Heterogeneity of molecular forms of dipeptidyl peptidase-IV and fibroblast activation protein in human glioblastomas

Ivana Matrasova, Petr Busek, Eva Balaziova, Aleksi Sedo

Background and Aims. Proteolytic enzymes contribute to the progression of various cancers. We previously reported increased expression of the proline specific peptidases dipeptidyl peptidase-IV (DPP-IV) and its closest paralogue fibroblast activation protein (FAP) in human glioblastomas. Here we analyze the molecular heterogeneity of DPP-IV and FAP in glioblastomas.

Methods. ELISA, isoelectric focusing, 1D and 2D electrophoresis followed by WB or enzyme overlay assay were utilized to analyze DPP-IV and FAP isoforms. Cell fractionation using a Percoll gradient and deglycosylation with PNGase F were performed to analyze the possible basis of DPP-IV and FAP microheterogeneity.

Results. Molecular forms of DPP-IV with an estimated molecular weight of 140-160 kDa and a pI predominantly 5.8 were detected in human glioblastoma; in some tumors additional isoforms with a more acidic (3.5-5.5) as well as alkaline (8.1) pI were revealed. Using 2D electrophoresis, two to three molecular forms of FAP with an alkaline (7.0-8.5) pI and an estimated MW of 120-140 kDa were identified in glioblastoma tissues. In glioma cell lines *in vitro,* several isoforms of both enzymes were expressed, however the alkalic forms present in glioblastoma tissues were not detected. Removal of N-linked oligosaccharides decreased the estimated molecular weight of both enzymes; the overall pattern of molecular forms nevertheless remained unchanged.

Conclusion. Several isoforms of DPP-IV and FAP are present in glioblastoma tissue. The absence of alkaline isoforms of both enzymes in glioma cell lines however suggests that isoforms from other, most likely stromal, cell types contribute to the overall pattern seen in glioblastoma tissues.

Key words: brain tumor, CD26, DPP-4, FAP, isoenzyme, seprase

Received: January 10, 2017; Accepted with revision: March 17, 2017; Available online: April 26, 2017
https://doi.org/10.5507/bp.2017.010

Institute of Biochemistry and Experimental Oncology, 1st Faculty of Medicine, Charles University in Prague, U Nemocnice 5, 12853 Prague 2, Czech Republic
Corresponding author: Aleksi Sedo, e-mail: aleksi@cesnet.cz

INTRODUCTION

Malignant gliomas are the most frequent primary tumors of the central nervous system[1]. Of these, glioblastomas (grade IV gliomas) represent the most malignant form with almost 100% mortality, aggressive and diffuse infiltrative growth and limited response to therapy[2]. As reviewed previously, several proteases were found to be deregulated in gliomas and critically contribute to the disease progression[3].

The almost ubiquitously expressed dipeptidyl peptidase-IV (DPP-IV, EC 3.4.14.5) and its closest paralogue fibroblast activation protein (FAP, EC 3.4.21.B28), characteristically expressed in the cancer and remodeling tissues, were implicated in the pathogenesis of various cancers. The functional consequence of their dysregulation may be cancer specific[4-6], possibly depending both on the available substrates of particular protease and on the cell population expressing the enzymes within the given tumor. The post-proline hydrolytic activity of DPP-IV and FAP is important for the processing of biologically active peptides and in the case of FAP also for the remodeling of the extracellular matrix[7,8]. Both proteases are involved in the regulation of cell differentiation, adhesion and migration by their proteolytic activity and also non-hydrolytic interactions[9,10]. Our previous reports demonstrated that the expression DPP-IV and FAP is increased in glioblastomas[11,12]. The data on their pathogenetic role in brain tumors is limited- DPP-IV inhibits glioma cell growth, in large part independently of its enzymatic activity[13], and FAP likely influences the interaction of glioma cells with the surrounding extracellular matrix (our unpublished results and ref.[6]).

DPP-IV and FAP are type II- transmembrane proteins that are enzymatically active as homodimers with a molecular weight (MW) typically of 220-240 kDa and 170 kDa, respectively[14,15]. Enzymatically active soluble forms of both enzymes lacking the transmembrane region are found in blood plasma, and in the case of DPP-IV also in other bodily fluids, but their origin is largely speculative[16,17]. In addition to these forms which are probably the result of shedding from the plasma membrane by an unknown protease, substantial molecular heterogeneity has been reported for both DPP-IV and FAP from various sources, and these molecular forms are thought to have unique pathophysiological functions. A spectrum of pI forms of DPP-IV was described in human plasma[18,19] and placenta[20]. Multiple molecular forms of DPP-IV were

also identified in normal and cancer lung tissues[21] and in glioma cells *in vitro*[22,23]. Interestingly, chemically induced differentiation of C6 glioma cells was accompanied by changes in the proportion of the DPP-IV isoforms[23]. In T lymphocytes, Kahne et al. identified 11 immunoreactive molecular forms of DPP-IV with pI range between 3.5 and 5.9. In addition, the expression pattern and subcellular localization of these isoforms was affected by the mitogenic stimulation of the cells[24]. The data on recombinantly produced DPP-IV suggest that the heterogeneity of molecular forms of DPP-IV may in part be a consequence of the differential glycosylation of the enzyme[25].

In contrast to DPP-IV for which only one mRNA has been reported[26], two alternative splicing forms of FAP were found in melanoma cells: one encoding a 97 kDa full-length transmembrane monomeric form and a shortened form encoding the 50–70 kDa C terminal part of the protein containing the catalytic region, but lacking a large part of the N terminal including the whole transmembrane and intracellular domain[27]. Furthermore, transgenic FAP lacking the cytoplasmic and transmembrane domains was converted into 50-70 kDa forms by putative EDTA-sensitive activators and the resulting shortened forms of FAP exhibited up to a 7 fold increase in the gelatinolytic activity[28]. In addition, the FAP amino acid sequence contains 5 potential glycosylation sites[15], which may give rise to the existence of molecular forms similarly to DPP-IV. Their presence has nevertheless not been explored so far. Our study for the first time analyzes the molecular forms of DPP-IV and FAP in human glioma tissues and their possible origin.

MATERIAL AND METHODS

Brain tissue samples

Brain tissue samples were collected from 51 patients undergoing astrocytic tumor resection, non-tumorous brain specimens were obtained from 15 patients in whom brain surgery was performed for drug-resistant temporal lobe epilepsy[12]. Written informed consent was obtained from the patients before their entry into the study according to the guidelines of institutional Ethics Committee conducted in accordance with the Declaration of Helsinki. The tumors were graded in compliance with the 2007 WHO Classification Criteria. Tissue samples, clear of macroscopic vessels and necrosis, were frozen on solid CO_2 and then stored at -80 °C.

Human glioma cell lines

U138MG, U87MG and U118MG (WHO grade IV, acquired from Cell Line Services, Germany) were cultured on the Nunc tissue plastic (Thermo scientific, Langenselbold, Germany) in the Dulbecco's modified Eagle's medium (DMEM; Sigma, Prague, Czech Republic) supplemented with 10% foetal calf serum (FCS; Sigma) under a humidified (90%) atmosphere of 5% CO_2 and 95% air at 37 °C.

Non-denaturing, non-reducing SDS polyacrylamide electrophoresis (SDS PAGE)

Tissue samples were homogenized in ice-cold phosphate buffered saline (PBS) pH 6.0 with an Ultra-Turrax homogenizer fitted with an S8N-5G probe (IKA, Staufen, Germany) to a final 15% w/v concentration. Homogenates were mixed 1:1 with a lysis buffer (10 mM Tris-HCl pH 7.5, containing 1 mM EGTA, 1 mM Na_2EDTA, 1% Triton X-100, 0.1% SDS, and 10% glycerol) supplemented with protease inhibitors (final concentration pepstatin A 25 μM, AEBSF 200 μM, E-64 50 μM) and centrifuged at 27 000 g, 4 °C for 30 min. Total cell lysates (10 x 10^6 cells/mL) were prepared on ice in a lysis buffer supplemented with protease inhibitors. To preserve the native enzyme structure and enzymatic activity, all samples were analyzed under non-reducing and non-denaturing conditions in discontinuous (4% stacking, 7% resolving) 1.5mm gels. Samples were mixed with 4x Laemmli sample buffer, 40 μg of total protein assayed according to Lowry[29] were loaded on the gels. The separation was carried out in an electrode buffer containing 25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.3 at constant voltage (60 V for 30 min followed by 140 V for 90 min).

ELISA

The DPP-IV and FAP proteins were assayed in tissue lysates by DuoSet DPP-IV and DuoSet FAP ELISA kits (DY1180 and DY3715, R&D Systems, Abingdon, UK) according to the manufacturer´s instructions. Briefly, the 96-well transparent plate was coated by capture antibodies diluted in PBS (anti DPP-IV, cat.no. 842127, 2 μg/mL and anti FAP, cat.no. 842997, 1 μg/mL; overnight incubation at room temperature (RT)). After washing and blocking the plate in 1% bovine serum albumin in PBS, the samples were applied for two hours. After washing, biotinylated detection antibodies (1 μg/mL) and streptavidin-horseradish peroxidase were applied. Substrate Reagent Pack (DY999, R&D Systems) was used for the visualization. The reaction was terminated by 2M sulphuric acid. The absorbance of samples was measured at 450 nm using the microplate reader Sunrise (Tecan, Malmedorf, Switzerland). The measured absorbance values were corrected by subtracting the absorbance values obtained at a reference wavelength of 570 nm. The resulting differential absorbance values were used for the constructing the calibration curves and data evaluation.

Western blot analysis (immunoblotting)

Gels were equilibrated in Bjerrum and Schafer-Nielsen transfer buffer containing 20% methanol. Proteins were transferred onto the PVDF membrane using a semidry blotting system (Bio-Rad, USA). PVDF membranes were thoroughly rinsed in 0.05% Tween 20 in Tris Buffer Saline pH 7.5 (0.05% TTBS) and blocked in 5% non-fat dry milk (NFDM) prior to incubation with the primary antibodies at 4°C, overnight. Membrane washing in TTBS was followed by incubation with a horse-reddish peroxidase conjugated secondary antibody at RT for 60 min. The used antibodies are listed in Table I. The blots were de-

Table 1. Antibodies used for immunoblotting.

Blocking Agent	Primary Antibody	Species/ clone/ Company	Dilution	Secondary Antibody	Species/ clone Company	Dilution
5% NFDM	Anti-Human DPP-IV	Rat, E19, Vitatex USA	1:1000	PreAdsorbed Anti-Rat, ab6257	Goat, Abcam UK	1:20000
5% NFDM	Anti-Human FAP	Rat, D8, Vitatex USA	1:5000	PreAdsorbed Anti-Rat, ab6257	Goat, Abcam UK	1:20000
5% NFDM	Anti-GAPDH	Rabbit, Merck-Millipore USA	1:1000	Anti-Rabbit	Sheep, Amesrham UK	1:20000
5% NFDM	Membrane Fraction WB Cocktail	Ab140365 Abcam, UK	1:500	Secondary Antibody Cocktail	Ab140365 Abcam, UK	1:500

NFDM= non-fat dry milk

veloped using Luminata Forte (Merck-Millipore, USA) and exposed to a photographic film (Hyperfilm™ ECL, Amersham).

DPP-IV enzyme overlay assay

The exopeptidase activity of DPP-IV was visualized by an enzyme overlay assay using the fluorogenic substrate 7-(Glycyl-L-Prolylamido)-4-methylcoumarin (H-GP-AMC, final concentration 100 μM). Gels were equilibrated in phosphate buffer pH 7.5, a cellulose acetate membrane impregnated with the substrate was placed on the top of the gel and covered by a glass plate to prevent evaporation. The signal was visualized after 30 min incubation at 37 °C on a transluminator (VilberLourmat, France) with an excitation wavelength of 360 nm.

Isoelectric focusing and 2D electrophoresis

Tissue samples or cells (10 million/mL) were mechanically homogenized in the sample/rehydration buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% w/v BioLyte 3/10 ampholytes, protease inhibitors) and incubated on an orbital shaker for 30 min at RT. Samples were cleared by centrifugation (27 000 g, 20 °C for 30 min). Protein concentration was determined by the Bradford assay (Bio-Rad, USA) according to the manufacturer's instructions. 150 μg of total protein was loaded onto 7 cm long Immobilized pH gradient (IPG) strips (pH 3-10 or pH 4-7, Bio-Rad, USA), passive rehydration was performed at RT, for 12-16 h. Isoelectric focusing (IEF) was performed under the following conditions: 250 V for 20 min (linear ramp); 4 000 V for 120 min (linear ramp); 10 000 VH at 4 000 V (rapid ramp). After isoelectric focusing, the IPG strips were incubated in the equilibration buffer I (6 M urea, 30% glycerol, 2% CHAPS, 50 mM Tris pH 6.8 and 1% DTT) for 15 min followed by incubation in the equilibration buffer II (6 M urea, 30% glycerol, 2% CHAPS, 50 mM Tris pH 6.8 and 2.5% IAA) for additional 15 min. IPG strips were then either used for the visualization of the exopeptidase activity of DPP-IV by an enzyme overlay assay, or the separation on 8% polyacrylamide gels in the second dimension was performed at 140V.

Deglycosylation by peptide-N-glycosidase F

Deglycosylation by peptide-N-glycosidase F (PNGase F, E.C. 3.5.1.52, Sigma Aldrich, USA) was performed according to the manufacturer's instructions with the following modifications: cell lysates were prepared as described above and 20 μg of total native protein was subjected to deglycosylation by 0.01 unit of PNGase F at 37 °C for 24 h. Control samples were incubated without PNGase F under the same conditions. The samples were subsequently mixed with the 4x Laemmli sample buffer and separated using non-denaturing, non-reducing SDS PAGE.

Cell fractionation using continuous Percoll gradient

Cells were mildly homogenized in ice-cold isotonic medium (40 million/mL, 250 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA, 3 mM $MgCl_2$) pH 7.6 supplemented with protease inhibitors in a 2 mL Dounce-tissue grinder (SigmaAldrich, Mexico) for 5 min. Nuclei were removed by centrifugation at 250 g, 4 °C for 10min, the supernatant was applied on top of a 4 mL of 27.4% Percoll (Pharmacia, Sweden). Ultracentrifugation was performed at 65 000 g, 4 °C for 60 min (MTX 150 Sorvall, rotor S560-ST, ThermoScientific). 50 μL fractions were collected and individual subcellular compartments were identified using the Membrane Fraction WB Cocktail (Abcam, UK). For IEF, the samples were diluted 1:4 using the sample/rehydration buffer.

Immunocytochemistry

Cells were cultured on glass coverslips, fixed by 1:1 aceton:methanol for 2 min at RT. Nonspecific binding was blocked by 10% fetal calf serum plus 1% bovine serum albumin in Tris-buffered saline for 1 hour. The samples were incubated with the monoclonal primary antibodies anti-DPP-IV (clone MA 261, 1:100) or anti-FAP (clone F19, 56 μg/mL) overnight at 4°C, followed by AF 488 donkey anti-mouse IgG (A2120, ThermoFisher Scientific, 1:500, 1 h at RT). 400 μM ToPro (ThermoFisher Scientific) or 50 ng/mL Hoechst 33258 (Sigma-Aldrich, St. Louis, USA) added to the solution of the secondary antibodies were used for nuclear counterstaining. The primary antibodies were omitted in the staining controls. Slides were mounted in Aqua Polymount (Polysciences, Eppelheim,

Germany) and viewed on the Olympus IX 81 confocal microscope equipped with the 488, 543 and 633 nm lasers (FluoView 300, Olympus, Prague, Czech Republic).

RESULTS

Expression of DPP-IV and FAP increases with increasing glioma grade

The expression of DPP-IV and FAP was analyzed in high-grade gliomas (grade IV; n=39, grade III; n=5), low-grade gliomas (grade II; n=7) and non-tumorous brain tissue (pharmacoresistant epilepsy; n=15). The concentration of both DPP-IV and FAP as determined by ELISA was significantly higher in glioblastomas (Fig. 1); for DPP-IV, the quantity determined by ELISA correlated with the DPP-IV enzymatic activity in tissue homogenates (r=0.79, $P<0.05$, data not shown). These data confirmed our previously reported results[11] in an independent patient cohort.

Molecular forms of DPP-IV and FAP in gliomas

All 66 patient samples were simultaneously analyzed using western blotting and an enzyme overlay assay after electrophoretic separation under non-denaturing, non-reducing conditions. The levels of DPP-IV and FAP were below the detection limit in all samples of the normal brain tissue (data not shown), but both molecules were detectable in grade III and IV gliomas (Fig. 2A**).** Substantial molecular heterogeneity of DPP-IV was present with up to three DPP-IV immunoreactive bands with an estimated molecular weight in the range of 140-160 kDa. The corresponding DPP-IV hydrolytic activity sensitive to a DPP-IV inhibitor was detected in the majority of these cases, suggesting the presence of enzymatically active molecular forms of canonical DPP-IV. Interestingly, in several samples containing only a single DPP-IV immunopositive band on WB, little or no enzymatic activity could be detected using the enzyme overlay assay (Fig. 2A). In contrast to several isoforms of DPP-IV, western blot analysis of the high-grade glioma tissue samples revealed only one molecular form of FAP with an electrophoretic mobility corresponding to 120-140 kDa (Fig. 2A).

Representative glioblastoma samples containing enzymatically active DPP-IV were further analyzed using isoelectric focusing. This confirmed the presence of several molecular forms of DPP-IV with the predominant isoform having pI 5.8. In some of the tumors additional isoforms with a more acidic (3.5-5.5) as well as alkaline (8.1) pI were detected (Fig. 2B). FAP immunodetection after 2D electrophoresis revealed the presence of two to three forms of FAP with an alkaline (7.0-8.5) pI and an estimated MW of 120-140 kDa (Fig. 2C).

Similarly to glioblastoma tissues, enzymatically active isoforms of DPP-IV with an electrophoretic mobility between 140-160 kDa were revealed in three permanent glioma cell lines (Fig. 2A). Using isoelectric focusing, the DPP-IV hydrolytic activity with a corresponding immunopositivity was detected predominantly at pI 5.8 with a MW of 140 kDa (Fig. 2D). In U118MG cells, which have the highest DPP-IV expression (data not shown), additional forms were detectable in the acidic region. FAP immunopositivity was only identified at an acidic pI between 4.2-4.8 (MW 140 kDa) and 5.8-6.2 (MW 140-200 kDa) in the three glioma cell lines.

Possible basis of the heterogeneity of DPP-IV and FAP molecular forms in glioma cells

Our previous results and literature data show that the majority of DPP-IV and FAP is present in the membrane

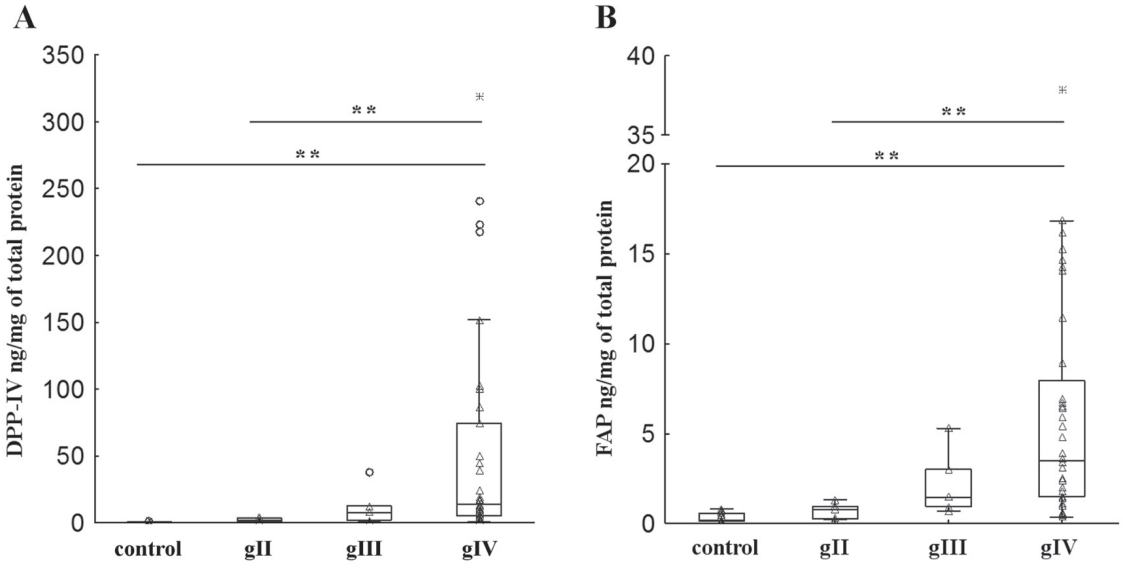

Fig. 1. DPP-IV and FAP expression in grade II-IV gliomas and non-tumorous brain tissue (pharmacoresistant epilepsy). Protein concentration of A) DPP-IV and B) FAP was assayed using ELISA. $^{**}P < 0.01$, Kruskal-Wallis test. Horizontal line- median, boxes- 25–75%, whiskers- range of non-remote values, white triangles- source data, white circles- remote values, asterisks- extremes.

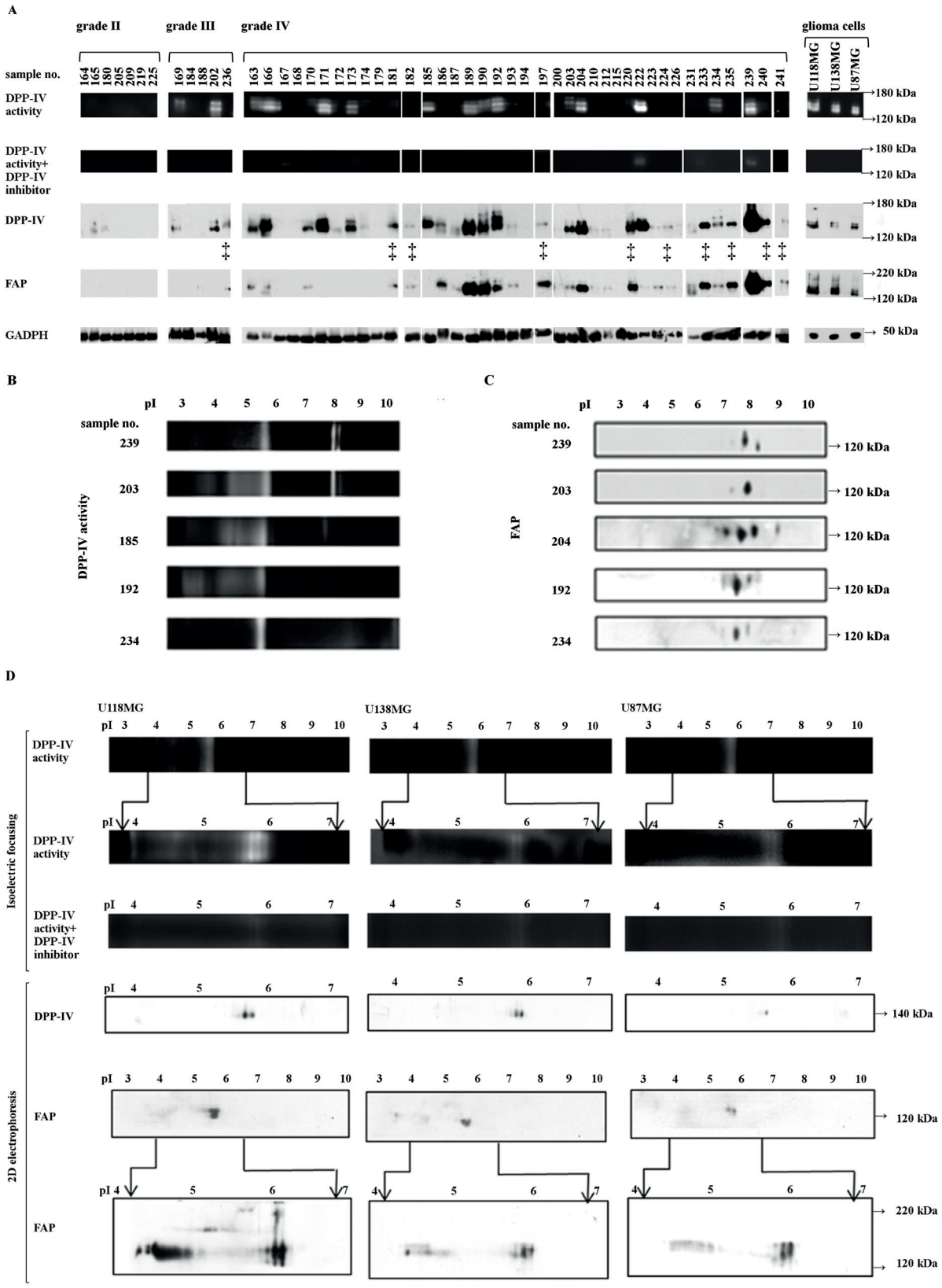

Fig. 2. Molecular forms of DPP-IV and FAP in human gliomas and glioma cell lines. A) Separation of DPP-IV and FAP molecular forms using SDS PAGE under non-denaturing and non-reducing conditions. DPP-IV enzymatic activity was detected using membrane overlay assay with and without a specific DPP-IV inhibitor. Immunodetection of DPP-IV, FAP and GAPDH was performed after transfer of the proteins to a PVDF membrane. Double dagger- tissues with a single DPP-IV band on WB without corresponding detectable DPP-IV enzymatic activity. GAPDH- glyceraldehyde phosphate dehydrogenase. B) Detection of the DPP-IV enzymatic activity in glioblastomas after separation of the molecular forms by isoelectric focusing. C) Immunodetection of FAP in glioblastomas after separation of the molecular forms by 2D electrophoresis. D) Analysis of DPP-IV and FAP molecular forms in human glioma cell lines using isoelectric focusing and 2D electrophoresis.

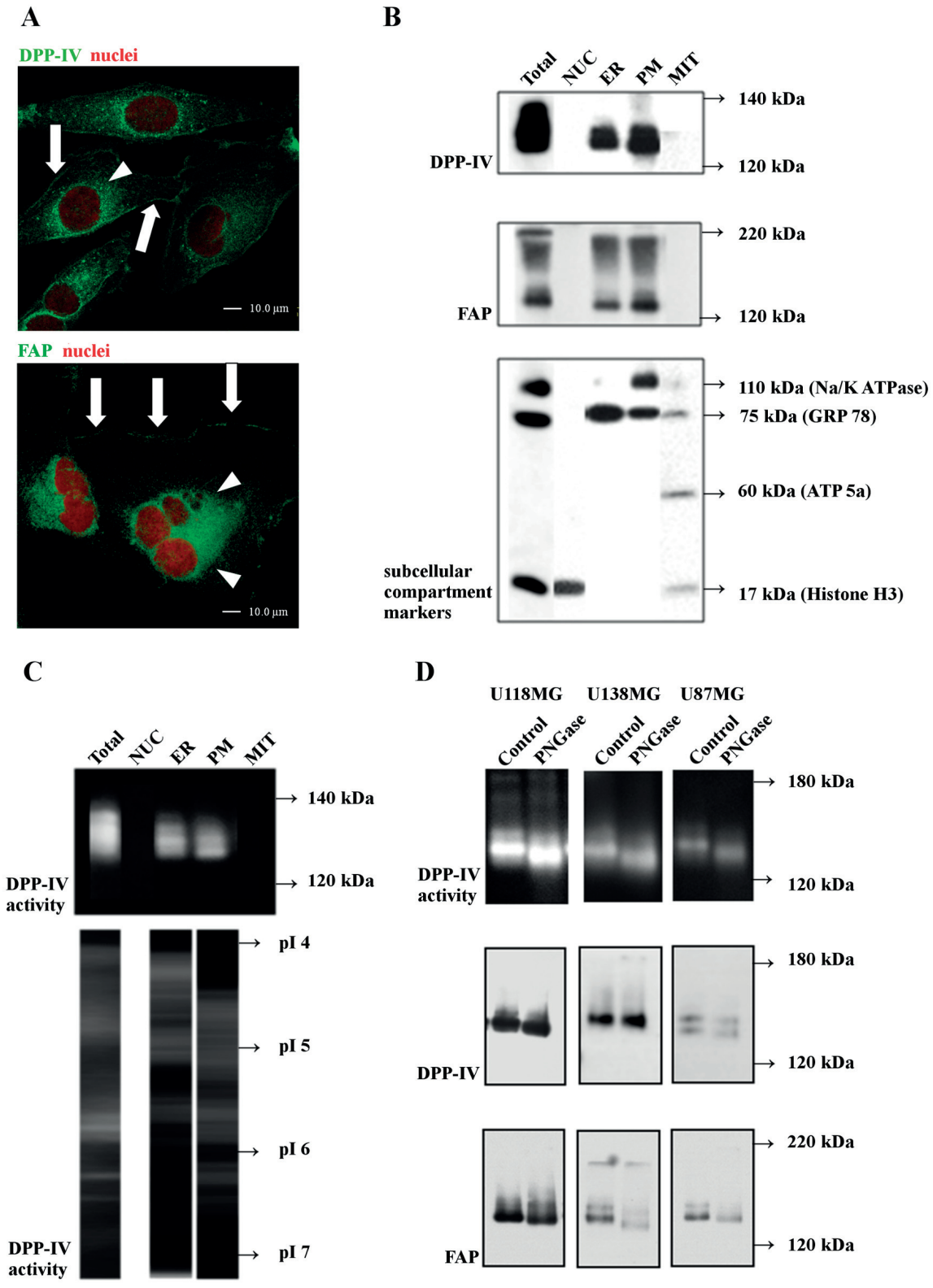

Fig. 3. Possible basis of the heterogeneity of DPP-IV and FAP molecular forms in glioma cells. A) Immunocytochemical visualization of DPP-IV and FAP in the plasma membrane (arrows) and intracellularly (arrow heads) in U118MG glioma cells. B) Immunodetection of DPP-IV, FAP and compartment markers in subcellular fractions in U118MG glioma cells. NUC= nuclear fraction, ER= endoplasmic reticulum fraction, PM= plasma membrane fraction, MIT= mitochondrial fraction. C) Detection of DPP-IV hydrolytic activity in subcellular fractions after SDS PAGE, and isoelectric focusing in U118MG glioma cells. D) Effect of deglycosylation on the electrophoretic mobility of DPP-IV and FAP molecular forms expressed in human glioma cell lines. PNGase F= peptide N-glycosidase F

fraction, possibly in the form of high molecular weight oligomeric complexes[30,31]. However, in addition to the plasma membrane, both DPP-IV and FAP are also localized in various intracellular compartments[32,33] (Fig. 3A). To assess whether the heterogeneity of molecular forms of DPP-IV and FAP reflects the presence of specific isoforms in subcellular compartments, cell fractionation using a continuous Percoll gradient was performed. Western blotting (Fig. 3B) and isoelectric focusing (Fig. 3C) confirmed that DPP-IV and FAP were dominantly present in the plasma membrane and the endoplasmic reticulum fractions. The estimated MW corresponded to the 140-160 kDa (DPP-IV) and 120-200 kDa (FAP) observed in glioma tissues. DPP-IV in the endoplasmic reticulum had dominantly an acidic pI between 4.4 and 5.1, while the pI of DPP-IV in the plasma membrane was more alkaline (4.8-5.8).

We further evaluated whether differential glycosylation might contribute to the heterogeneity of molecular forms of both molecules. Upon deglycosylation with PNGase F the estimated molecular weight decreased by approximately 10 kDa in comparison to non-treated samples. The overall pattern of molecular forms of both DPP-IV and FAP nevertheless remained unchanged (Fig. 3D). The removal of N-linked saccharides by PNGase F did not influence the hydrolytic activity of DPP-IV (data not shown).

DISCUSSION

Since the discovery of the multifunctional dipeptidyl peptidase-IV, several other proteases possessing similar enzymatic activity or sequence homology have been described and classified as "Dipeptidyl peptidase-IV activity and/or structure homologues" (DASH) (ref.[34]). Of these, DPP-IV and its closest paralogue FAP are dominantly plasma membrane localized and were implicated in cancerogenesis, most probably by interacting with local regulatory as well as structural molecules present in the cancer microenvironment[35,36].

The presence of heterogeneous molecular forms of DPP-IV observed in various biological sources, including transformed astrocytic cells[21,22], led us to presume the existence of specific isoforms of both enzymes in glioblastoma.

In the current study, we observed a significantly higher expression of DPP-IV and FAP proteins in high-grade gliomas compared to the grade II astrocytoma and the normal brain tissue, which confirmed our previous results[11]. There was a wide intertumoral variability in the expression of both molecules, possibly reflecting their differential expression in individual molecular subtypes of glioblastoma (ref.[12] and our unpublished data) and our unpublished data. FAP immunopositivity was detectable by WB in most of the high-grade glioma samples, but was absent in the DPP-IV negative gliomas. This is consistent with ours as well as other authors' reports suggesting the co-expression and possible coregulation of DPP-IV and FAP in glioma cells and tissues[11,31], human pancreatic alpha cells[37] and in some cancer cell lines[38]. The coexpression of DPP-IV and FAP was also described in endothelial cells, where both molecules are part of proteolytically active heteromeric aggregates with a molecular weight of 820 kDa, promoting cell migration and invasion[30].

We further demonstrate the presence of several enzymatically active forms of canonical DPP-IV in most of the high-grade gliomas. Using non-denaturing SDS PAGE, we show that the migration pattern of DPP-IV present in glioblastomas corresponds well with the one observed in glioblastoma cell lines. Interestingly, the pattern consisting of three molecular forms of the enzyme activity was restricted to the samples which in parallel exhibited more than one band of DPP-IV immunopositivity. This may be explained by the fact that the samples without detectable activity in the enzyme overlay assay had lower quantity of DPP-IV (measured by ELISA) and thus their enzymatic activity may be below the detection limit of the method. However, since we could not assess the specific enzymatic activity of individual DPP-IV isoforms, we cannot definitely exclude the existence of hydrolytically inactive isoform of DPP-IV in glioblastomas.

Isoelectric focusing revealed several isoforms of DPP-IV in glioblastomas and glioma cell lines, typically having an acidic pI in similar ranges as described for DPP-IV in human lymphocytes[24]. Interestingly, alkalic isoforms were detected in several tissue samples but were not observed in any of the analyzed glioma cell lines. Thus, it is possible to presume that these alkalic isoforms might originate from other cellular source of DPP-IV within the glioblastoma tissue. Indeed, we previously described the expression of DPP-IV enzymatic activity in the microvasculature and mononuclear-like cells in glioblastomas[39]. Alternatively, transformed glial cells could express different isoforms of DPP-IV when being in direct contact with other elements of the tumor microenvironment.

Immunodetection of FAP after native electrophoresis demonstrated only a single form, while the 2D electrophoretic separation revealed several isoforms of FAP in glioma tissue as well as in glioma cell lines. Previous studies reported a pI of 5 for FAP isolated from the human melanoma cell line LOX[14], which corresponds with our results in glioma cells. Nevertheless to the best of our knowledge, the substantial molecular heterogeneity of FAP isoforms has not been observed so far. Interestingly, the isoforms detected in glioblastoma tissues exhibited more alkaline pI compared to the glioma cell lines. Similarly to DPP-IV, these isoforms may originate from the stromal cells expressing FAP in glioblastomas[12] or reflect the differences in FAP isoforms expressed by glioma cells *in vitro* and *in situ*.

Our previous results and literature data show that the majority of DPP-IV and FAP is present in the membrane fraction, possibly in the form of high molecular weight oligomeric complexes[30,31]. However, in addition to the plasma membrane, both DPP-IV and FAP are also localized in various intracellular compartments[32,33]. The expression pattern of individual MW isoforms of the enzymes was not substantially different between plasma membrane and endoplasmic reticulum, although acidic pI isoforms

of DPP-IV were more prevalent in the endoplasmatic reticulum than in the plasma membrane fraction.

The molecular mechanisms responsible for the heterogeneity of DPP-IV and FAP remain to be identified. Removal of N-linked polysaccharides from both DPP-IV and FAP using PNGase did not influence the proportion of their isoforms, but only slightly shifted the whole pattern toward to the lower molecular weights. Similarly, literature data show that deglycosylation has no effect on the molecular heterogeneity of DPP-IV isolated from human leukocytes or placenta[20,24]. The DPP-IV hydrolytic activity measured in the samples treated with PNGase F remained comparable to the control samples (data not shown), supporting the conclusion of Aertgeerts et al that deglycosylation of DPP-IV does not to affect itsr hydrolytic activity[15,40]. Overall these data suggest that differential glycosylation does not play a major role in generating the variability of DPP-IV and FAP.

CONCLUSION

Using enzymatic and immunochemical methods, we describe for the first time that DPP-IV and FAP are present in various isoforms in high grade gliomas with a varying pattern in individual tumors. Part of this variability corresponds with the patterns observed in glioma cell lines; nevertheless the absence of alkalic isoforms of both enzymes in the glioma cell lines suggests possible contribution of the stromal cells to the pattern observed in glioblastoma tissues. The microheterogeneity of both enzymes is most probably not due to differential glycosylation.

Acknowledgement: This work was supported by Charles University projects GA UK No 67612 and UNCE 204013, Ministry of Health of the Czech Republic, grant No 15-31379A and EATRIS-CZLM2015064.

Author contributions: IM: manuscript writing, figures, data collection, analysis and interpretation;
PB: manuscript writing, data collection, analysis and interpretation; EB: data collection and analysis;
AS: manuscript writing, data interpretation.

Conflict of interest statement: The authors state that there are no conflicts of interest regarding the publication of this article.

REFERENCES

1. Taylor LP. Diagnosis, treatment, and prognosis of glioma: five new things. Neurology 2010; 75(18 Suppl 1):S28-32.
2. Hou LC, Veeravagu A, Hsu AR, Tse VC. Recurrent glioblastoma multiforme: a review of natural history and management options. Neurosurg Focus 2006;20(4):E5.
3. Busek P, Prevorovsky M, Krepela E, Sedo A, Glioma-Associated Proteases, in Glioma Cell Biology. 2014, Springer. p. 317-395.
4. Wesley UV, Tiwari S, Houghton AN. Role for dipeptidyl peptidase IV in tumor suppression of human non small cell lung carcinoma cells. Int J Cancer 2004;109(6):855-66.
5. Busek P, Stremenova J, Sedo A. Dipeptidyl peptidase-IV enzymatic activity bearing molecules in human brain tumors-good or evil? Front Biosci 2008;13:2319-26.
6. Mentlein R, Hattermann K, Hemion C, Jungbluth AA, Held-Feindt J. Expression and role of the cell surface protease seprase/fibroblast activation protein-alpha (FAP-alpha) in astroglial tumors. Biol Chem 2011;392(3):199-207.
7. Mentlein R. Dipeptidyl-peptidase IV (CD26)--role in the inactivation of regulatory peptides. Regul Pept 1999;85(1):9-24.
8. Park JE, Lenter MC, Zimmermann RN, Garin-Chesa P, Old LJ, Rettig WJ. Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts. J Biol Chem 1999;274(51):36505-12.
9. Chen WT, Kelly T, Ghersi G. DPPIV, seprase, and related serine peptidases in multiple cellular functions. Curr Top Dev Biol 2003;54:207-32.
10. Gorrell MD, Wang XM, Park J, Ajami K, Yu DM, Knott H, Seth D, McCaughan GW. Structure and function in dipeptidyl peptidase IV and related proteins. Adv Exp Med Biol 2006;575:45-54.
11. Stremenova J, Krepela E, Mares V, Trim J, Dbaly V, Marek J, Vanickova Z, Lisa V, Yea C, Sedo A. Expression and enzymatic activity of dipeptidyl peptidase-IV in human astrocytic tumours are associated with tumour grade. Int J Oncol 2007;31(4):785-92.
12. Busek P, Balaziova E, Matrasova I, Hilser M, Tomas R, Syrucek M, Zemanova Z, Krepela E, Belacek J, Sedo A. Fibroblast activation protein alpha is expressed by transformed and stromal cells and is associated with mesenchymal features in glioblastoma. Tumour Biol 2016;37(10):13961-71.
13. Busek P, Stremenova J, Sromova L, Hilser M, Balaziova E, Kosek D, Trylcova J, Strnad H, Krepela E, Sedo A. Dipeptidyl peptidase-IV inhibits glioma cell growth independent of its enzymatic activity. Int J Biochem Cell Biol 2012;44(5): 38-47.
14. Pineiro-Sanchez ML, Goldstein LA, Dodt J, Howard L, Yeh Y, Tran H, Argraves WS, Chen WT. Identification of the 170-kDa melanoma membrane-bound gelatinase (seprase) as a serine integral membrane protease. J Biol Chem 1997;272(12):7595-601.
15. Aertgeerts K, Levin I, Shi L, Snell GP, Jennings A, Prasad GS, Zhang Y, Kraus ML, Salakian S, Sridhar V, Wijnands R, Tennant MG. Structural and kinetic analysis of the substrate specificity of human fibroblast activation protein alpha. J Biol Chem 2005;280(20):19441-4.
16. Cordero OJ, Imbernon M, Chiara LD, Martinez-Zorzano VS, Ayude D, de la Cadena MP, Rodriguez-Berrocal FJ. Potential of soluble CD26 as a serum marker for colorectal cancer detection. World J Clin Oncol 2011;2(6):245-61.
17. Keane FM, Yao TW, Seelk S, Gall MG, Chowdhury S, Poplawski SE, Lai JH, Li Y, Wu W, Farrell P, Vieira de Ribeiro AJ, Osborne B, Yu DM, Seth D, Rahman K, Haber P, Topaloglu AK, Wang C, Thomson S, Hennessy A, Prins J, Twigg SM, McLennan SV, McCaughan GW, Bachovchin WW, Gorrell MD. Quantitation of fibroblast activation protein (FAP)-specific protease activity in mouse, baboon and human fluids and organs. FEBS Open Bio 2013;4:43-54.
18. Krepela E, Kraml J, Vicar J, Kadlecova L, Kasafirek E. Demonstration of two molecular forms of dipeptidyl peptidase IV in normal human serum. Physiol Bohemoslov 1983;32(6):486-96.
19. O'Mullan P, Craft D, Yi J, Kim C, Gelfand CA. High-Resolution Isoelectric Separation of Dipeptidyl Peptidase IV Facilitates Molecular Understanding of Posttranslational Modifications, Enzyme Activity, and a Link to Type II Diabetes. Journal of Clinical Ligand Assay 2007;30(3):90.
20. Puschel G, Mentlein R, Heymann E. Isolation and characterization of dipeptidyl peptidase IV from human placenta. Eur J Biochem 1982;126(2):359-65.
21. Sedo A, Krepela E, Kasafirek E, Kraml J, Kadlecova L. Dipeptidyl peptidase IV in the human lung and spinocellular lung cancer. Physiol Res 1991;40(3):359-62.
22. Medeiros Mdos S, Balmforth AJ, Vaughan PF, Turner AJ. Hydrolysis of atrial and brain natriuretic peptides by the human astrocytoma clone D384 and the neuroblastoma line SH-SY5Y. Neuroendocrinology 1991;54(3):295-302.
23. Sedo A, Malik R, Krepela E. Dipeptidyl peptidase IV in C6 rat glioma cell line differentiation. Biol Chem 1998;379(1):39-44.
24. Kahne T, Kroning H, Thiel U, Ulmer AJ, Flad HD, Ansorge S. Alterations in structure and cellular localization of molecular forms of DP IV/CD26 during T cell activation. Cell Immunol 1996;170(1):63-70.
25. Baer JW, Gerhartz B, Hoffmann T, Rosche F, Demuth HU. Characterisation of human DP IV produced by a Pichia pastoris expression system. Adv Exp Med Biol 2003;524:103-8.
26. Bauvois B, Djavaheri-Mergny M, Rouillard D, Dumont J, Wietzerbin J. Regulation of CD26/DPPIV gene expression by interferons and retinoic acid in tumor B cells. Oncogene 2000;19(2):265-72.

27. Goldstein LA, Chen WT. Identification of an alternatively spliced seprase mRNA that encodes a novel intracellular isoform. J Biol Chem 2000;275(4):2554-9.
28. Chen D, Kennedy A, Wang JY, Zeng W, Zhao Q, Pearl M, Zhang M, Suo Z, Nesland JM, Qiao Y, Ng AK, Hirashima N, Yamane T, Mori Y, Mitsumata M, Ghersi G, Chen WT. Activation of EDTA-resistant gelatinases in malignant human tumors. Cancer Res 2006;66(20):9977-85.
29. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193(1):265-75.
30. Ghersi G, Zhao Q, Salamone M, Yeh Y, Zucker S, Chen WT. The protease complex consisting of dipeptidyl peptidase IV and seprase plays a role in the migration and invasion of human endothelial cells in collagenous matrices. Cancer Res 2006;66(9):4652-61.
31. Balaziova E, Busek P, Stremenova J, Sromova L, Krepela E, Lizcova L, Sedo A. Coupled expression of dipeptidyl peptidase-IV and fibroblast activation protein-alpha in transformed astrocytic cells. Mol Cell Biochem 2011;354(1-2):283-9.
32. Mori Y, Kono K, Matsumoto Y, Fujii H, Yamane T, Mitsumata M, Chen WT. The expression of a type II transmembrane serine protease (Seprase) in human gastric carcinoma. Oncology 2004;67(5-6):411-9.
33. Zhang M, Xu L, Wang X, Sun B, Ding J. Expression levels of seprase/FAPalpha and DPPIV/CD26 in epithelial ovarian carcinoma. Oncol Lett 2015;10(1):34-42.
34. Sedo A, Malik R. Dipeptidyl peptidase IV-like molecules: homologous proteins or homologous activities? Biochim Biophys Acta 2001;1550(2):107-16.
35. Iwata S, Morimoto C. CD26/dipeptidyl peptidase IV in context. The different roles of a multifunctional ectoenzyme in malignant transformation. J Exp Med 1999;190(3):301-6.
36. Ge Y, Zhan F, Barlogie B, Epstein J, Shaughnessy J, Jr., Yaccoby S. Fibroblast activation protein (FAP) is upregulated in myelomatous bone and supports myeloma cell survival. Br J Haematol 2006;133(1):83-92.
37. Busek P, Vanickova Z, Hrabal P, Brabec M, Fric P, Zavoral M, Skrha J, Kmochova K, Laclav M, Bunganic B, Augustyns K, Van Der Veken P, Sedo A. Increased tissue and circulating levels of dipeptidyl peptidase-IV enzymatic activity in patients with pancreatic ductal adenocarcinoma. Pancreatology 2016;16(5):829-38.
38. Wesley UV, Albino AP, Tiwari S, Houghton AN. A role for dipeptidyl peptidase IV in suppressing the malignant phenotype of melanocytic cells. J Exp Med 1999;190(3):311-22.
39. Mareš V, Stremenová J, Lisá V, Kozáková H, Marek J, Syrůček M, Šoula O, Šedo A. Compartment-and malignance-dependent up-regulation of γ-glutamyltranspeptidase and dipetidylpeptidase-IV activity in human brain gliomas. Histology and histopathology 2012;27(7):931.
40. Aertgeerts K, Ye S, Shi L, Prasad SG, Witmer D, Chi E, Sang BC, Wijnands RA, Webb DR, Swanson RV. N-linked glycosylation of dipeptidyl peptidase IV (CD26): effects on enzyme activity, homodimer formation, and adenosine deaminase binding. Protein Sci 2004;13(1):145-54.