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Compartment- and malignance-dependent up-regulation of γ-glutamyltranspeptidase and dipetidylpeptidase-IV activity in human brain gliomas

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Summary. γ-Glutamyltranspeptidase (GGT, syn. γ-Glutamyltransferase) and dipeptidylpeptidase-IV (DPP-IV) activity participates in metabolic and growth control of normal and tumor cells by processing biologically active peptides. Here, we report on up-regulation of these enzymes in human brain gliomas determined by catalytic enzyme histochemistry and immuno-cytochemistry. Higher activity of GGT was found in 50%, 68% and 81% of WHO grade II, III and IV tumors, respectively. The process started at/near the micro-vasculature, from where it spread to the parenchyma. On average, the enzyme activity in grade II, III and IV gliomas exceeded controls 2.0, 3.0 and 3.5-fold, respectively. Up-regulation of DPP-IV-like activity also started at the microvasculature, but mainly in pericytes and mononuclear-like cells around the vessels and dispersed in the parenchyma. Marked elevation of this enzyme activity, comprising also tumor parenchyma, occurred only in grade IV glioblastomas (65% patients; 3.6 times above controls) which can, therefore, help in their differentiation from grade III gliomas. The increase of total DPP-IV-like activity also included its two enzymatic homologs, the canonical DPP-IV/CD26 and FAP-1α. The increase in GGT is supposed to be a tumor grade dependent response of microvasculature and tumor astrocytes to stress induced by tissue hypoxia and/or the metabolic aberrancies. The increase in DPP-IV-like activity in high-grade tumors can be attributed to inflammatory/scavenging processes performed by the mononuclear-like cells and, in glioblastomas, also to regressive changes in the structure and function of the microvasculature and tumor parenchyma, including astrocyte stress response. The inverse relationship between DPP-IV-like activity and Ki67 in most glioblastomas and shorter survival time of patients with low activity of this enzyme also suggest its anti-oncogenic effects.

Key words: Brain gliomas, γ-glutamyltranspeptidase (syn. γ-glutamyltransferase), Dipeptidyl peptidase-IV, Tumor microvasculature, Reactive astrocytes, Oxidative/metabolic stress

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Introduction

The pathobiology of human brain tumors is still not understood well enough to guarantee effective therapy and reliable prognosis (Kleihues et al., 2000; Riemenschneider et al., 2010; Jung et al., 2011). A new insight into their growth behavior is provided by studies of cell surface proteases. The growth invasiveness of tumor cells can be enhanced, for instance, by matrix-degrading metalloproteases, a urokinase-type plasminogen activator and some cysteine proteases (Krepela, 2001; Levicar et al., 2003; Zamecnik et al., 2004). The proliferation and migration of tumor cells are also affected by some ectoenzymes with dipeptidyl peptidase-IV-like (DPP-IV-like) activity (e.g. DPP-IV/CD26 and fibroblast activation protein-1α) *via* processing of specific peptides, changing their biological activity, affinity to cognate receptors or interactions with structural proteins of the extracellular matrix (Busek et al., 2004). Tumor growth also depends on the balance of tissue-hypoxia-induced reactive oxidative species (ROS, NOS etc.) and the effectiveness of antioxidative

mechanisms, especially the level of the tripeptide γ-glutamyl-cysteinyl-glycin (glutathione, GSH). Its intracellular synthesis depends on the pool of cysteine released from the extracellular GSH cleaved to γ-glutamyl and cysteinyl-glycine residues by γ-glutamyltranspeptidase (GGT, EC 2.3.2.2, CD224, syn. γ-Glutamyltransferase). The catabolic GSH intermediates can further influence the proliferation/apoptosis balance via their pro-oxidative effects (del Bello et al., 1999; Paolicchi et al., 2002; Corti et al., 2010). Moreover, the hypoxia-activated GGT-positive astrocytic vascular end-feet and pericytes were shown to initiate the angiogenesis preceding disassembly of capillary walls in the normal brain (LaManna et al., 2004; Kaur et al., 2005). Besides its anti-oxidative/anti-hypoxic functions, GGT can confer higher growth potential to tumors by a higher uptake of some amino acids and the transport and reduction of iron ions (Dominici et al., 2003; Hawkins et al., 2006). The faster growth of GGT-rich cells was shown in several types of tumor cells in cultures, as well as in epithelial and lymphoid tumors in situ (Hanigan et al., 1999a). Up-regulation of GGT protein was also observed in human high-grade gliomas by immunocytochemistry (Schafer et al., 2001).

DPP-IV-like enzymatic activity is inherent to several molecular species, the DPP-IV Activity and/or Structure Homologs (DASH) (Sedo and Malik, 2001). Besides canonical DPP-IV/CD26 (EC. 3.4.14.5), the DASH family includes fibroblast activation protein-1alpha (FAP-1α), DPP8 and DPP9. They specifically process oligopeptides with proline or alanine at the penultimate position, such as some chemokines, neuropeptides, incretins, etc., followed by changes in their biological effects (Lambeir et al., 2003; Busek et al., 2004). Cell behavior can also be affected by the formation of DASH member heterodimers, as shown e.g. by the increased migration and invasiveness of endothelial cells in cultures caused by DPP-IV/CD26 and FAP-1α (Ghersi et al., 2006). Similarly to GGT, DPP-IV-like activity is up-regulated in several types of tumors in situ (Sedo et al., 2008), including brain gliomas (Stremenova et al., 2007), and is also inducible by hypoxia (Dang et al., 2008). Noteworthy is the fact that GGT and DPP-IV-like activities are newly appearing targets for pharmacotherapy of some non-neuronal tumors (Castro et al., 2002; Sato and Dang, 2003).

Studies of both these enzyme activities in human gliomas are, however, rare and comparison of their data is limited by the great inter-/intra-tumor heterogeneity and the differences in the methods applied for their detection. As a consequence, their role in growth behavior of brain tumors is still unclear. Here, we report on the grade of malignance- and tissue-compartment-dependent up-regulation of both these enzymes. We show that the process starts at tumor microvasculature and spreads to its parenchyma in high-grade gliomas. Functionally, up-regulation of both enzymes is supposed to reflect the cell/tissue response to the tissue hypoxia- and gene mutation-induced oxidative and metabolic stress. Moreover, high DPP-IV-like activity in glioblastomas could be used as an auxiliary diagnostic factor, and the ratio of the two enzymes for prediction of post-operation survival time for a subgroup of glioblastoma patients.

Materials and methods

Patients and diagnostic cytology

The study involved 125 patients who underwent surgery for a clinically diagnosed brain tumor. For controls, we used peritumoral tissues dissected therapeutically from 8 patients and tissue from the amygdalo-hippocampal regions of 11 patients treated surgically for drug-resistant epilepsy. All samples were collected in accordance with the guidelines of the WHO CIOMS and the Ethics Committee of the Hospital Na Homolce, Prague. Formalin-fixed (10%) and paraffin-embedded perioperational material stained with hematoxylin and eosin was used for the diagnosis and evaluation of the degree of vascularization and extend of necrosis using a 3-tiered semiquantitative scale.

Catalytic histochemistry of GGT and DPP-IV (Lojda, 1981)

Cryostat sections were prepared from 3 to 5 perioperational micro samples from various tumor depths (approx. 100 mg each, Bright Instrument Co, Ltd., UK) and were fixed in acetone and chloroform (1:1, 2 min, 4°C). For GGT, the slides were incubated in solutions of glutamyl-4-methoxy-naphthylamide (2.0 mg in 10 ml PBS, Bachem, Germany) and glycyl-glycine (20 mM, Sigma, USA) as a γ-glutamyl acceptor at pH 7.4 and 4°C for 18 h. For DPP-IV, a solution of L-glycyl-L-proline-4-methoxy beta-naphthylamide hydrochloride (2.5 mg in 10 ml PBS, Sigma, USA, pH 7.4 and 4°C for 18 h) was used. For both enzymes, incubation occurred in the presence of Fast Blue B (235 mg in 10 ml PBS, Fluka, USA). The slides were washed in 4% paraformaldehyde and tap water and mounted in water-miscible media. No counterstaining was applied so as not to alter the color and intensity of the histochemical reaction. Cytological details were visualized by phase contrast microscopy. The intensity of staining was semiquantitatively evaluated in a bright field (Axioplan, Opton, obj. 20x) using a 5-tiered arbitrary unit (a.u.) scale. The intra- and inter-tumor heterogeneity of tumor specimens was approximated by "weighted average staining intensity" values (WASI) which incorporate proportionally the areas stained to a given intensity. According to the WASI values, the patients were arbitrarily classified into three subgroups referred to as Low (L), Medium (M) and High (H) intensity stained groups. For GGT, the thresholds for L, M and H subgroups were <1.5, 1.5 - 3.0 and 3 - 5, respectively. For DPP-IV, these values were <0.5, 0.5 -

2.0 and >2 for the L, M and H subgroups, respectively. The L values corresponded to the staining of non-tumor controls.

Immunocytochemistry

DPP-IV/CD26, FAP-1α GFAP and carbonic anhydrase-IX (CA-IX) were detected in cryostat sections (see above) pre-incubated in 3% heat-inactivated bovine fetal serum for 20 min followed by an overnight incubation at 4°C either with mouse monoclonal anti-human DPP-IV/CD26 antibody (1:100, clone M-A261, Acris, Germany), mouse monoclonal anti-human FAP-1α (F11-24, 1:200, Alexis Biochemicals, Germany) or mouse anti-GFAP monoclonal antibody (GF 01, 1:50, Exbio, Prague, CR). This was followed by 2-hr incubation in anti-mouse IgG-FITC (1:200, Sigma, USA) or anti-mouse IgG-Alexa Fluor 488 conjugate (1:400, InVitrogen, USA). CA-IX was detected by polyclonal rabbit antibody (1:500, Abcam, UK) and anti-rabbit IgG-FITC (1:40, Sigma, USA). The slides were evaluated microscopically (Axioplan, Opton, obj. 40x). Intensity of fluorescence (except GFAP) was scored on a 5-tiered arbitrary unit scale (see above for catalytic histochemistry).

Expression of Ki67, p53, EGFR, CD68 and UCHL-1 was detected in paraffin-embedded material by the following primary monoclonal antibodies (Mabs): mouse anti-human Ki67 Mab (1:100, clone MIB-1, Dako, Denmark), mouse anti-human p53 Mab (1:100, clone DO-7, Dako, Denmark), rat anti-human EGFR Mab (1:100, pharmDX kit Dako, Denmark or 1:200, Acris, Germany), mouse anti human T-cell UCHL-1 Mab (1:100, Dako, Denmark) and the mouse anti-human macrophage Mab CD68 (1:100, Dako, Denmark). This was followed by peroxidase-DAB terminal staining (EnVision+Dual Link System-HRP, Dako, Denmark). The expression of Ki67 and p53 was scored as a percentage of stained cells. EGFR was evaluated semiquantitatively on a 3-tiered scale in arbitrary units. In controls, the primary antibody was omitted.

Statistics

The data were evaluated using the Kruskal-Wallis test, Mann-Whitney U tests and the Spearman correlation coefficient using the program Statistica 9.0. Patient survival was evaluated by Cox proportional hazards regression model and the K-means cluster analysis.

Results

Patients and tumor characteristics

The histological classification of tumors and the number, gender and age of 125 patients with brain gliomas of WHO grade II-IV and 19 non-tumor control samples are reviewed in Table 1.

GGT activity

In non-tumor control brain tissue, GGT activity was present in slim capillary segments and juxtavascular, pericyte-like cells, while the staining of parenchyma was

Table 1. Glioma diagnoses and patients' age.

Grade WHO	Diagnosis	Male*	Female*	Age (range) ** [years]
Non-tumor	Pharmacoresistant epilepsy	4	7	40.0 (22-54)
	Adjacent non-tumor tissue	4	4	54.0 (46-67)
Grade II	Fibrillary astrocytoma	5	7	35.0 (29-58)
	Oligoastrocytoma	3	3	40.0 (19-51)
	Oligodendroglioma	2	3	40.0 (38-48)
	Pleomorphic Xanthoastrocytoma	1		53.0
Grade III	Anaplastic astrocytoma	15	5	50.0 (25-68)
Grade IV	Glioblastoma multiforme	46	35	56.2 (26-83)

Tumor grades WHO II - IV; *: The number of patients; **: median and its min/max values.

Table 2. The number of tumors with different GGT and DPP-IV-like enzymatic activities, and expression of DPP-IV/CD26 and FAP-1α.

Glioma Grade		GGT L/ M/ H*	DPP-IV-like L/ M/ H*	DPP-IV/CD26 L/ M/ H*	FAP-1α L/ M/ H*
Non-tumor	Pat. No.	15/ 4/ 0	10/ 4/ 0	11/ 1/ 0	8/ 3/ 0
	(%)	(79/ 21/ 0)	(71/ 29/ 0)	(91/ 0 / 0)	(73/ 27/ 0)
Grade II	Pat. No.	12/ 9/ 3	16/ 2/ 0	5/ 2/ 0**	3/ 1/ 0**
	(%)	(50/ 38/ 12)	(89/ 11/ 0)	(71/29/ 0)	(75/ 25/ 0)
Grade III	Pat. No.	6/ 10/ 3	14/ 3/ 2	5/ 3/ 1	6/ 3/ 0
	(%)	(32/ 52/ 16)	(74/ 16/ 10)	(56/ 33/ 11)	(67/ 33/ 0)
Grade IV	Pat. No.	14/ 33/ 28	24/ 29/ 15	12/ 9/ 5	14/ 8/ 4
	(%)	(19/ 44/ 37)	(35/ 43/ 22)	(46/ 35/ 19)	(54/ 31/ 15)

Pat. No: number of patients; * L: low; M: medium; H: high activity/expression (see Material and Methods); ** Pleomorphic xanthoastrocytoma grade II with extreme DPP-IV/CD26 and FAP-lα not included.

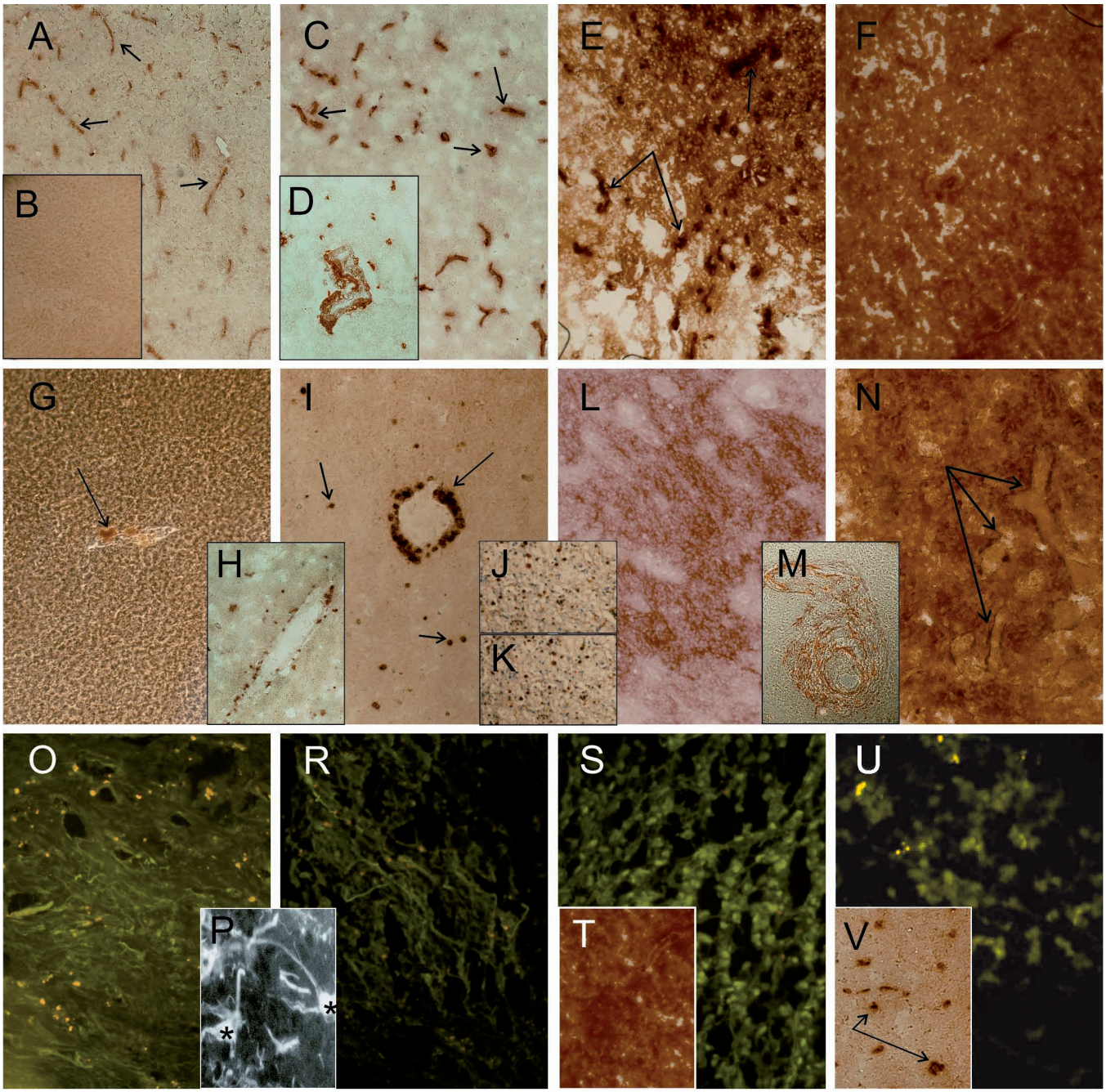

Fig. 1. The catalytic- and immunocytochemistry of human brain gliomas. **A.** Non-tumor control brain tissue with low GGT activity in capillaries (arrows). **B insert.** Negative staining control. **C.** Grade II tumor with increased GGT activity in hyperplastic microvasculature (arrows). **D insert.** A detail of hyperplastic vessel. **E.** Grade III tumor with high GGT activity in a segment of parenchyma and hyperplastic capillaries (arrows). **F.** Grade IV tumor with high and extensive GGT activity. **G.** DPP-IV-like activity in a solitary perivascular cell (arrow) in non-tumor brain sample. **H insert, I.** DPP-IV-like activity in perivascular and solitary mononuclear-like cells (arrows) in grade III astrocytoma. **J, K inserts.** CD68 and UHCL1 antibody-stained mononuclear-like cells in grade III astrocytomas, respectively. **L.** Medium DPP-IV-like activity in grade IV tumor parenchyma. **M insert.** Spindle/smooth muscle-like DPP-IV-like stained cells in/around the hyperplastic vessels. **N.** High DPP-IV-like activity in parenchyma of grade IV glioblastoma with dilated and densely stained capillaries (arrows). **O and R.** DPP-IV/CD26 and FAP-1· in grade IV glioblastoma with fiber-like stained texture, respectively. **P insert.** The hypertrophic GFAP stained astrocyte fibers (asterisks) in grade IV glioblastoma. **S.** High expression of carbonic anhydrase-IX in grade IV glioblastomas associated with high GGT activity (**T insert**). **U.** Absence of carbonic anhydrase-IX in grade IV glioblastomas associated with low GGT activity mainly in vascular profiles (arrows, **V insert**). Axioplan, Opton. A-V except inserts: Optical magnif. 10x (obj. Opton Plan-Neofluar 20x, digital camera Olympus DP70 with 2/3 inch chip and 0.5 intermediate lens), digit. magnif. 8.0; Inserts: Optical magnif. 20x (obj. Opton Plan-Neofluar 40x, digital camera Olympus DP70 with 2/3 inch chip and 0.5 intermediate lens), digit. magnif. 4.2; inserts J, K digit. magnif. 3.0.

much lower or negative (Fig. 1A). In tumors, the GGT staining was *prima facie* higher in all WHO grade tumors. Increased activity appeared already in grade II tumors, mainly in their microvasculature (Fig. 1C,D). The relatively wide staining of the capillary walls suggests the presence of GGT both in the endothelial cells, pericytes and the astrocytic end-feet enveloping brain capillaries. In high-grade tumors, the GGT staining irregularly spread from the vessels to the surrounding parenchyma (Fig. 1E). In some grade IV glioblastomas, there was high activity throughout the whole histological specimen except for necrotic foci (Fig. 1F). In some places, a high GGT activity was apparent in fiber-like structures resembling the hypertrophic processes of reactive astrocytes. In some glioblastomas, GGT activity was, however, low or limited only to small tissue segments. GGT staining of the whole tumor/sample, as assessed by the weighted average (WASI) values, exceeded the most frequent control values (<1.5) in 50% of glioma grade II patients (12 of 24). In grade III and IV tumors, the increase occurred in 68% (13 of 19) and 81% of patients (61 of 75), respectively. The average staining intensity in all grade II, III and IV tumors exceeded controls 2.0, 3.0 and 3.5-fold, respectively (Fig. 2A).

DPP-IV-like enzymatic activity, expression of DPP-IV/CD26 and FAP-1α protein

In non-tumor brain tissue, the extent and intensity of DPP-IV-like staining was, as compared to GGT, much lower and expressed only in rarely occurring solitary perivascular/pericyte-like cells, while the endothelial lining was stained only exceptionally and at low intensity (Fig. 1G,H). Similarly, brain parenchyma was unstained or only stained in some samples at threshold intensity.

In low grade II gliomas, DPP-IV-like activity was low, except for a few very juxtavascular cells. In grade III and IV gliomas, the frequency of these cells, including pericyte- and mononuclear-like stained cells, increased and was significantly higher in 21% and 56% of patients, respectively (Fig. 1H,I). As shown in parallel sections in the paraffin-embedded tissue by UCHL-1 and CD68 antibodies, the tumors rich in DPP-IV-like positive mononuclear cells also possessed numerous T-lymphocytes and macrophages (Fig. 1, inserts J,K). In glioblastomas, the staining of vascular apparatus markedly increased (Fig. 1H,D) and the DPP-IV-like activity trimmed capillaries were often dilated and filled with thrombi or proliferating endothelium (Fig. 1N). DPP-IV-like activity occurred also in the spindle shaped- and smooth muscle- or pericyte-like cells in/around the hyperplastic vessels. In tumor parenchyma the weak DPP-IV-like activity (above the most frequent WASI values in controls 0.5 a.u.) started appearing in small segments of grade III tumors in 5 of 19 patients. However, the average staining intensity in this group was not significantly different from controls or grade II

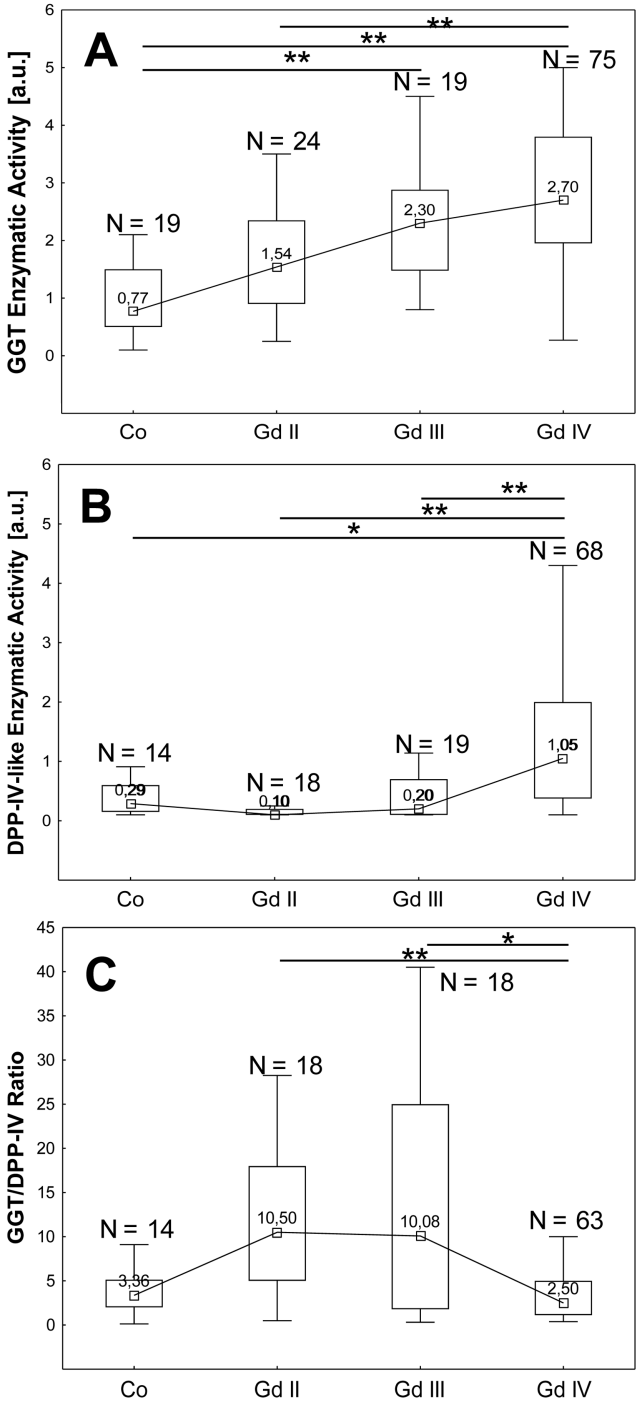

Fig. 2. A. γ-glutamyltranspeptidase (GGT). **B.** Dipeptidyl peptidase-IV (DPP-IV)-like activity. **C.** GGT/DPP-IV ratio in non-tumor control brain tissue and (Co) and grade (Gd) II – IV gliomas. Squares: Medians; Boxes: 25-75% percentile of measured values; Bars: Minimal and maximal values; asterisk: p<0.01, double asterisk: p<0.01; Kruskal-Wallis test.

gliomas (Fig. 2B). A significant increase of DPP-IV-like activity was found only in glioblastomas (Fig. 1N, Fig. 2B) in 44 of 68 patients, in which the average staining intensity exceeded controls 3.6-fold (WASI values, $p<0.05$, Fig. 2B). The DPP-IV-like positive fibers resembling processes of the hypertrophic and GFAP-positive/astrocytes occurred at some places (Fig. 1P).

Immunocytochemistry of canonic DPP-IV/CD26 showed that its expression in non-tumor brain tissue was low (WASI less than 0.5 a.u.). Higher values were, however, found in 20 of 43 examined tumors, including 2 of 7 (28%) of grade II-, 4 of 9 (44%) grade III-, and 14 of 26 (54%) of grade IV-samples (Table 2).

Similarly, the expression of FAP-1α was low in most non-tumor brain tissue samples. In tumors, it exceeded the most frequent control values in 16 of all 39 examined tumors, including 25% grade II (1 of 4, except one pleomorphic xanthoastrocytoma with extremely high value), 33% grade III (3 of 9) and 46% (12 of 26) grade IV gliomas (Table 2).

In some glioblastoma samples/segments, DPP-IV/CD26 and FAP-1α positive fiber-like structures (Fig. 1O, R) appeared, and occasionally they were close to capillaries. There was a significant correlation between the average staining intensity of DPP-IV/CD26 and FAP-1α expression (WASI values) in all grade tumors, including the grade IV group itself ($R=0.47$, $p<0.01$ and $R=0.43$, $p<0.05$, respectively). DPP-IV/CD26 and FAP-1α significantly correlated also with total DPP-IV-like activity in all grade tumors ($R=0.47$, $p<0.01$ and $R=0.39$, $p<0.01$, respectively). This correlation was weaker in glioblastomas for FAP-1α.

GGT and DPP-IV vs. Ki67, EGFR, vascularization and necrotization

Conventional diagnostic parameters, including p53, Ki67, vascularization, incidence of necrotic foci and EGFR expression (Table 3), increased with WHO tumor grade, except for p53. As shown in Fig. 3, up-regulation of GGT activity was *quasi* similar to Ki67, EGFR and vascularization. The course of up-regulation of DPP-IV-like activity in tumors of increasing grades was different and coincided only with necrotization, i.e. both these parameters significantly increased only in glioblastomas. In these tumors, the DPP-IV-like activity was often inversely proportional to Ki67 (see also below).

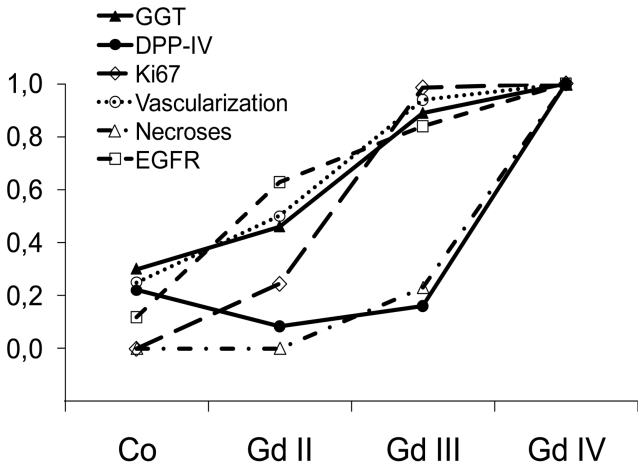

Fig. 3. Relationship among GGT, DPP-IV-like activity, vascularization, necrosis, Ki67 antigen and EGFR in gliomas of grade (Gd) II - IV. Normalized values 0 – 1.0 (maximal value). Co: Control non-tumor samples.

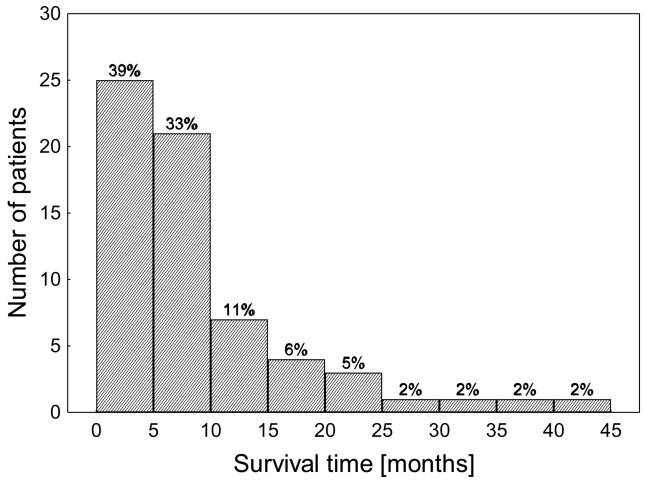

Fig. 4. Frequency distribution of the survival time of glioblastoma patients.

Table 3. Immunohistochemistry of p53, Ki67, vascularization, necrosis and EGFR in gliomas.

Glioma Grade	p53[A] [%]	Ki67[B] [%]	Vascularization[C] [a.u.]	Necrosis[D] [a.u.]	EGFR[E] [a.u.]
Non-tumor	ND	ND	0.00	0.00	0.13
Grade II	43.00	4.00	1.00	0.00	1.25
Grade III	20.00	10.00	1.92	0.25	1.58
Grade IV	25.00	20.00	2.00	1.50	1.93

All values: Medians; ND: Not determined; Upper index A-E: Regression coefficients for the corresponding columns (Spearman correlation): A=0.03, $p>0.05$; B=0.39, $p<0.01$; C=0.58, $p<0.01$; D=0.72, $p<0.01$; E=0.47, $p<0.01$

Expression of carbonic anhydrase-IX (CA-IX)

CA-IX, a cell surface zinc metalloenzyme, was examined as a marker of the tissue hypoxia-caused oxidative stress. Expression of this enzyme, examined only in glioblastomas, correlated with GGT catalytic activity, i.e. it was higher in samples with high GGT and vice versa in tumors with low GGT (Fig. 1S, insert T, and U, insert V, respectively).

Survival of glioblastoma patients

Post-operation survival time traced in 61 grade IV glioblastoma patients ranged from 1 to 42 months (median 7.0 months, Fig. 4). None of the applied histopathological diagnostic criteria, including p53, Ki67, the degree of tumor vascularization and necrotization, patients age, EGFR expression and tumor localization, significantly correlated with patient post-operation survival time (Cox proportional hazards regression model), nor was there a significant correlation between GGT or DPP-IV-like activity and the post-operation survival time. However, there was a significant inverse relationship between the DPP-IV-like activity and Ki67 values (R=0.30, p<0.05, Fig. 5).

Survival of patients on the post-operation oncological chemo-radiotherapy was longer than of those who were not eligible for this treatment (median 8.0 months, N=50 vs. 4.0 months, N =11). Except for the significantly higher perioperational DPP-IV-like activity in the oncologically treated patients (p<0.05), there were no significant differences in other parameters between these groups (Table 4).

K-means cluster analysis of the GGT/DPP-IV ratios and survival time (Fig. 6) revealed a subgroup of patients (6 of 54, 11.1%) surviving for a markedly shorter (7.5±3.5 months, 6 of 54, 11.1%) and longer time (23.0±5.7 months, 10 of 54 patients, 18.5%, Fig. 6, quadrants A and C, respectively, and Table 5). It should be noted that there was no longer-surviving patient with the GGT/DPP-IV ratio comparable to cluster A (Fig. 6, right upper empty quadrant D). The incidence of patients with (+) and without (-) oncological chemo-radiotherapy was similar in both these clusters (5+/1- and 9+/1-). There was also no difference between the age of patients in these two subgroups (Table 5). However, the average

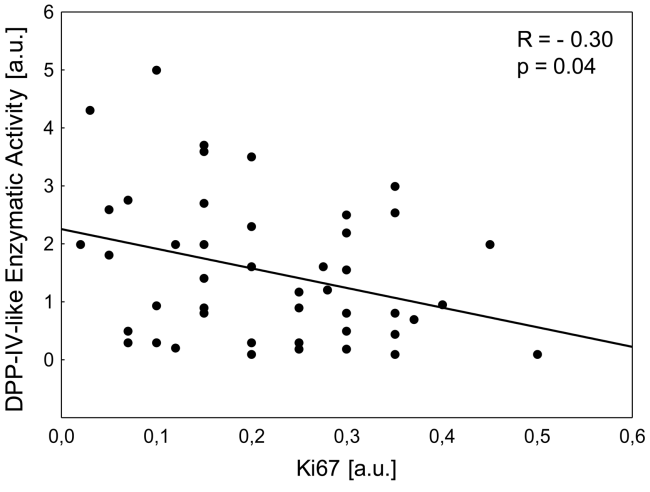

Fig. 5. DPP-IV-like activity vs. Ki67 in the glioblastoma patients.

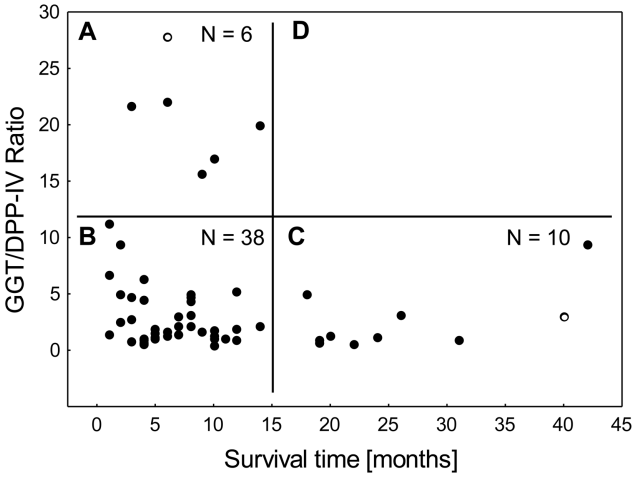

Fig. 6. GGT/DPP-IV ratio vs. survival time of patients with glioblastomas. Quadrants A-D determined by K-means cluster analysis. **A.** Short-term surviving patients (<15 months) with high GGT/DPP-IV ratio due to low DPP-IV-like enzymatic activity. **B.** The main group of short-term surviving patients with low GGT/DPP-IV ratio. **C.** A minor group of long-term surviving patients (> 15 months) with low GGT/DPP-IV ratio. **D.** Absence of long-term surviving patients with high GGT/DPP-IV ratio values.

Table 4. Differences between introductory i.e. perioperational values of Ki67, vascularization, necrosis, age, GGT and DPP-IV-like enzyme activities, and survival time in glioblastoma patients with or without post-operation oncological chemo-radiotherapy (CHRT).

Subgroups	N	Ki67 [%]	Vascularization [a.u.]	Necrosis [a.u.]	Age [years]	GGT [a.u.]	DPP-IV-like * [a.u.]	GGT/DPP-IV Ratio *	Survival ** [months]
CHRT	50	0.2±0.1	2.0±0.7	1.8±1.2	57.0±11.3	2.8±1.1	1.4±1.2	1.9±5.7	8.0±8.2
Non-CHRT	11	0.2±0.1	2.0±0.2	1.0±1.2	58.0±12.6	2.3±1.5	0.4±0.4	5.0±7.7	4.0±10.7

All values: Median ± SD; Mann Whitney U test, ** p<0.01, * p<0.05.

Table 5. Diagnostic histopathological criteria, GGT and DPP-IV-like enzyme activities and survival time in glioblastoma patients in subgroups A and C selected by GGT/DPP-IV ratio (Fig. 6).

Subgroups (Fig. 6)	Ki67 [%]	Vascularization [a.u.]	Necrosis [a.u.]	Age [years]	GGT [a.u.]	DPP-IV-like ** [a.u.]	GGT/DPP-IV Ratio **	Survival ** [months]
A	35.0±10.3	2.5±0.5	1.5±0.8	54.0±10.6	2.2±1.3	0.1±0.1	20.8±3.9	7.5±3.5
C	25.0±11.1	2.0±0.7	1.0±1.0	57.0±11.6	2.5±1.1	1.2±1.4	1.2±2.6	23.0±5.7

All values: Median ± SD; Mann Whitney U test, ** $p<0.01$.

DPP-IV-like activity in the short and longer surviving clusters of patients (A and C, respectively) differed 12-times. Expression of Ki67, tumor vascularization and necrotization was inversely proportional to DPP-IV-like activity in most glioblastoma patients of quadrant A and C (Fig. 6); the differences in medians of these parameters did not, however, reach statistical significance (Table 5).

Discussion

The study showed that up-regulation of GGT started already in low-malignance grade II tumors, mainly at/near the microvasculature. The relatively wide staining profiles of the capillary walls suggest that increased activity of GGT also initially comprised their astrocytic end-feet envelopes. In high-grade gliomas the GGT activity spread to glial/astrocytic parenchyma. Increased activity of GGT, or its immunochemically detected protein, was reported earlier in tumors of prostate, ovary, mammary gland, kidneys, liver, lungs, lymphatic cells, etc. (Hanigan et al., 1999a). Primarily the enzyme is supposed to participate in GSH metabolism stimulated by oxidative and metabolic stress caused by insufficient blood supply and the tumor inherent aberrancies of metabolic pathways (Mikkelsen et al., 2002; Proescholdt et al., 2005; Jung et al., 2011), and others. In our study, oxidative stress in glioblastomas was confirmed by increased expression of carbonic anhydrase-IX and, moreover, it was found to be proportional to GGT activity. Participation of GGT in the anti-oxidative and detoxification processes, together with other functions of GGT, such as enhanced transport of amino acids and iron ions (Dominici et al., 2003; Hawkins et al., 2006), can support the survival and proliferation of glioma cells. The absence of up-regulation of the antioxidative GGT/GSH system in the subgroup of high-grade tumors with low GGT, observed in our and an earlier immunocytochemical study (Schafer et al., 2001), could be substituted by other anti-oxidative pathways such as, e.g., the activation of superoxide dismutases, thioredoxin systems, neuroglobin synthesis or activation of prototypical oxidation stress-responsive NF-kappaB (Arner and Holmgren, 2000; Djavaheri-Mergny et al., 2002; Emara et al., 2009). Correlation of GGT activity with the degree of vascularization found in the present study suggests that GGT in gliomas can also be engaged in the defense to hypoxia via angiogenesis triggered by the GGT-rich pericytes, the process demonstrated in normal hypoxic brain (LaManna et al., 2004).

Compared to GGT, DPP-IV-like staining was lower, less extensive and shifted to high-grade gliomas, namely glioblastomas. It also started at the microvasculature, but with prevalence in the perivascular and extravasated mononuclear-like cells. The immune phenotype of these cells was confirmed in gliomas in this and earlier studies (Kleihues et al., 2000; Pro and Dang, 2004; Heimberger et al., 2008) and their DPP-IV positivity suggests activation of their immune/scavenging and adhesive properties (Lojda, 1981; Chen and Kelly, 2003; Kikkawa et al., 2003), including facilitation of their transit from the blood. The role of the marked up-regulation of DPP-IV-like activity in the microvasculature and tumor parenchyma of glioblastomas requires further study. It also included its two enzymatic homologs, the canonical DPP-IV/CD26 and FAP-1·. The latter were shown to support angiogenesis in cultures by participating in the breakdown of extracellular collagen and their heterodimers can stimulate proliferation and migration of endothelial cells (Zukowska-Grojec et al., 1998; Ghersi et al., 2006; Gonzalez-Gronow et al., 2008; Sato et al., 2011). Similar effects may have taken part in the activation of angiogenesis in high-grade gliomas shown in the present (Fig. 3) and earlier studies (Kleihues et al., 2000). Up-regulation of DPP-IV-like activity in the parenchyma of high-grade tumors, mainly glioblastomas, seems to concern mainly astrocytes. This is supported by the enzyme positivity in fibers resembling processes of reactive astrocytes. Increased activity of DPP-IV-like activity, as well as that of GGT, was also observed earlier in transformed astrocytes in cultures treated with cytostatics or radiation (Mares et al., 2003, 2009). Therefore, up-regulation of both these enzymes may belong to a common enzymatic adaptive response of tumor astrocytes to oxidative and metabolic stress.

DPP-IV can also exert anti-oncogenic effects by inhibiting migration and proliferation of tumor cells by cleavage of pro-oncogenic peptides, e.g. SDF-1α or substance P (Christopherson et al., 2002; Palma, 2006; Arscott et al., 2009) or promotion of cell adhesion to

extracellular fibronectin and collagen (Kikkawa et al., 2003). Slower tumor growth was also observed in mouse neuroblastoma transplants expressing transgenic DPP-IV *in situ* (Arscott et al., 2009) and prostate cancer cells in cultures. Higher DPP-IV activity was reported for less malignant renal tumors and melanomas (Wesley et al., 1999; Varona et al., 2010). The tumor growth suppressive effect of DPP-IV-like activity in the present study is demonstrated by an inverse correlation of the enzyme activity and Ki67 in most glioblastoma patients. It is also supported by shorter survival time of glioblastoma patients with high GGT/DPP-IV ratio (Fig. 6 cluster A), due to low DPP-IV-like activity. The significance of high DPP-IV-like activity in longer surviving patients (Fig. 6 cluster C) requires further study, including searching for other factors, because a high value of this enzymatic activity occurred also in some short surviving patients (Fig. 6 cluster B).

In conclusion, the study showed the grade of malignance- and tissue-compartment-dependent up-regulation of GGT and DPP-IV-like enzyme activity in human gliomas. In high-grade gliomas, the process of up-regulation of these enzymes started in the microvasculature and spread to the parenchyma, including reactive astrocytes. The data suggests that GGT supports the growth of all grades of gliomas by participating in glutathione metabolism and possibly also in angiogenesis via the GGT-rich and hypoxia sensitive pericytes. The GGT/GSH-mediated suppression of oxidative stress could, however, result in increased resistance of gliomas to chemo- and radiotherapy reported for some carcinomas, lymphatic tumors (Hanigan et al., 1999b) and also glioma cells in culture (Santin et al., 2009). The role of DPP-IV-like activity in human gliomas requires further elucidation. It seems to participate in the inflammatory/scavenging processes performed by the mononuclear-like cells, starting in grade III gliomas. In glioblastomas, DPP-IV-like activity can also be engaged in the regressive changes of the microvasculature and tumor parenchyma. Moreover, its antioncogenic/antiproliferative effect is suggested by its inverse correlation with Ki67 in most, though not all glioblastomas, as well as in the worse post-operation prognosis of patients with the lowest values of this enzyme activity, namely, when accompanied by high GGT. Finally, up-regulation of the two enzymes can serve as a marker of reactive tumor astrocytes and the high DPP-IV-like activity also as an auxiliary criterion for their differentiation from grade III gliomas.

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