2018.

Head of the **Defense Committee:** Antal, Miklós, MD, PhD, DSc

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The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 1:30 PM, 01 June 2018.

1. INTRODUCTION

Neoplastic diseases of the central nervous system (CNS) can be classified into two major groups based upon their origin. Primary tumors are those that originate from the CNS itself, whereas secondary tumors are metastases of other types of tumors in the CNS. The most common primary malignant CNS tumors are gliomas, approximately 75% of all CNS malignancies are of glial origin. Astrocytomas contribute to an outstanding proportion within this group, as almost 2/3 of all primary malignant brain tumors are astrocytomas. Incidence and other epidemiologic characteristics of the patients depends on the histologic type. The cumulative incidence of all astrocytoma is 4.5/100 000, low grade astrocytomas are more common among younger patients, while high grade gliomas mostly present in later ages (median age at diagnosis for grade III glioma is 53 years, and 64 years for grade IV). The importance of gliomas does not come from their incidence – even though the relative incidence is high – but it originates from other characteristics of the disease. Astrocytomas do not have early or specific symptoms, therefore they are usually diagnosed rather late. The vast majority of tumors show a diffuse growth pattern, thus surgical removal is extremely difficult, especially in case of high grade tumors. Besides, the tumor cells show only a limited sensitivity to radio- and chemotherapy, so oncologic treatment is also a challenge. It is important to note that a significant amount of the patients is still at working age, so patient outcome in astrocytomas is of concern not only for medical providers, but is relevant from a social-economic point of view as well.

This PhD thesis aims to study the most common and the most malignant primary malignant brain tumor, called glioblastoma (GBM). Treatment of glioblastoma patients has undergone major development. The Stupp protocol (surgical resection of the tumors followed by concurring temozolomide chemo- and radiotherapy) has improved patient survival. Further survival benefit might be gained from bevacizumab, a VEGF inhibitor antibody, which can be

given after tumor recurrence whilst on temozolomide therapy, in Hungary. These therapies has significantly lengthened previous progression free and overall survival times, however, GBM remained a challenging disease both for the patients and their families, as well as health care system caring for them, negatively influencing the quality of life of GBM patients and today, unfortunately, inevitably leading to the death of those affected by the disease.

However, there is a notable heterogeneity – even in neoplastic standards – among GBM patients, certain group of patients respond to treatment much better than others, their progression free and overall survival is well above average. Yet, there is no reliable method in clinical use to assess disease prognosis. Some factors are known to be associated with a more favorable outcome, but these are not really usable for individual cases. These favorable prognostic markers include a younger age, male gender, a higher Karnofsky Performance Scale point, and a smaller tumor size. In everyday clinical practice, these are of limited benefit. In the past years, a considerable amount of information has been gathered about astrocytoma, especially glioblastoma molecular oncology which led to changing the classification of gliomas. The IDH mutation status of the tumors is of outstanding importance, as it is well known how greatly and positively it influences prognosis. The frequent hypermethylation of the MGMT gene promotor is also noteworthy, as it leads to gene silencing, and thus to increased temozolomide treatment efficacy. MGMT methylation status is therefore more of a predictive marker. The numerous described mutations of other genes and signaling pathways, however, as of today, seems to remain clinically less relevant, unfortunately.

1.1. Extracellular matrix in the central nervous system and its connection to glioma invasion

The extensive invasion capacity of tumor cells contributes greatly – besides the limited radio-chemosensitivity – to the aggressive clinical behavior of GBM. The source of this invasive character of tumor cells lies mostly in tumor extracellular matrix (ECM). Changes in ECM quantity and quality are well known in many types of tumors, including GBM, and contribute to the development of a *pro-cancer niche*, in which astrocytoma cells can infiltrate the neighboring brain tissue.

Neurons, glial cells and blood vessels make up the bulk of CNS volume. Extracellular space is only approximately 15-25% of the total volume, and contains ECM composing molecules, ions, cytokines, growth factors, neurohormones, and metabolites. ECM composition differs greatly in brain compared to other parts of the human body. The major ECM component in CNS is hyaluronic acid (HA). HA is a giant, space-filling glucose-amino-glycan (GAG). It binds to the surface receptors CD44 and CD168/RHAMM and greatly defines tissue structure, while also playing a regulative role in many cellular processes (cell division, adhesion, motility). Protein-bound carbohydrates, i.e. proteoglycans are abundant in the brain. Heparan-sulphate proteoglycans (HSPGs), for example syndecan, is present in large quantities. Another large group of proteoglycans, chondroitin-sulphate proteoglycans (CSPGs) are usually expressed during brain development and regeneration. Aggrecan, versican, neurocan or brevican belong to this group. Their main role is to bind HA and other ECM components. Tenascins are also notable proteoglycans in the brain, which play a role in cell division, migration, and morphogenesis. Fibrillary ECM components, however, are much less frequent in the brain. These include collagens, laminin and fibronectin, and they bind to transmembrane receptors via integrins. Unlike elsewhere, the structural background of ECM and the tissue itself is not made up of collagens and other fibrillary proteins but of mostly HA. Besides these components, there

are enzymes, like matrix metalloproteinases and ADAMTSs present in the ECM which are responsible for the dynamic changes of ECM.

Today, it is known that ECM is not only a major determiner of tissue morphology, but it is a surprisingly dynamically changing and versatile component of the extracellular compartment, affecting many aspects of cellular function. ECM therefore not only fills the extracellular space, but plays a role in many cellular processes, including cell division, survival, adhesion, or migration. Besides, it also functions as some sort of depot for growth factors and cytokines, influencing cell function further. Because of these mentioned reasons, extracellular matrix literature today deals with the matrix present in the extracellular space as a functional unit, rather than the classical, descriptive, 'sum of all components' approach. Thus this functional extracellular matrix includes not only the GAGs, PGs and fibrillary proteins and ECM enzymes present, but the receptors on the cellular surfaces that bind them. Even more, there are articles regarding the connection of tumor invasion and ECM published which include the cytokines and growth factors stored in the ECM.

The altered ECM creates a niche that is fit for cancer stem cells (CSC). To our best knowledge, these cells are responsible for the development and sustenance of gliomas, by e.g. anchoring the CSCs and creating a special, so called pro cancer niche, polarizing cells and ensuring asymmetrical cell division. Some components of ECM are certainly connected to the invasion capacity of tumor cells, too. These are not only present in a greater amount than normal, contributing to a pro-invasive character of the tumor, but play a modulating role, too. It is, therefore, clear, that there is a cross-talk between tumor cells, tumor ECM and non-tumor cells and ECM, which cross-talk helps tumor cells to modify how non-tumor cells work to provide a pro-invasive compartment.

Peritumoral infiltration, however, is not only observed in GBM, but in other, lower grade astrocytomas, too. For this reason, tumor samples of various grades of astrocytomas were included in our research, analyzing their composition according to different approaches. The amount of ECM components influencing glioma invasion was then measured not only in astrocytomas, but in order to translate results correctly, non-tumor brain tissue was also used as controls, as well as metastatic brain tumor tissues, to compare results with other malignant neoplastic tumors of different histologic origin. The interpretation of results has been driven by the principal of clinical usefulness; methods of molecular biology were used to answer questions of clinical value, so the research that has been done belongs to the field of translational medicine and aims to bridge basic medical sciences and clinical medicine.

2. AIMS

The aim of this research was to examine the changes in the quantity of ECM components in various grades of astrocytoma based upon the role of extracellular matrix, mostly described in glioblastoma. It was analyzed, whether there is a correlation between the expression pattern (i.e. the invasion spectrum) of invasion-related ECM components (i.e. the invasion panel) and the invasiveness of various histological classes. It was also tested, whether the invasion spectrum shows any correlation with astrocytoma grade. To answer these questions, the amount and expression pattern of ECM components were researched in various grades of astrocytoma, as well as primary and secondary brain tumor samples. Furthermore, it was studied, whether ECM composition and the invasion spectrum are connected to patient outcome in case of a given grade, hence bearing prognostic relevance. This question was answered by investigating correlations between expression data and clinical factors.

3. METHODS

3.1. Patients and tumor samples

During the research, intraoperatively taken flash-frozen tumor samples from the Brain Tumor and Tissue Bank at the Department of Neurosurgery, University of Debrecen Clinical Center were used and analyzed using methods of molecular biology, along with flash-frozen non-tumor samples for controls. Immunohistochemistry staining was done on formaline fixed, paraffine embed tissue samples of the same patient, collected from the archives of the Department of Pathology, University of Debrecen. The research was authorized by the National Research Ethical Committee (Authorization No. 514550-2/2015/EKU), each patient has signed and informed consent form, stating that the tumor- and brain tissues removed can be collected, stored and used for research. Samples were evaluated by a neuropathologist in each case to provide accurate histologic diagnoses.

3.1.1. Analysis of the ECM composition in CNS malignancies of different origin

The extracellular matrix analysis of primary and secondary malignant tumors was done in 41 GBM and 22 non-small cell lung cancer (NSCLC) brain metastasis samples, along with 63 non-tumor control brain tissue. Tissues were analyzed by a neuropathologist to confirm the original diagnosis. The mRNA expression of a total of 40 ECM components was measured, protein measurements were done for 20 ECM components. Sufficient amount of tumor cells in the samples was confirmed by measuring Ki-67 expression in each sample.

3.1.2. Analysis of the ECM composition in various grades of astrocytoma

Changes in the molecules of the invasion panel in astrocytoma samples with varying degree of invasiveness, i.e. astrocytomas of various grades were compared in 94 WHO grade I-II-III-IV astrocytoma samples and 54 non-tumor brain tissue control. 20 ECM components were

measured both on transcriptional and translational level. The ECM components were selected after thorough study of literature and preliminary data of the research group. To confirm glial origin of the tumor, GFAP gene expression was measured. Ki67 expression was measured to confirm sufficient amount of malignant cells in the samples.

3.1.3. Analysis of the ECM composition in glioblastoma samples with different prognosis

To study the role of ECM molecules in prognosis, 26 GBM samples were selected. 20 ECM molecules were measured in each samples. Patients had received the same treatment protocol, i.e. at least subtotal tumor resection, followed by concurring chemo-irradiation, followed by temozolomide monotherapy, according to the Stupp protocol. Patients had been given bevacizumab monotherapy if tumor progression while on chemotherapy had occurred, as long as patient status had allowed so. Samples had been acquired from the tumor mass removed during first surgeries. Mean age of patients was 58.69 ± 8.01 years (min: 43 years, max: 75 years). Patients were divided in two groups using overall survival (OS) data, patients with an OS of 23 months or shorter were sorted into group A (n=12), patients with an OS longer than 23 months were put into group B (n=14). 23 months as a selection parameter was decided upon literature data and mean survival time in departmental data. A neuropathologist confirmed the diagnosis in each case, glial origin was verified by GFAP expression and sufficient amount of malignant cells was double-checked by Ki67 gene expression.

3.2. Measuring mRNA expression

The mRNA expression level of the ECM components was determined through real-time quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR). Flash-frozen tissue samples were first pulverized and then homogenized using TriReagent (Invitrogen, USA). Total RNA was isolated from TriReagent lysates according to the manufacturer’s instructions. A NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, USA) was used to

measure the quantity and purity of RNA. In the next step, reverse transcription was performed to convert total RNA to single-stranded cDNA with the help of a High-Capacity cDNA Archive Kit with RNasin (Applied Biosystems, USA). The cDNA was then loaded onto a microfluidic card (cDNA from 100 ng of total RNA per port). An Applied Biosystems 7900HT real-time PCR system with Micro Fluidic Card upgrade (Applied Biosystems, USA) was used to perform TaqMan low-density array (TLDA) experiments. The Micro Fluidic Cards were analyzed with SDS 2.1 software as relative quantification studies, and the C_t (Cycle threshold) values were exported for further analysis. β -actin and glyceraldehyde 3-phosphate dehydrogenase housekeeping genes were used as inner standard, GAPDH was used as reference genes to calculate the dC_t value for each gene. Expression values were calculated using the comparative C_t method.

3.3. Measuring protein levels

Concentrations of the transcribed proteins were measured with a mass spectrometer to uncover expressional changes as described previously. Tissue homogenization for protein analysis was performed as described in the case of RNA purification. However, a lysis buffer containing 50 mM Tris, 1 mM EDTA, 17 mM beta-mercaptoethanol, and 0.5% Triton-X100™ was used in this case for tissue lysis. The protein content was measured using the Bradford method, and equal amounts of proteins were used for in-solution trypsin digestion. The selected reaction monitoring (SRM)-based targeted proteomic method was developed for relative protein amount determination. For protein concentration estimation, the area under the curve of the acquired spectra was calculated, and the SRM spectra were used for AUC calculations in which the intensity of the signal exceeds 500 cps. Data integration was conducted with the help of the Analyst 1.4.2 software (Applied Biosystems; Carlsbad, CA, USA) based on the curve shape determined from pilot analyses

3.4. Immunohistochemistry

Immunohistochemistry staining was used to study IDH1 mutation status and Ki67 protein expression during the analysis of correlation between ECM components and prognosis. The 4- μ m-thick sections from flash-frozen sample corresponded formalin-fixed, paraffin-embedded blocks were stained for the R132H mutant specific IDH1 mouse monoclonal antibody (DIA H09) (Dianova, Hamburg, Germany) and Ki-67/MIB-1 mouse monoclonal antibody (M7240) (Dako, Glostrup, Denmark) according to the manufacturer's instructions, using a 1:50 and 1:200 dilution for IDH1 and Ki-67, respectively.

3.5. Statistical analysis

The mRNA and protein measurement data, as well as the clinical data of patients were analyzed using various statistical methods. To compare the amount of each ECM component, two samples t-test, Mann-Whitney test (in case of significantly different standard deviation) or one-way ANOVA (in case of 3 or more groups) were used. Statistical classifiers (linear discriminant analysis, nearest neighbor search, J48 pruned tree and locally weighted learning) were used to compare the invasion panel as a whole. Patients' clinical data was compared using two samples t-test, Mann-Whitney test, and χ^2 -test, while Kaplan-Meier survival analysis was performed to compare survival data of patient groups with different prognosis. Statistical classification was done using Weka v3.6 statistical program (University of Waikato, Hamilton, New-Zealand), all the other statistical testing was performed using GraphPad Prism v7.00 (GraphPad Software, La Jolla, CA, USA) statistical program.

4. RESULTS

4.1. Comparing the ECM composition of primary and secondary malignant brain tumors

4.1.1. Results of mRNA expression measurements

The amount of invasion-related ECM molecules differs significantly in tumor and non-tumor samples. Non-tumor brain tissue and GBM samples expressed CD44, cadherin-N, cadherin-N2, collagen I α 1, III α 1, IV α 1 and VI α 1, EGFR, ErbB4, fibronectin, hyaluronan synthase-1 and -2, HMMR (CD168), integrin- α 9, - β 1 and - β 3, laminin- α 4, - β 1 and - β 2, MMP-2 and -9, perlecan, tenascin-C and -R mRNA in significantly different amounts. NSCLC brain metastasis samples showed significant differences in the expression of agrin, brevican, cadherin-N, cadherin-P, collagen I α 1, III α 1, IV α 1 and VI α 1, ErbB4, fibronectin, hyaluronan synthase-1, HMMR (CD168), integrin- α 5, - α 11, - β 1 and - β 3, laminin- β 2, matrilin-2, neurocan, neuroglycan-C, perlecan, syndecan-1 and -4, as well as tenascin-R mRNA compared to non-tumor brain. Primary and secondary brain tumors not only differed from non-tumor brain tissue greatly, but from each other, too. Expression levels of agrin, brevican, CD44, cadherin-N2, cadherin-P, EGFR, integrin- α 5, - α 9 and - α 11, matrilin-1, MMP-9, neurocan, neuroglycan-C, syndecan-1, -3 and -4, along with tenascin-C mRNA revealed significant differences in tumor samples of different origin.

The mRNA expression pattern separates the three groups from one another well using statistical classifiers. Linear discriminant analysis marked cadherin-N, collagene IV, ErbB2, hyaluronan synthase-2, integrin α 3, - α 5 and - α 9, MMP-9, and syndecan molecules as key points of group separation. After cross-validation, the method identified 92.3% of all samples.

4.1.2. Results of protein expression measurements

Non-tumor brain and GBM differed significantly in the protein level of EGFR, ErbB2, integrin- β 1, laminin- α 4 and - β 1, MMP-2 and -9. Metastatic tumor samples differed significantly from non-tumor brain only in the amounts of integrin- α 7 protein. Primary and secondary brain tumors expressed significantly different amounts of integrin- α 7 and - β 1, MMP-9 and neurocan. Protein expression studies partially confirmed results of mRNA measurements. Data from mRNA and protein expression studies proved to be concordant during post hoc analyses. Both the transcriptional and translational measurements revealed differences above the level of significance in case of seven components altogether (EGFR, integrin β -1, laminin α -4 and β -1, MMP-2 and -9, as well as neurocan).

Protein expression based invasion spectra were also analyzed by statistical classifiers. 84.7% of all samples was correctly identified by the method, positive predictive value was 0.843. Key molecules of the classifier included Erb-B1, Erb-B3, integrin- α 2, - α 3 and - β 1, laminin- α 1 and - α 4, MMP-2 and MMP-9, along with tenascin-R.

4.2. Comparing the ECM composition of various grades of astrocytomas

4.2.1. Results of mRNA expression measurements

The amount of invasion-related extracellular matrix components did not show any statistically verifiable difference when compared individually. The expression pattern of the molecules in the invasion panel – i.e. the invasion spectrum – did reveal marked differences among distinct grades. The differences in the expression pattern among the grades were found to be great enough so that linear discriminant analysis (LDA) could separate the grades. The expression pattern of non-tumor brain tissue was found to be especially distinct from astrocytoma samples. Furthermore, among astrocytoma samples, the invasion spectrum of glioblastoma samples exhibited striking differences compared to other grades of astrocytoma. LDA marked the

following molecules as key molecules during the analysis: brevican, cadherin-N2, fibronectin and integrin- β 1. LDA performed with two neighboring grade, given the characteristics of the analysis, resulted in the better separation of groups. These two-group LDAs implied the key role of more molecules, highlighting differences between two neighboring grade. mRNA expression pattern allowed LDA to classify samples into one of the five groups. The accuracy of the classification was 50.8% if all studied groups (non-tumor brain and WHO grade I-II-III-IV astrocytomas) were included, two-group LDAs performed with an accuracy of 80% or above. This most likely resulted from the general properties of statistical classifiers, i.e. an increase in the number of groups to be classified always reduces accuracy.

4.2.2. Results of protein expression measurements

Protein expression studies found no statistically verified differences in the amount of individual molecules. Expression pattern, however, was found to be distinct, linear discriminant analysis separated the analyzed groups based upon the expression of the molecules in the invasion panel, much the same as the mRNA invasion spectrum. LDA of all the groups classified samples with an accuracy of 29.2%, key molecules of the classification included brevican, ErbB2, HMR (CD168), integrin- α 1, laminin- α 4 and - β 1, MMP-2, MMP-9, and syndecan. Proteomic analysis of grade III samples resulted in non-reproducible measurements, therefore we opted these out of the LDA, which limits the interpretation of the results from LDA of all samples. Two-group LDAs performed very well, nonetheless, the accuracy of the classification was at least as high as those of the mRNA invasion spectra.

4.3. Comparing the ECM composition of grade IV astrocytoma (glioblastoma) samples with different prognosis

4.3.1. Results of patient characteristics analysis

Progression free survival (PFS) in the patient group with worse prognosis (Group A) was 6.0 ± 5.7 months, whereas PFS in Group B was 14.6 ± 9.8 months. Mean overall survival (OS) in Group A was 13.4 ± 8.3 months, and 35.7 ± 13.3 months in Group B. Both PFS and OS was significantly different (PFS $p=0,04$, OS $p<0,001$). Mean age of patients did not differ significantly in the two prognostic group (61.3 ± 5.88 vs 56.43 ± 9.06 years, $p=0,12$). Homogeneity analysis of the patient groups revealed no statistical differences in terms of lobular localization or lateralization of the tumor. ($p=0.52$ and $p=0.92$, respectively). Mean tumor size was found to be similar as well (49.3 ± 20.8 vs. 43.53 ± 17.7 mm, $p=0.42$). Rate of reoperations due to tumor recurrence was found to be statistically indifferent ($7/12$ v $12/14$, $p=0.13$). These results show that patients in different prognostic groups do not differ from each other statistically, the only difference is in survival times which were used as the selection criteria and cannot be explained by differences in clinical characteristics of the patients.

4.3.2. Results of immunohistochemistry staining

IDH1 mutation, a favourable prognostic marker, was identified in 25% and 28.5% of the samples in Group A and Group B, respectively. The proportion of IDH1-positive samples did not differ significantly in the two groups ($p=0.84$). The Ki-67/MIB1 labeling index (LI) was also determined. Average LI was 12.5 ± 6.09 for Group A and 18.61 ± 15.11 for Group B; the difference is not statistically different ($p=0.20$).

4.3.3. Results of mRNA expression measurements

The amounts of the 20 measured ECM components did not show significant differences in the tumor samples. Certain molecules (e.g. brevican, collagen III, EGFR, MMP-2, neurocan, or tenascin-C) appeared to show marked differences but these were found to be statistically insignificant. On the other hand, small individual differences added up and resulted in significant differences between the two groups. Statistical classifier J48 pruned tree analyzed the invasion spectra of samples and was able to separate them according to prognostic groups. The method identified 84.6% of the samples correctly, performed better in the group with better prognosis, but the positive predictive value was higher (0.9) in the prognostic group with shorter survival, underscoring the clinical significance of the method. Brevican and integrin- β 1 were selected as key molecules for separation. The first decision on the decision tree was done using integrin- β 1 levels, so it seems the role of this molecule might be more important.

4.3.4. Results of protein expression measurements

Protein level analysis of molecules in the invasion panel resulted in findings similar to those of the mRNA panel. Protein amounts were indifferent when the ECM components were analyzed individually, the expression pattern, however, showed characteristic differences. Brevican, EGFR and tenascin-C showed greater differences, findings were concordant with mRNA results. Cadherin-N2, as well as laminin- α 4 and - β 1 were also found to be markedly different, but these findings were not found during mRNA measurements. The classifier was also able to differentiate based upon the protein invasion spectra with good accuracy. 85.7% of the samples were identified correctly, the positive predictive value was again higher in Group A, whereas sensitivity was higher in Group B.

5. DISCUSSION

Astrocytomas are the most common central nervous system malignancies. Patient care is a challenge for the medical team providing treatment, which roots in, besides limited radio- and chemosensitivity, the invasiveness of tumor cells. This capacity to invade peritumoral brain and grow diffusely is so important that the newest WHO classification categorizes astrocytomas (and the much alike oligodendrogliomas) based upon the growth pattern and not the original cell phenotype. Research focusing on the peritumoral infiltration of astrocytomas center around the extracellular matrix. Alteration in the quantity and quality in the molecules of the ECM and in the cell-surface receptors binding these molecules are well described in many types of tumors, while more and more is known in case of gliomas. The aim of this research was to analyze the invasion-related ECM molecules in order to better understand what lies in the background of invasiveness of astrocytomas, especially high grade astrocytomas. The expression pattern (i.e. invasion spectrum) of the invasion-related ECM molecules (i.e. invasion panel) was studied in order to find out how ECM changes in astrocytomas, to see if there are certain molecules that are mainly responsible for the invasiveness of gliomas, and to understand differences in the different invasive potential in low and high grade gliomas. It was tested if the invasion spectrum can be used in clinical practice, besides describing ECM composition in tumors, whether it correlates with histological diagnosis or patient outcome, for example.

WHO grade I-II-III-IV astrocytoma samples, as well as NSCLC brain metastasis samples and non-tumor brain tissue controls were analyzed with methods of molecular biology to answer these questions.

5.1. The invasion spectrum in primary and secondary malignant brain tumors shows marked differences

Findings strongly suggest that the composition of the extracellular matrix, especially amounts of invasion-related ECM components is not mainly dependent on tumor dignity, albeit correlates with malignancy, of course. CNS tumors that are both malignant but originate from different tissues were found to have very different composition of ECM, and both tumors showed major differences compared to non-tumor brain. Glioblastoma and metastatic samples both expressed 24-24 molecules, a total of 34 ECM components in a different amount than non-tumor brain on transcriptional level. There were nine ECM components whose altered and statistically significant mRNA expression was followed by a congruent change in protein levels, even more, in four cases (EGFR, laminin- α 4, - β 1, MMP-2) even protein level changes were found to be significant. Significant mRNA changes were followed by congruent (but nonsignificant) changes in protein levels in case of three ECM components in metastatic samples. GBM and metastatic samples expressed 17 ECM molecule mRNA statistically significant, identical protein changes followed in case of six components, which were significant in only one protein (neurocan). The finding that tumor and non-tumor ECM in the brain differ from each other is not surprising, however our findings clearly show that metastatic and glioblastoma samples differ from non-tumor brain in different ECM components. Our results also reveal that the invasion spectra of malignant primary and secondary brain tumors differs greatly. There are differences in amount the molecules and receptors binding the cells to the matrix and in the types of the interlinking molecules of the matrix itself. These alterations are so characteristic that it was possible to identify the histologic group of the sample using the invasion spectrum alone.

5.2. The expression pattern of ECM components in tumors of the same histologic group with a different grade display characteristic differences

Differences in the ECM composition of tumors of the same histologic group (i.e. astrocytoma) with different grade, on the other hand, are not so striking. Even though the amount of most invasion panel molecules is different, the variations were found to be insignificant statistically. This is in accordance with the concept that views astrocytomas, especially diffusely growing astrocytomas as entities on the same spectrum of disease, glioblastoma being one end-point and diffuse astrocytoma being the other one; while the non-diffuse pilocytic astrocytoma can be found elsewhere, separately. The classification of astrocytomas based upon histologic phenotype only is considered obsolete, it is needed to identify at least IDH mutation status, too. The correlation of histologic phenotype with molecular examinations and prognosis is best seen in how IDH status and not WHO grade correlates patient outcome in astrocytomas, WHO grade II-III. A similar tendency is seen in case of anaplastic astrocytoma and glioblastoma. And even when individual ECM components did not show significant differences in astrocytoma samples of various grade, the expression pattern was found to be significantly different. It seems that the differences seen in individual molecule levels that stay below the level of significance add up and cannot be ignored when the invasion spectrum, a characteristic of the sample as a whole, is studied. Samples of the neighboring grades were separated with very good accuracy based upon the invasion spectrum. Furthermore, linear discriminant analysis was able to identify some key molecules, too, and differences in the amount of these molecules was a main determinant in sample separation, even if these differences were not the most striking ones. Key molecules include integrin and laminins that bind onto them, fibronectin, and proteoglycans that interlink other matrix components like brevican neurocan or versican, as well as tenascins that bind tumor cells to ECM. Protein invasion spectrum analysis confirms and expands these findings. A good example is the identification of laminin- $\alpha 4$ and $-\beta 1$ chain as key molecules, as it is

known that these laminin chains, along with the γ chain, make up laminin-8 (laminin-411), an ECM component that has been previously described to play a role in glioma invasion, and in our research, the amount of either one or both chains seemed to be crucial for group separation. The connection between tumor grade and the invasion spectrum, thus can be easily seen. The practicality and importance of the connection is great, as treatment protocols depend on tumor grade and histology, and the method can aid neuropathologists to grade tumors correctly. This may be especially hard when only a limited amount of tissue sample is available, e.g. because only biopsy had been performed. In such cases, GBM and anaplastic astrocytomas, or grade II and grade III tumors can be hard to distinguish. Furthermore, molecular biology of the tumors seems to be more important than histologic phenotype when assessing prognosis, and the invasion spectrum gives information on the molecular composition of the tumors. Which ECM components are expressed in greater amounts and which are decreased? How are the components correlated to each other? Today, oncotherapy of gliomas is of anti-proliferative nature, but there are studies in phase II or phase III testing anti-invasive agents. Supplementing anti-proliferative treatment with anti-invasive drugs would allow us to decrease the amount of alkylating agent used today, leading to fewer and more tolerable adverse events that often set back treatment. A new target may even make it possible to resect tumors completely, or decrease recurrence rate following resection.

5.3. The invasion spectrum correlates with prognosis in glioblastoma

Studying tumor samples of glioblastoma patients revealed that the composition of extracellular matrix correlates with patient outcome. Literature data suggests that younger age, male gender and smaller tumor size has a weak association with a more favorable prognosis. However, previous and current research on patient data in our department failed to find such correlation when comparing patients with different prognosis. The prognostic role of MGMT methylation status and IDH mutations are well known in literature. Identifying the MGMT methylation status has more consequential role in the elderly (>65 years), as with unmethylated MGMT promotor in elderly one should consider the risks and benefits of using temozolomide, and possibly decreasing dosage or omitting the drug. IDH mutation is not frequent in glioblastoma, and even though the mutation has a clear correlation with survival, which is rather strong for an individual with the mutation, the mutation is a weak marker in larger population due to its low frequency. Nonetheless, as IDH mutant patients carry the same point mutation in the vast majority of cases, testing for the mutation is cost efficient, simple and is part of everyday routine due to its diagnostic relevance. The used separation criteria of 23 months is above mean overall survival times of standard Stupp protocol. It is a result of patients receiving additional targeted oncotherapy (bevacizumab) upon tumor recurrence, making mean overall survival at the Department of Neurosurgery, University of Debrecen Clinical Center 23 months. Patients involved in the study received targeted therapy in both groups equally, however, some patients had very poor prognosis, others, even without receiving bevacizumab, lived much longer. The rate of IDH mutation did not differ in the two groups, neither did the clinical data of patients. “Classical” prognostic marker thus were not helpful in deciding whether patients would have worse or better prognosis.

The composition of the ECM, however, proved to be different in the two groups. Individual molecules did not show significant differences, the expression pattern, i.e. the invasion spectrum, though, was clearly distinct in patients with poor or better prognosis. The prognosis of approximately 85% of patients was identified correctly, accuracy tests of the method confirmed usability. Notably, the positive predictive value of the method was higher for patients with worse prognosis in cases of both mRNA and protein invasion spectra, underscoring clinical benefits, as the analysis selects patients who need even more attention and more frequent follow-ups. It is important to mention that the invasion spectrum is not only a method for assessing patient outcome. In the near future, hopefully, the key molecules that were selected by the classifiers could serve as targets for novel oncotherapies. Further added value lies in the information the analysis reveals, as it is a tool to understand the molecular composition of the tumor. The high costs of targeted oncotherapy, as well as the rare, but severe adverse events stress the careful selection of the right patient population to receive a given targeted therapy in order to gain maximal treatment benefit. Helping to choose the right patient population for targeted therapies in the future therefore creates a sort of economic benefit, too, in using the invasion spectrum, as it displays whether the target of a given drug is expressed in the patient's tumor sample or not. The inefficacy of bevacizumab therapy is a good example for this approach.

5.4. Extracellular matrix molecules playing a key role in glioma invasion

The extracellular matrix of the brain is unique compared to other parts of the body. Its composition shows major differences, e.g. hyaluronic acid is the major component and not fibrillary proteins, or many components are seldom outside the central nervous system (brevican, cadherin-N2). ECM in non-tumor brain tissue is organized in a way that cellular motility, tissue plasticity and rapid changes in the ECM are limited to a certain extent. On the other hand, glioma cells are able to break through this limit and scatter around in the central nervous system but not outside of it, as it is a typical sign in glial tumors that they do not invade normal intracranial arteries and distant metastases are never formed. Tumor cells take an active part in re-shaping the ECM, partially by synthesizing the tumor ECM, and partially by modulating the function of non-tumor cells, as the altered microenvironment influences how non-tumor cells work.

A characteristic trait of the altered ECM in glioma is that the role and amount of hyaluronic acid (HA) is increased manifold. As a proof of this, it was found in our research that GBM expresses an increased amount of hyaluronan synthase-2 compared to normal brain. A significant difference was also found in CD44, a major HA receptor, as well as in co-receptor CD168 (HMMR) expression. The latter also plays an important role in separating low and high grade gliomas. These findings are in accordance with previous knowledge regarding HA and glioma invasion.

Lecticans that interact with HA are members of a chondroitin-sulphate proteoglycan family and their expression is also increased. A “brain specific” member is brevican, along with versican and neurocan, their amount is greatly increased in astrocytomas, their expression correlates with tumor grade. Brevican was identified as a key molecule by classifiers multiple times, and an increased expression of neurocan and versican can be observed in higher grade gliomas, too.

These molecules playing a role in cellular adhesion, migration and division have been mentioned before by other research groups regarding glioma invasion, however, the connection between tumor grade and their expression is a novel finding. The molecules bind fibrillary ECM components which are normally present in the brain in smaller quantities only. In gliomas, however, the amount of fibrillary ECM is increased. It was found that there is an increased expression of fibronectin, collagens and certain laminins (e.g. $\alpha 4$ and $\beta 1$ subchain containing laminin-8) compared to normal brain in gliomas, and this increase is grade-dependent. Migrating cells use these components as a migration pathway during invasion.

Tumor cells bind to components of the ECM using cell-surface receptors. The role of integrins in tumor invasion is well known, in our research integrins- $\alpha 1$, - $\alpha 3$, - $\alpha 5$ and - αV , along with - $\beta 1$ - $\beta 3$ was found to play a role in invasion. The role of integrin- $\beta 1$ seems to be remarkable, as its expression increases by grade, it was found to be a key molecule in the separation of many groups, and literature data supports these findings. Laminins and integrins that bind them correlate with patient outcome, according to our findings, something that has not been previously described. Furthermore, the expression of other surface molecules, including syndecans, tenascins and cadherins are also correlated with tumor grade and increased invasiveness, as confirmed by our data and literature findings.

The role of matrix metalloproteinases in the dynamic transformations of the matrix is without question. The amount of MMP-2 correlates with tumor grade, confirming previous data. Expression is greatly increased in gliomas, and the excessive amount of MMP-2 is even capable to modulate cell function.

6. SUMMARY

Astrocytomas are glial tumors, the vast majority of malignant primary CNS tumors are of astrocytic origin. Treatment and prognosis of the various types of astrocytomas show great differences among the histologic groups. The one with the worst prognosis is the most common type, glioblastoma comprises approximately 50% of all malignant CNS tumors. Survival varies between 16 to 24 months, patients show a great degree of heterogeneity in terms of survival. Previous research suggests that patient age, gender, tumor size correlate with patient outcome, however, our previous and present findings could not confirm this, possibly because these prognostic markers are considered weak. Currently, there is no prognostic marker in everyday clinical use.

This dissertation is therefore based upon analyses that have been performed to study the hypothesized correlation between the molecular composition and the clinical classification of tumors. The connection of histopathology and molecular composition has been analyzed, as well as the relationship of molecular composition and overall survival of patients with different prognosis but with no other differences. To answer the questions, qRT-PCR, mass-spectrometry and immunohistochemistry methods were used.

Based upon our findings, it can be stated that primary and secondary intracerebral malignancies have different extracellular matrix compositions. The invasion-related molecules of ECM have different expressional patterns in various grades of astrocytoma and this can be used to identify tumor grade. The expressional pattern is also different in tumor samples of patients with poor or better prognosis, which was correctly assessed with an 85% success rate. Analyzing the invasion spectrum would help neuropathologists in identifying tumor grade, clinicians in assessing patient outcome and scientists in gaining extra information of gliomas.

6.1. Novel scientific findings

1. Primary and secondary malignant central nervous system tumors and non-tumor brain have great differences in extracellular matrix composition
2. The invasion spectrum, i.e. the expression pattern of invasion-related extracellular matrix molecules of primary and secondary malignant central nervous system tumors are significantly different.
3. Central nervous system malignancies of the same histopathologic group (e.g. astrocytomas) show distinct ECM composition in various grades, both on transcriptional and translational level. Thus, invasion spectrum is a grade-specific characteristic marker of the tumor that allows separation of different tumor grades.
4. Tumors of same histopathology and grade (e.g. glioblastoma) show great heterogeneity in patient outcome which cannot be explained by differences in clinical factors.
5. Analyzing the invasion spectrum reveals the key role of multiple molecules in tumor invasion (e.g. brevican, integrin- β 1, laminin- α 4)
6. The invasion spectrum can be used as a prognostic marker because it identifies glioblastoma samples with poor and better prognosis with good accuracy.

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List of publications related to the dissertation

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