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ORIGINAL ARTICLE

Effect of Concomitant Radiochemotherapy on Invasion Potential of Glioblastoma

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Abstract Glioblastoma (GBM) is the most common primary brain tumor in adults with inevitable recurrence after oncotherapy. The insufficient effect of "gold standard" temozolomide-based concomitant radiochemotherapy may be due to the inability to prevent tumor cell invasion. Peritumoral infiltration depends mainly on the interaction between extracellular matrix (ECM) components and cell membrane receptors. Changes in invasive behaviour after oncotherapy can be evaluated at the molecular level by determining the RNA expression and protein levels of the invasion-related ECM components. The expression of nineteen ECM molecules was determined at both RNA and protein levels in thirty-one GBM samples. Fifteen GBM samples originated from the first surgical procedure on patients before oncotherapy, and sixteen GBM samples were collected at the second surgery due to local recurrence after concomitant chemoirradiation. RNA expressions were measured with qRT-PCR, and protein levels were determined by quantitative analysis of Western blots. Only MMP-9 RNA transcript level was reduced ($p < 0.05$) whereas at protein level, eight molecules showed changes concordant with RNA expression with significant decrease in brevican only. The results suggest that concomitant radiochemotherapy does not have sufficient impact on the expression of invasion-related ECM components of glioblastoma, oncotherapy does not significantly affect its invasive behavior. To avoid the spread of tumors into the brain parenchyma, supplementation of antiproliferative treatment with anti-invasive agents may be worth consideration in oncotherapy for glioblastoma.

Keywords Radiochemotherapy · Temozolomide · Extracellular matrix · Invasion · Glioblastoma

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Introduction

Gliomas derived from glial cells that support neurons in the central nervous system play a pivotal role in primary brain tumors [1]. The World Health Organization grades these tumors on a I–IV scale according to their appearance and degree of aggressiveness, with grade IV corresponding to the most malignant type, that is, glioblastoma multiforme (GBM). Responsible for more than half of all gliomas, such tumors are rapidly progressing and aggressive and have an extremely poor prognosis [2]. Despite complex and intense treatment, recurrence is inevitable and causes relatively rapid death. By now, GBM is irreversible, and the treatment goal is to delay progression in order to preserve the patient's quality of life as long as possible [3]. One of the main causes of repeated recurrence is the tumor's aggressively infiltrative nature, which

makes radical surgical resection impossible and reduces the effectiveness of focal brain radiotherapy.

The most radical resection possible and subsequent radio- and chemotherapy are the fundamentals of the treatment. Postoperative concomitant chemoradiotherapy, now used as the "gold standard" worldwide, was introduced in 2005 and increased the median survival rate from less than a year to 14.6 months [4]. Nevertheless, there are great efforts to explore new target-based agents that can solve the problem of inevitable recurrence. The target of many of these potential therapies is the extracellular matrix (ECM), because peritumoral infiltration of glioma cells is regulated mainly by ECM molecules (collagens, proteoglycans, laminins, hyaluronan, and synthesizing and degrading enzymes), which create an active dynamic medium [5]. Alteration in ECM composition plays a key role in cell movement, which can be observed by determining the expressional changes of the relevant molecules [6].

Once the oncotherapy alters the expression of ECM molecules, it may affect the infiltrative potential of the tumor. Potent reduction of peritumoral invasion could increase the chances for radical tumor resection of recurrent GBM. In this study, the effect of the standard chemoirradiation therapy for GBM on the tumor ECM was evaluated by determining mRNA and protein expression of invasion-related ECM compartments in untreated and recurrent glioblastoma samples.

Materials and Methods

In this project, thirty-one human brain tumor samples removed during neurosurgical operation were tested. Each sample was histologically verified as GBM by an experienced neuropathologist. The fresh frozen tissue samples were selected from the Neurosurgical Brain Tumor and Tissue Bank of Debrecen; fifteen samples originated from patients with newly diagnosed GBM without any oncotherapy, and sixteen samples were excised from recurrent tumors after chemoradiotherapy (Table 1). After removal, the brain tissues were immediately frozen in liquid nitrogen and stored at −80 °C until processing. The research was performed with the permission of the Hungarian Ethical Committee, and every patient signed an informed consent form before the operation. In the tissue samples, the gene expression profile of nineteen invasion-related molecules at both RNA and protein levels were determined. The list of the investigated molecules was composed by the results of our previous studies and contained proteoglycans, transmembrane proteins and degrading enzymes [7, 8] (Table 2). Real time quantitative reverse transcriptase polymerase chain reaction (RT-QPCR) and the comparative C_t method were used to determine the quantity of mRNA of selected molecules, following the same method described previously [9, 10].

Table 1 Details of oncoterapy in the patients of post-treatment group. RT: 30 × 1,8Gy focal brain radiotherapy, CRT: concomitant chemoradiation (RT + 7 5 mg/m^2 daily administration of temozolomide), TEM: temozolamide monotherapy (1st cycle: 150 mg/m^2, then 200 mg/m^2)

Patient No.	Treatment
Patient 1	RT + 2 TEM
Patient 2	CRT + 6 TEM
Patient 3	CRT
Patient 4	RT + 4 TEM
Patient 5	CRT + 6 TEM
Patient 6	CRT + 6 TEM
Patient 7	CRT + 6 TEM
Patient 8	CRT
Patient 9	CRT + 2 TEM
Patient 10	CRT + 6 TEM
Patient 11	CRT
Patient 12	CRT + 2 TEM
Patient 13	RT + 15 TEM
Patient 14	CRT
Patient 15	RT + 15 TEM
Patient 16	CRT + 1 TEM

To evaluate the expression of the respective proteins, a proteomic assay was performed. First, tissue homogenization was performed with lysis buffer 50 mM Tris, 1 mM EDTA, 17 mM beta-mercaptoethanol, and 0.5 % Triton X-100. The protein concentration was measured with the Bradford method [11]. The relative amount of proteins was determined by the selected reaction monitoring (SRM)-based targeted proteomic

Table 2 List of examined molecules, gene names and assay probes used for RNA analysis

Gene name	Assay Probe
Brevican	BCAN-Hs00222607_m1
Cadherin-N2	CDH12-Hs00415843_m1
CD168	HMMR-Hs00234864_m1
Collagen type III alpha1	COL3A1-Hs00164103_m1
Erb B2	ERBB2-Hs00170433_m1
Fibronectin	FN1-Hs00277509_m1
Integrin alpha1	ITGA1-Hs00235030_m1
Integrin alpha3	ITGA3-Hs00233722_m1
Integrin alpha7	ITGA7-Hs00174397_m1
Integrin beta1	ITGB1-Hs00559595_m1
Laminin alpha4	LAMA4-Hs00158588_m1
Laminin beta1	LAMB1-Hs00158620_m1
Matrix metalloproteinase-2	MMP2-Hs00234422_m1
Matrix metalloproteinase-9	MMP9-Hs00234579_m1
Neurocan	NCAN-Hs00189270_m1
Syndecan-1	SDC1-Hs00174579_m1
Tenascin-C	TNC-Hs00233648_m1
Tenascin-R	TNR-Hs00162855_m1
Versican	VCAN-Hs00171642_m1

method [12–16]. By measuring the area under the curve of the SRM spectra using software analysis (Analyst 1.4.2), we calculated the quantitative protein levels of the samples.

For statistical analysis, the Mann-Whitney *U* test was performed to highlight the significant alterations in the expressional changes. The level of significance was $p < 0.05$. We also established 95 % confidence intervals.

Results

mRNA Transcription

The comparison of gene expression in treated and untreated samples showed that the mRNA level of twelve of the examined nineteen molecules exhibited underexpression after treatment: brevican, collagen type III alpha-1, fibronectin, intergrin alpha-1, integrin alpha-7, laminin alpha-4, laminin beta-1, matrix metalloproteinase-9, neurocan, syndecan-1, tenascin-R, and versican. Increased expression was detected in the case of seven molecules: cadherin-N2, CD168, erb-B2, integrin alpha-3, integrin beta-1, matrix metalloproteinase-2, and tenascin-C (Fig. 1). The underexpression of matrix metalloproteinase-9 with fold change of 0.21 was significant (*p value*: *0.006*, *95 % CI*: *0.13–0.26*).

Protein Translation

The quantitative protein analysis showed that the level of twelve proteins decreased in the post-treatment samples: brevican, cadherin-N2, CD168, collagen type III alpha-1, integrin alpha-3, integrin alpha-7, integrin beta-1, laminin beta-1, matrix metalloproteinase-2, matrix metalloproteinase-9, neurocan, and tenascin-R. Protein concentration increased after treatment in the case of five molecules: erb-B2, fibronectin, integrin alpha-1, laminin alpha-4, and versican. Syndecan-1 and tenascin-C could not be detected in the post-treatment samples. After oncotherapy, the level of brevican exhibited a significant decrease (*protein level*: *-4432.44*, *p value*: *0.006*, *95 % CI*: *-8857.43 – -7.46*) (Fig. 2).

Comparing mRNA and Protein Expression

The analysis of the expressional data at both RNA and protein levels detected concordant changes in eight cases (Fig. 3). Consequent post-treatment underexpression was seen in the case of brevican, collagen type III alpha-1, integrin alpha-7, laminin beta-1, matrix metalloproteinase-9, neurocan, and tenascin-R. In contrast, the transcription and translation of erb-B2 was lowered after oncotherapy. The statistical analysis indicated only two significant changes: matrix metalloproteinase-9 at the RNA level and brevican at the protein level.

Discussion

Until 2005, treatment of GBM was limited to surgical resection and subsequent radiotherapy [17–19]. Later, therapeutic strategies expanded to include chemotherapeutic agents, which were used alone or combined with irradiation. In the "pre-temozolomide" era, several drugs were used to treat GBM—platinoids, taxanes, topoisomerase inhibitors, and

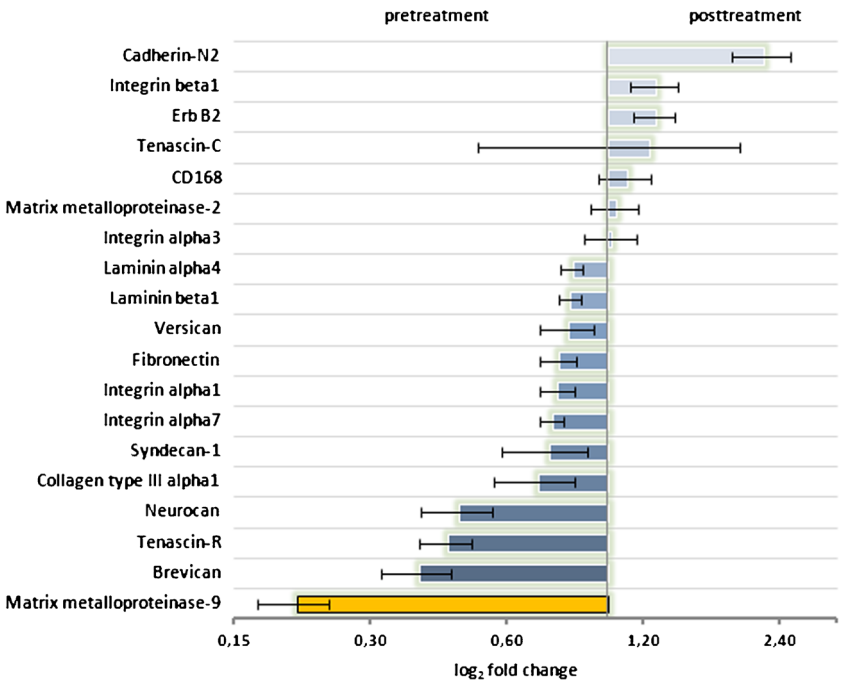

Fig. 1 RNA fold change of the ECM molecules in pre-treatment and post-treatment glioblastoma samples on log [2] scale with standard errors indicated. Lines trending towards left mean higher pre-treatment levels, while lines oriented towards right show overexpression after oncotherapy. Yellow color means significant change

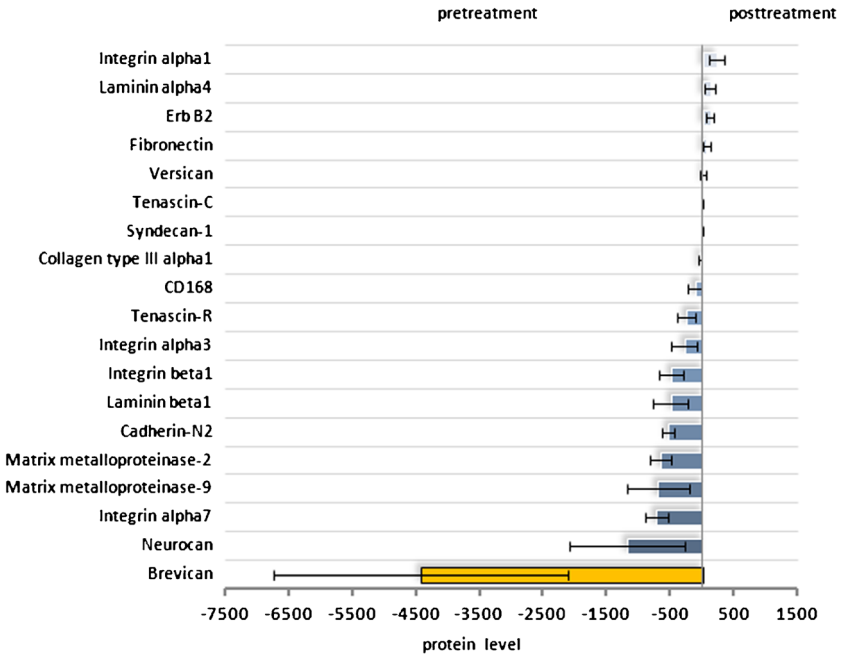

Fig. 2 Protein level alteration of studied molecules with standard errors before and after oncotherapy. The molecules are listed in order of the extent of change. Lines heading to left imply higher pre-treatment levels, while lines trending towards right mean overexpression after chemoirradiation. Yellow color indicates significancy

other alkylating agents—but since 2005 temozolomide has been considered the gold standard [20]. Temozolomide alkylates the DNA of the tumor cells, which leads to single- and double-strand breaks performed by DNA repair enzymes, which in turn results in activation of apoptosis pathways [21–23]. Combined with radiotherapy, it is used daily in the concomitant phase, and five days/month afterward in the monotherapy phase [24]. The adjuvant effect in concomitant therapy is due to the radiosensitization of temozolomide, which supplements its alkylating effect with the DNA fragmentation brought about by irradiation [25].

Recurrence of GBM occurs practically in every patient, independently of the combinational use of radio- and chemotherapy and macroscopically total surgical resection. The inevitable recurrence of the tumor adjacent to the resection cavity is due to the invasive nature of GBM. As GBM progresses, the tumor cells deeply infiltrate the brain tissue, making total resection impossible and reducing the effect of focal brain radiotherapy. Both chemo- and radiotherapy develop their antiproliferative effect through inhibition of DNA replication; it can thus be assumed that they have no relevant effect on the molecular mechanisms of invasiveness [26]. Therefore, it has

Fig. 3 Expressional alterations of the studied ECM molecules at RNA and protein levels between untreated and treated glioblastoma samples with *p* values. Gray lines show concordant change at both levels, the yellow boxes indicate significant changes

Molecules	RNA		PROTEIN		RNA	PROTEIN
	fold change	p value	protein level	p value		
Matrix metalloproteinase-9	**0,21**	**0,006**	-696,52	0,767	↓	↓
Brevican	0,38	0,149	**-4432,44**	**0,006**	↓	↓
Tenascin-R	0,44	0,260	-248,43	0,597	↓	↓
Neurocan	0,47	0,244	-1174,38	0,716	↓	↓
Collagen type III alpha1	0,70	0,540	-35,47	0,806	↓	↓
Syndecan-1	0,74	0,678	0,00	0	↓	-
Integrin alpha7	0,75	0,161	-720,12	0,113	↓	↓
Integrin alpha1	0,78	0,228	218,80	0,575	↓	↑
Fibronectin	0,78	0,395	68,87	0,753	↓	↑
Versican	0,82	0,859	1,70	0,909	↓	↑
Laminin beta1	0,83	0,185	-499,08	0,223	↓	↓
Laminin alpha4	0,84	0,374	124,27	0,766	↓	↑
Integrin alpha3	1,02	0,621	-279,55	0,307	↑	↓
Matrix metalloproteinase-2	1,04	0,859	-660,13	0,093	↑	↓
CD168	1,10	0,737	-116,92	0,482	↑	↓
Tenascin-C	1,24	0,984	0,00	0	↑	-
Erb B2	1,27	0,429	121,35	0,503	↑	↑
Integrin beta1	1,28	0,199	-496,82	0,112	↑	↓
Cadherin-N2	2,20	0,334	-538,95	0,166	↑	↓

become necessary to determine the expression pattern of invasion-related molecules and show the effect of oncotherapy on the expressional changes. At present, there is insufficient knowledge regarding the details of the molecular mechanisms of the concomitant therapy or its effect on tumor infiltration, and all the relevant studies have been performed on cell cultures, not on human brain tissue. The present study determined the potential changes caused by the standard oncotherapy at RNA and protein levels on the expression of invasion-related molecules in glioblastoma. The expressional changes of nineteen ECM and tumor cell membrane components were determined in GBM samples before and after concomitant radiochemotherapy. In the post-treatment samples, only the mRNA level of MMP-9 and the protein concentration of brevican showed a significant decrease. All the other molecular changes were not significant.

Because MMP-9 is a matrix-degrading enzyme, its level in glioblastoma tends to increase compared to nontumor tissue [27]. Trog D. et al. examined the effect of temozolomide and irradiation on GBM cell lines, and they found a significant increase of metalloproteinase in the surviving cells, which correlated with GBM's aggressive and infiltrative nature [28]. In our human samples, the mRNA level of MMP-9 decreased remarkably after oncotherapy; however, much lower expressional decrease was detected in protein concentration. This represents the major effect of antitumor agents on DNA replication, which has only a minor impact at the protein level.

Brevican is a brain-specific ECM proteoglycan. The upregulation of its expression has a large positive effect on the proliferation and motility of glial cells, supporting cell invasion and thus the formation of invasive gliomas [29–32]. At the molecular level, brevican increases the expression of numerous cell adhesion molecules, enhances the secretion of fibronectin before establishing contact with it, and promotes EGFR activation [33]. Nakada M. et al. and Held-Feindt J. et al. found a clear correlation between brevican expression and the activity of cell invasion [34, 35]. In our study, the significantly decreased level of brevican due to oncotherapy indicates that concomitant chemoirradiation has some effect on tumor invasion.

Conclusion

Among the nineteen invasion-related ECM molecules and cell membrane receptors, significant expressional change after oncotherapy for glioblastoma was observed in only two cases: MMP-9 and brevican. These results suggest that the concomitant radiochemotherapy does not significantly influence the invasion behavior of glioblastoma cells in the peritumoral area. Thus, the infiltrating cells of the recurrent tumor evade the radical resection as well as the stereotactic radiosurgical elimination. These findings can help to clarify the fundamental reasons why the oncotherapy for GBM is finally unsuccessful. Moreover, they underline the great need for developing new targeted anti-invasion therapies.

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Compliance with Ethical Standards

Conflict of Interest The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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