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ANATOMICAL PATHOLOGY

Fibroblast activation protein as a potential theranostic target in brain metastases of diverse solid tumours

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Summary

Brain metastases are a very common and serious complication of oncological diseases. Despite the vast progress in multimodality treatment, brain metastases significantly decrease the quality of life and prognosis of patients. Therefore, identifying new targets in the microenvironment of brain metastases is desirable. Fibroblast activation protein (FAP) is a transmembrane serine protease typically expressed in tumour-associated stromal cells. Due to its characteristic presence in the tumour microenvironment, FAP represents an attractive theranostic target in oncology. However, there is little information on FAP expression in brain metastases.

In this study, we quantified FAP expression in samples of brain metastases of various primary origin and characterised FAP-expressing cells. We have shown that FAP expression is significantly higher in brain metastases in comparison to non-tumorous brain tissues, both at the protein and enzymatic activity levels. FAP immunopositivity was localised in regions rich in collagen and containing blood vessels. We have further shown that FAP is predominantly confined to stromal cells expressing markers typical of cancer-associated fibroblasts (CAFs). We have also observed FAP immunopositivity on tumour cells in a portion of brain metastases, mainly originating from melanoma, lung, breast, and renal cancer, and sarcoma. There were no significant differences in the quantity of FAP protein, enzymatic activity, and FAP+ stromal cells among brain metastasis samples of various origins, suggesting that there is no association of FAP expression and/or presence of FAP+ stromal cells with the histological type of brain metastases.

In summary, we are the first to establish the expression of FAP and characterise FAP-expressing cells in the microenvironment of brain metastases. The frequent upregulation of FAP and its presence on both stromal and tumour cells support the use of FAP as a promising theranostic target in brain metastases.

Key words: Brain metastases; tumour stroma; fibroblast activation protein; cancer-associated fibroblasts; theranostics.

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INTRODUCTION

Brain metastases are the most frequent intracranial malignancies, occurring in approximately 25% of patients with metastatic tumours. Tumours which most commonly metastasise to the brain originate from the lung, breast, melanocytes, or gastrointestinal tract.[1] Brain metastases reduce quality of life and significantly increase morbidity and mortality in patients with advanced-stage cancer.[2] Therapy of brain metastases is multidisciplinary, combining surgery, radiation therapy, laser interstitial thermal therapy, or systemic therapies, including targeted cancer drugs. However, targeted therapies are available only for a limited subpopulation of cancer patients, and resistance frequently develops due to changes in the target molecule and high plasticity of cancer cells.[3,4] Despite multimodality treatment, the prognosis of patients with brain metastases remains poor, with an overall survival ranging between 3 and 47 months following the diagnosis of intracranial disease.[5] Early diagnosis and thorough follow-up of metastatic spread is an important factor in the management of brain metastases. Hybrid methods are being developed, combining anatomical and morphological information [magnetic resonance imaging (MRI), computed tomography (CT)] with assessment of the functional state by evaluating specific biochemical processes or selectively expressed molecules using radiopharmaceuticals detectable by single-photon emission computed tomography (SPECT) or positron emission tomography (PET).[6,7]

Furthermore, radiopharmaceuticals with therapeutic isotopes can be used to target the tumour microenvironment, leading to improved tumour control or even regression.[8]

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Hence, new targets enabling the application of compounds with therapeutic and diagnostic (theranostic) potential hold great promise for future strategies in cancer management. Targets in the tumour stroma offer an added benefit due to their relevance for a broad spectrum of tumour types.

Fibroblast activation protein (FAP, seprase, EC 3.4.21.B28) represents an emerging theranostic target in various cancers.[9] FAP is a type II membrane-bound glycoprotein exhibiting post-proline dipeptidyl peptidase and endopeptidase activity.[10,11] FAP expression is low or undetectable in most healthy adult human tissues. Exceptions include alpha cells of Langerhans islets,[12] multipotent bone marrow stromal cells (BM-MSC),[13] placenta, cervix, and uterine stroma.[14,15] A soluble form of FAP can also be detected in human plasma.[16] FAP expression is strongly upregulated in remodelling tissues, such as healing wounds and cancer. Several studies have linked higher FAP expression with an increase in local tumour invasion, lymph node metastasis and decreased overall survival[17] in non-small cell lung,[18] colorectal,[19,20] pancreatic,[21,22] hepatocellular,[23] and ovarian carcinoma[24] as well as osteosarcoma.[25] In breast cancer patients, FAP-positive stroma has been associated with a better prognosis,[26] but this has not been confirmed in other studies.[27,28] In solid tumours, FAP is dominantly expressed in activated fibroblasts, but in some cases also in cancer cells.[9] Hence, FAP is considered an attractive target for theranostic agents aimed at both the tumour microenvironment and cancer cells. Several preclinical and clinical FAP-targeting strategies have been developed using antibodies, immunoliposomes, vaccines, or chimaeric antigen receptor (CAR) T cell therapies (reviewed by Busek *et al.*[9]). The most novel and promising approach for FAP targeting is based on conjugates with highly specific low molecular weight FAP inhibitors, which specifically bind to the enzyme active site. Anti-FAP antibody mimetics ('anti-FAP iBodies') and radiopharmaceuticals utilising a FAP inhibitor (dubbed 'FAPIs') have recently been prepared by us and others.[29,30] FAPIs have been tested in various tumour types.[31] Small studies in gliomas[32–34] and a recent case study reporting the use of a therapeutic radiopharmaceutical FAPI in the treatment of advanced-stage breast cancer with brain metastasis,[35] suggest the potential use of FAPIs also in brain tumours. However, there is very little information on FAP expression and its enzymatic activity in brain metastases. The aim of this work was to determine the expression of FAP, an emerging theranostic target, in brain metastases of various origins and to identify the cell types that express FAP in the microenvironment of these malignancies.

MATERIAL AND METHODS

Patient samples

Tissue samples were obtained from patients with brain metastases (n=112) and pharmacoresistant epilepsy (PRE; n=17, controls) undergoing neurosurgical resection at the Department of Neurosurgery, Hospital Na Homolce, Prague, and Department of Neurosurgery and Neurooncology, First Faculty of Medicine, Charles University and Military University Hospital, Prague. Patient cohort characteristics are summarised in Table 1. Full informed consent was obtained from all donors prior to neurosurgical resection. The tumours were diagnosed and characterised according to the current World Health Organization (WHO) classification.[36] Formalin-fixed and paraffin-embedded (FFPE) blocks were used for the immunodetection of FAP (brain metastases n=112, PRE n=5) and Masson's trichrome staining (brain metastases n=106). Snap frozen brain metastases (n=58) and control PRE tissues (n=12) were stored at −78°C and used to prepare tissue lysates and frozen sections.

Preparation of tissue lysates and determination of total protein concentration

Tissue lysates were prepared by mechanical dissociation and chemical extraction as described previously.[37] Total protein concentration was determined using the DC Protein Assay Kit II (cat. no. 5000112; Bio-Rad Laboratories, USA) according to the manufacturer's instructions.

Determination of enzyme activity

Enzymatic activity of FAP was measured as described previously.[37] Briefly, a kinetic assay with 150 µM of N-(quinoline-4-carbonyl)-D-Ala-L-Pro-7-amido-4-methyl-coumarin as a fluorogenic FAP-specific substrate[38] prepared by standard Boc peptide chemistry[29] in black, flat bottom 96-well plates (Corning Costar, USA) was used. FAP assays were carried out at 37°C in a total reaction volume of 100 µL in phosphate-buffered saline (PBS) containing 8 mM NaH_2PO_4, 42 mM Na_2HPO_4 and 150 mM NaCl, pH 7.4. Fluorescence was measured in microplate fluorimeter Infinite M1000 (Tecan, Austria) using excitation and emission wavelengths/slits of 380/5 nm and 460/5 nm, respectively. During the assays, less than 3% of the initial FAP substrate concentration was cleaved. The FAP substrate on its own was stable in the PBS buffer, pH 7.4. Measurements were done in triplicate and calibrated with several concentrations of 7-amino-4-methylcoumarin (AMC) in the assay buffer.

Table 1 Characteristics of the patient cohort

Diagnosis/Origin of metastasis	n	Age at surgery[a]	Sex (male/female)
Brain metastases	112	63 (32–86)	40/72
Lung cancer	33	62 (48–81)	13/20
Breast cancer	23	62 (34–82)	0/23
Gastrointestinal cancer	19	67 (49–82)	10/9
Carcinoma of unknown primary	11	63 (32–75)	3/8
Melanoma	9	66 (47–86)	4/5
Renal cancer	7	62 (47–76)	6/1
Ovarian cancer	3	74 (60–79)	0/3
Sarcoma	2	64 (58–70)	2/0
Cervical carcinoma	1	66	0/1
Embryonal carcinoma	1	41	1/0
Bladder cancer	1	71	1/0
Primary peritoneal serous carcinoma	1	65	0/1
Salivary gland cancer	1	46	0/1
Pharmacoresistant epilepsy (control)	17	47 (22–63)	5/12

[a] Data are presented as median with range in parentheses.

ELISA

The FAP protein concentration was assayed in tissue lysates by a DuoSet FAP kit (Cat. No. DY3715; R&D Systems, USA) in accordance with the manufacturer's recommendations, as described previously.[37]

Western blot analysis

Western blot analysis was performed under both native and reducing and denaturing conditions as described previously[37,39] with the following modifications to allow total protein normalisation using the stain-free methodology.[40] Trihalocompound 2,2,2-trichloroethanol (TCE, T54801; Sigma-Aldrich, Germany) was added to polyacrylamide separation gels at a final concentration of 0.5% v/v.[41] Tissue lysates were mixed with 4× Laemmli buffer and loaded at 20 µg of total protein per lane in 1.5 mm, 7 cm mini gels (4% v/v stacking gel and 8% v/v separation gel). Samples were then electrophoresed in an electrode buffer containing 25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.3, at constant voltage (60 V, 30 min and then 140 V, 90 min).

After electrophoresis, gels were immediately removed from the glass plates and stain-free gel images were captured after 45 s activation at optimal automatic exposure using the ChemiDoc MP Imaging System (Bio-Rad, USA). Gels were subsequently equilibrated in Bjerrum and Schafer-Nielsen transfer buffer containing 20% methanol. Proteins were transferred onto low-fluorescent signal PVDF membranes (Merck-Millipore, Germany) at constant voltage (10 V, 60 min) using the semidry blotting system (Bio-Rad, USA). After protein transfer, PVDF membranes were imaged in the ChemiDoc MP Imaging System using optimal automatic exposure, and the signal in each lane was used for total-protein normalisation (Supplementary Fig. 1, Appendix A).[42]

For immunodetection, PVDF membranes were rinsed in TTBS (0.05% Tween 20, 100 mM Tris/HCl and 154 mM NaCl, pH 7.5) and blocked in EveryBlot Blocking Buffer (EBBB; Bio-Rad, USA) for 5 min at room temperature (RT) with agitation. Primary antibody (Rat Anti Human FAP D8; Applied DNA Sciences, USA) was diluted 5000× in full-strength EBBB and incubated at 4°C overnight. Membranes were then rinsed five times for 5 min with TTBS and incubated with an HRP-conjugated secondary antibody (ab6257; Abcam, UK) diluted 20,000× in EBBB for 60 min at RT with agitation. After rinsing the membranes six times with TTBS for 5 min, chemiluminescent signal was developed using Luminata Forte (Merck, Germany) and captured using the optimal automatic exposure in the ChemiDoc MP Imaging System. For relative quantification, the FAP protein signal was normalised to the total protein signal determined by the stain-free methodology using ImageLab Software (Bio-Rad, USA) according to the manufacturer's instructions.

Masson's trichrome staining

FFPE tissue sections of brain metastases were standardly deparaffinised. After the last wash in 50% ethanol, slides were incubated in Weigert's iron haematoxylin solution for 10 min, washed in tap water, incubated with xylidine ponceau for 5 min, and washed in distilled water. Afterwards, the slides were incubated for 5 min in phosphotungstic acid solution, followed by a 5-min incubation in Orange G solution. The slides were then washed in distilled water, incubated in aniline blue dye for 2 min, and left to dry. After drying, the slides were mounted, viewed, and photographed on an inverted microscope (Olympus IX70; Olympus, Japan) with camera (Canon EOS Rebel T7 DS126741; Canon, Japan) using the QuickPHOTO Microscope Software Version 3.2 (Promicra, Czechia). The slides were assessed by an experienced pathologist (MS), and Masson's trichrome staining was evaluated using a histomorphometric 4-tier scoring system (Table 2) similar to other works.[43]

Immunohistochemical staining

FFPE tissues of brain metastases were cut into 4 µm paraffin sections. Slides were standardly deparaffinised and rehydrated. Antigen retrieval was performed with an EDTA-based pH 9.0 epitope retrieval solution in a water bath (40 min at 95–100°C, cooling 20 min at RT), followed by a wash in distilled water and PBS. Blocking of endogenous peroxidase activity was done by incubating in a 3% solution of hydrogen peroxide for 15 min at RT. After blocking, the slides were washed in PBS and incubated with an anti-FAP primary rabbit monoclonal antibody (Ab207178, clone EPR20021; 1:250, overnight at 4°C; Abcam, UK) followed by a wash in PBS.

Table 2 Scoring of Masson's trichrome staining in tissue sections of brain metastases

Masson's trichrome score	Description
0	Negative, or positivity only in perivascular regions
1	Positivity in small septa, covering <30% of the visual field
2	Positivity in large septa, covering 30–60% of the visual field
3	Positivity in large septa covering >60% of the visual field

Afterwards, the slides were incubated with a ready to use EnVision+ Dual Link System-HRP system (30 min at RT; Agilent Technologies, USA), followed by a wash in PBS. FAP immunopositivity was visualised with a 3,3′-diaminobenzidine chromogenic substrate solution (20 min, RT). Slides were then washed in distilled water, incubated with 3% solution of Copper(II) sulphate (5 min, RT) and washed in distilled water. Counterstaining of nuclei was performed with Mayer's Haematoxylin Solution (3 min at RT; Sigma-Aldrich, USA), followed by a standard dehydration procedure. The slides were assessed by an experienced pathologist (MS), and FAP immunopositivity was evaluated in stromal and tumour cells using a 4-tier visual histomorphometric scoring system (Tables 3 and 4, respectively) similar to other works.[26,44] Representative images were photographed as described above.

Sequential double immunofluorescence labelling was performed in 10 µm frozen sections with minor modifications of a previously described protocol.[45,46] Briefly, after fixation with 4% paraformaldehyde (10 min, RT), permeabilisation using 0.1% Triton X-100 (5 min, RT) and blocking with 10% FBS and 1% bovine serum albumin in Tris-buffered saline (TBS; 60 min, RT), tissue sections were sequentially stained with antibodies against FAP, collagen type I, CD31, TE-7, αSMA, PDGRFβ, EpCAM, pancytokeratin and GFAP (Supplementary Table 1, Appendix A). After washing, slides were incubated with the corresponding Alexa Fluor 488- and 546-conjugated secondary antibodies (Supplementary Table 1, Appendix A). To counterstain cell nuclei, 400 µM ToPro (Thermo Fisher Scientific, USA) or 50 ng•mL^{-1}Hoechst 33258 (Sigma-Aldrich) were used. Immunostained sections were viewed and photographed using a FluoView 300-IX 81 confocal microscope (Olympus).

Table 3 Scoring of FAP+ stroma in tissue sections of brain metastases

Stromal FAP	Description
0	FAP+ stroma not present
1	FAP+ stroma in small areas, typically in perivascular regions
2	FAP+ stroma in perivascular regions and in septa around tumour cells
3	FAP+ stroma in perivascular and septal regions covering over 1/3 of the visual field

Table 4 Scoring of FAP+ cancer cells in tissue sections of brain metastases

FAP in cancer cells	Description
0	Tissue without FAP+ cancer cells
1	<30% FAP+ cancer cells per visual field
2	30–60% FAP+ cancer cells per visual field
3	>60% FAP+ cancer cells per visual field

Statistical analysis

Statistical analyses were performed with STATISTICA 12 (Tibco Software, USA) and GraphPad Prism 8.0.2 (GraphPad Software, USA). A p value <0.05 was considered statistically significant.

RESULTS

FAP expression is higher in brain metastases compared to non-tumorous brain tissue

The expression of FAP was determined in tissue lysates of brain metastases (n=58) of various primary origin. Tissue samples from neurosurgical interventions for pharmaco-resistant epilepsy (n=12) were used as a non-tumorous brain control. The concentration of FAP protein was more than 20 times higher in tumour tissues (Fig. 1A). To verify that enzymatically active FAP molecules are present in the microenvironment of brain metastases, we utilised a kinetic assay using a FAP-specific substrate. FAP enzymatic activity positively correlated with the levels of FAP protein determined by ELISA and was significantly higher in brain metastases compared to non-tumorous brain tissue. In our previous work, we have shown that FAP is overexpressed in grade IV primary brain tumours, glioblastomas.[37,45] The expression of FAP in brain metastases was also significantly higher compared to glioblastomas (Supplementary Fig. 2, Appendix A).

The expression of FAP in brain metastases was further confirmed by western blotting (Fig. 1C). Under denaturing, reducing conditions, FAP immunopositivity corresponding to FAP monomer was detected at 90 kDa in most brain metastases, whereas no immunopositivity was present in non-tumorous brain tissue. Densitometric analysis confirmed that FAP expression was significantly higher in comparison to non-tumorous brain tissue (Fig. 1D). In native western blotting, FAP immunopositivity with electrophoretic mobility corresponding to 100–140 kDa was detectable in a large proportion of brain metastases; in addition, high molecular weight forms were present in several tissues (Supplementary Fig. 3, Appendix A), similarly as observed in glioblastomas previously.[39]

FAP is typically present in the stroma of brain metastases

FAP staining was performed on FFPE sections of brain metastases (n=112) of various primary origin. FAP immunopositivity was detected in perivascular regions and in stromal septa surrounding areas with cancer cells, while no immunopositivity was observed in non-tumorous brain tissue (Fig. 2A,C). The perivascular localisation of FAP was further confirmed by fluorescent immunohistochemical double labelling of FAP and CD31 (platelet endothelial cell adhesion molecule; PECAM-1) as a canonical marker of endothelial cells (Fig. 2D).

In tumours outside the central nervous system, FAP expression is typically associated with remodelling and organisation of extracellular matrix proteins, especially

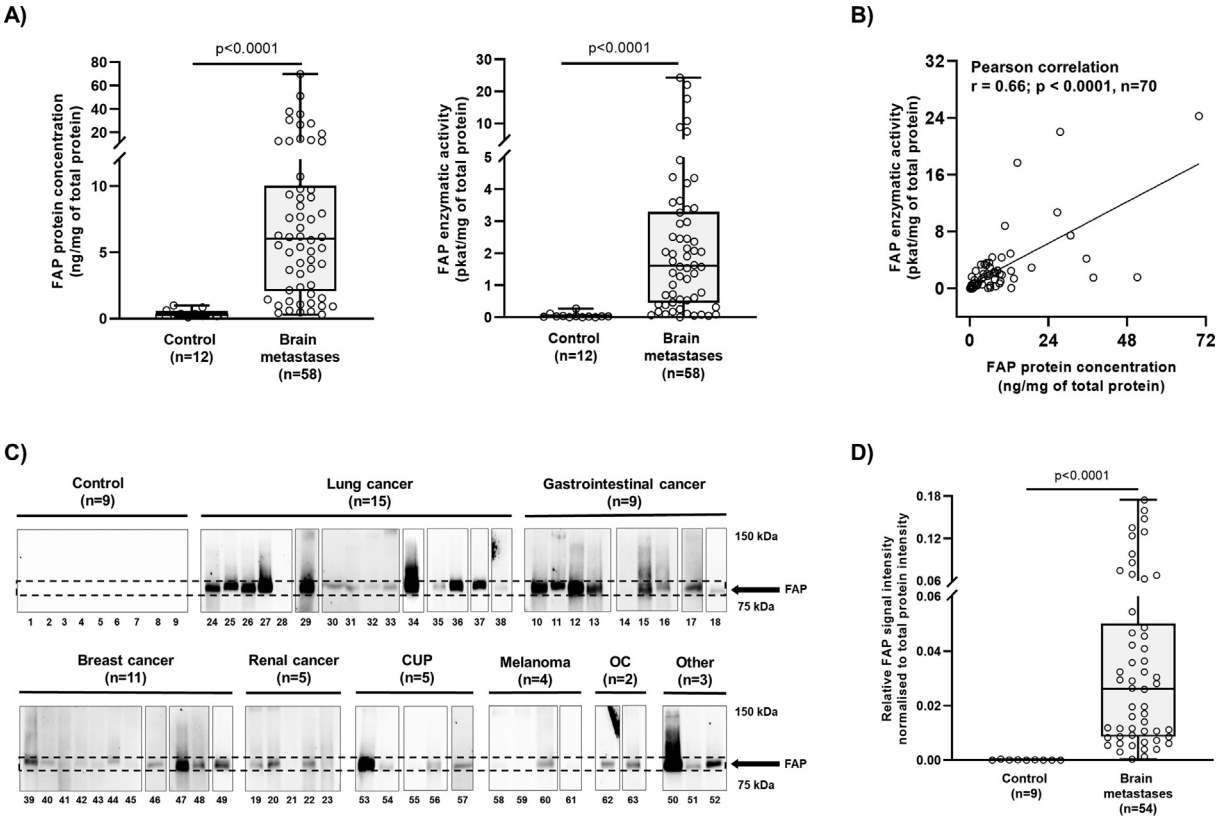

Fig. 1 FAP expression is significantly higher in brain metastases compared to non-tumorous brain tissue. (A) FAP protein (left panel) and FAP enzymatic activity (right panel) in brain metastases (n=58) and control non-tumorous brain tissues (n=12), p<0.0001, Mann–Whitney U test. (B) Correlation between FAP protein levels and FAP enzymatic activity (n=70); circles = source data. (C) Western blot analysis of FAP expression in control and brain metastasis tissues. CUP, carcinoma of unknown primary; OC, ovarian cancer; other, sarcoma (lane 50), primary peritoneal serous carcinoma (lane 51), bladder cancer (lane 52). (D) Quantification of western blot data normalised to total protein (stain-free method) in brain metastases (n=54) and control non-tumorous brain tissues (n=9), p<0.0001, Mann–Whitney U test. (A,D) Horizontal line = median; boxes = 25–75%; whiskers = range; circles = source data.

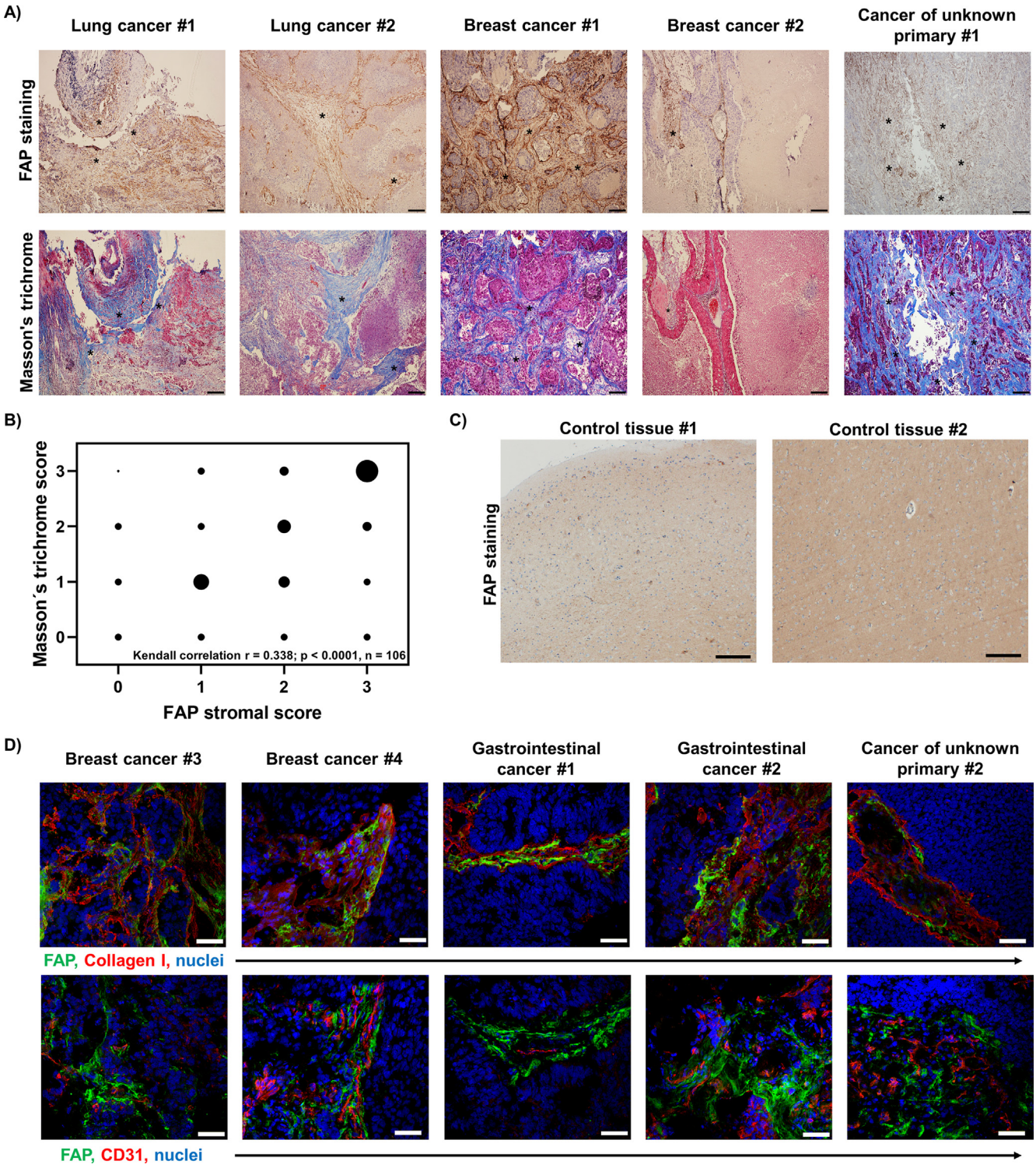

Fig. 2 FAP immunopositivity is typically found in stromal regions containing collagen and blood vessels. (A) FAP immunopositivity in septal and perivascular regions (arrows) and Masson's trichrome staining of the corresponding areas (asterisks); scale bars = 200 µm. (B) Correlation between Masson's trichrome score and FAP stromal score in tissue sections of brain metastases (n=106). Frequencies of overlapping points between two variables are illustrated by the size of the point markers. (C) No detectable FAP immunopositivity in control tissues (pharmacoresistant epilepsy); scale bar = 200 µm. (D) Immunohistochemical double labelling of FAP and collagen I or CD31, scale bars = 50 µm.

collagens.[47] We performed Masson's trichrome staining to determine the presence of collagens in tissue sections of brain metastases. Indeed, several brain metastases contained deposits of collagens, and the corresponding regions also showed FAP immunopositivity. Both the immunopositivity of FAP in stromal cells and Masson's trichrome staining positivity were scored (Tables 2 and 3) in FFPE sections of brain metastases (n=106) and exhibited a positive correlation (Fig. 2B). This observation was further confirmed by fluorescent immunohistochemical double labelling of FAP and collagen I, where FAP+ cells were typically surrounded by the immunopositivity of collagen I (Fig. 2D).

FAP+ stromal cells co-express other markers of cancer-associated fibroblasts

FAP is a canonical marker of cancer-associated fibroblasts (CAFs). After confirming FAP expression in perivascular regions and collagen-containing areas, we were interested whether FAP+ stromal cells express other markers of CAFs. Indeed, the microenvironment of brain metastases contained cells positive for FAP and other markers of CAFs: TE7, PDGFRβ, and αSMA (Fig. 3A). The FAP signal was localised in TE7+ cells, whereas only part of the FAP immunopositivity was co-localised with the signal of PDGFRβ or αSMA. Co-localisation of FAP and markers of epithelial cells

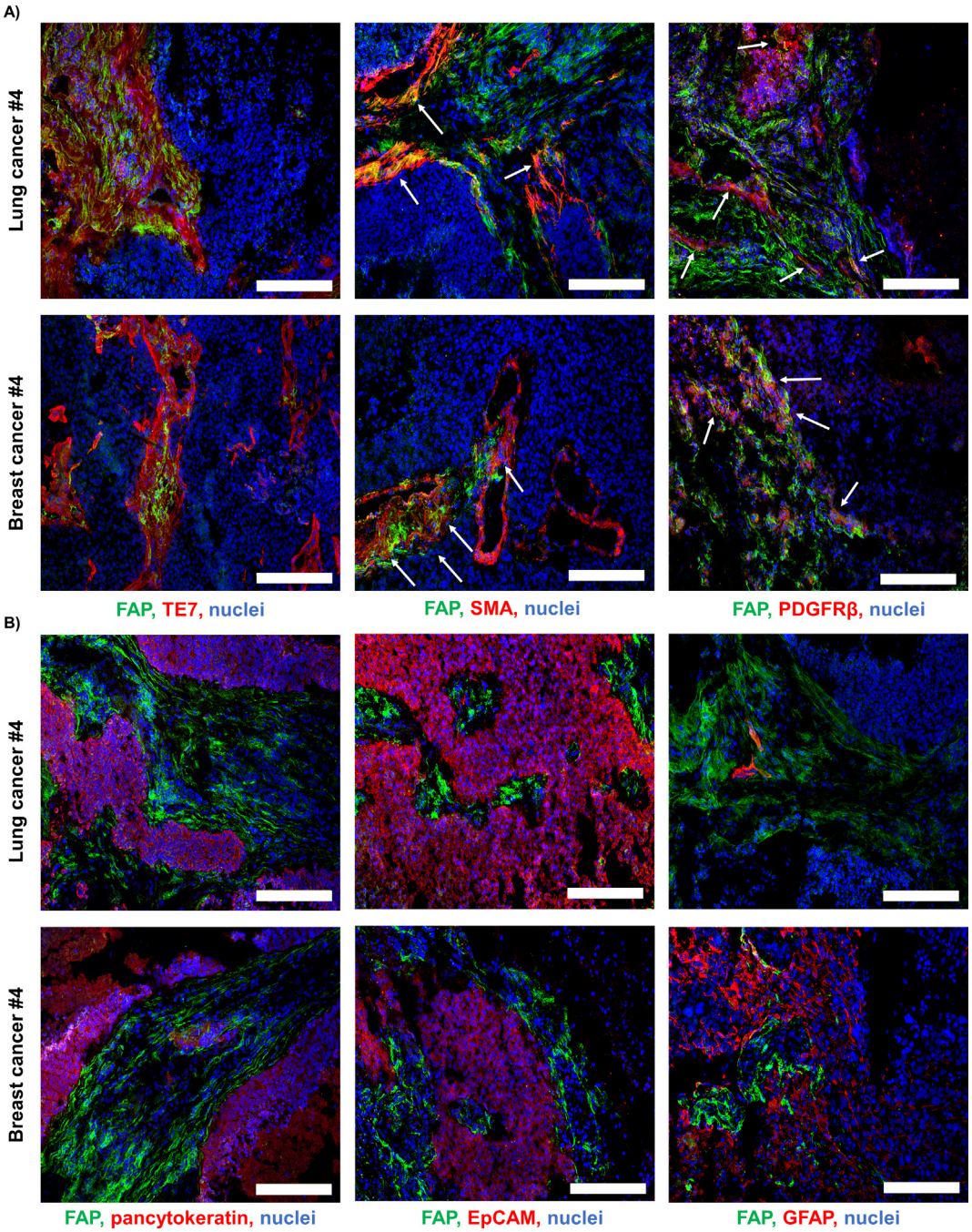

Fig. 3 Stromal FAP-expressing cells in brain metastases express markers of cancer-associated fibroblasts. (A) Immunohistochemical double labelling of FAP and other markers of cancer-associated fibroblasts, TE7, αSMA, PDGRFβ; arrows indicate double positive areas. (B) Immunohistochemical double labelling of FAP and markers of epithelial cells and astroglia, scale bars = 200 μm.

such as pancytokeratin or EpCAM were not observed in the vast majority of analysed tumours (Fig. 3B).

FAP expression and the proportion of FAP+ stroma are comparable among brain metastases of different histological origin

FAP protein level and FAP enzymatic activity were compared among brain metastases (n=58) in relation to their origin. There was no statistically significant association between FAP expression and the histological type of brain metastases (Fig. 4A). Further, FAP immunopositivity in stromal cells (Fig. 4B) was compared in FFPE sections of brain metastases (n=112). FAP+ stromal cells were detected in 100 (89%) of 112 tissues analysed. Similarly to the comparison of FAP enzymatic activity and FAP protein concentration, the quantity of FAP+ stromal cells was comparable in individual histological types of brain metastases (Fig. 4C).

FAP is expressed in cancer cells in a part of brain metastases

In addition to the prevailing stromal positivity of FAP, FAP was also detected in cancer cells in brain metastases originating from melanoma (7/9), lung (16/33), breast (6/23), gastrointestinal (2/19), renal (4/7) and ovarian cancer (1/3), carcinoma of unknown primary (3/11), salivary gland cancer (1/1), and sarcoma (2/2) (Fig. 5A). However, cells co-expressing FAP and the epithelial cell marker pancytokeratin were only rarely observed in brain metastases of carcinomas (Fig. 5B). The highest numbers of FAP+ cancer cells (positivity in more than 30% cancer cells) were observed in brain metastases of melanoma, lung, and breast cancer (Fig. 5D). As expected, due to their mesenchymal origin, brain metastases of two sarcomas analysed in this study showed strong FAP expression in most cancer cells (Fig. 5C).

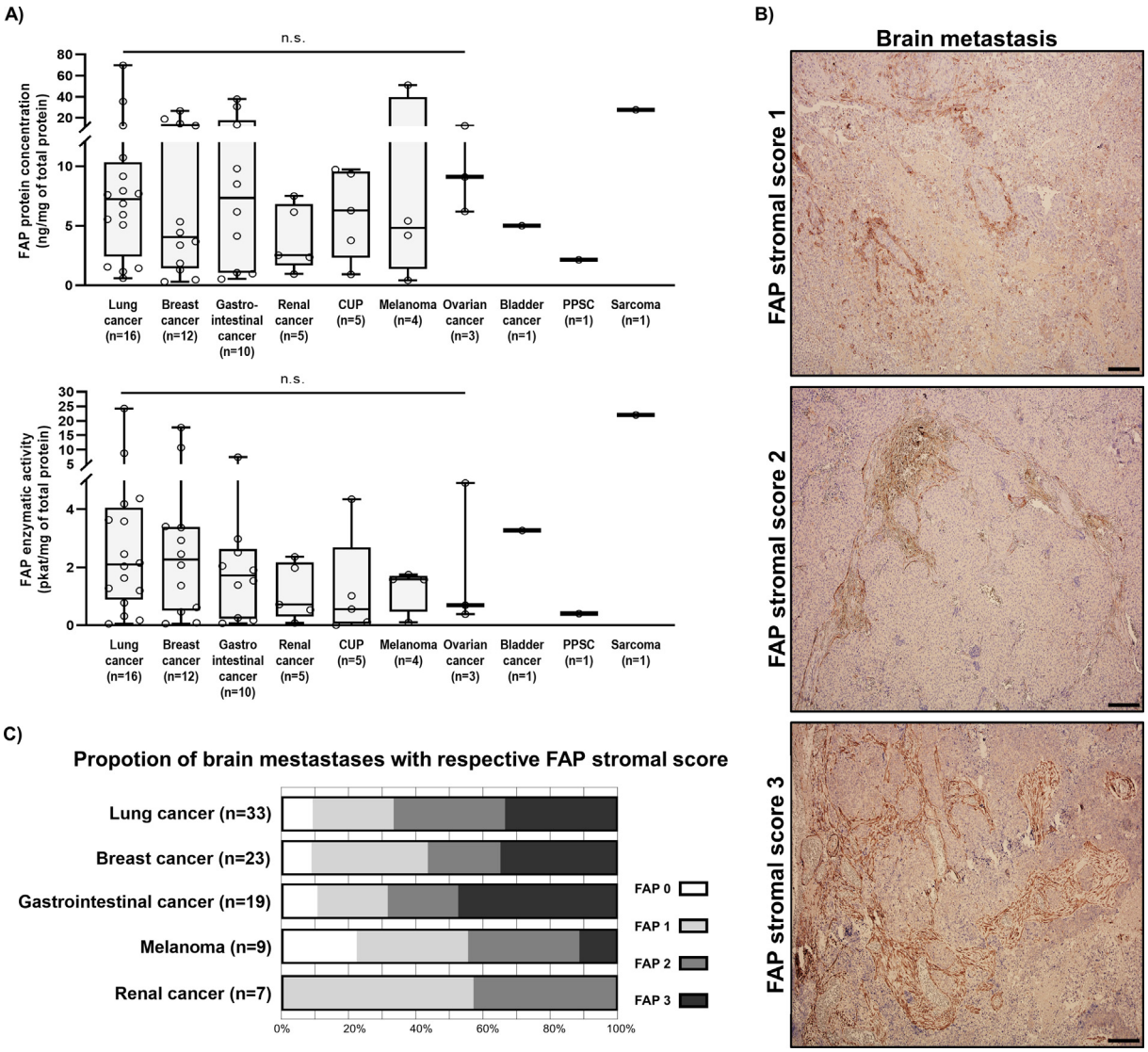

Fig. 4 FAP expression is similar among brain metastases of different primary origin. (A) Levels of FAP protein (upper panel) and enzymatic activity (lower panel) in brain metastases of different primary origin; CUP, carcinoma of unknown primary; PPSC, primary peritoneal serous carcinoma; ns, non-significant; p>0.05, Kruskal–Wallis test; horizontal line = median; boxes = 25–75%; whiskers = range of values; circles = source data. (B) Representative images of stromal FAP immunopositivity (score 1–3) in FFPE tissues; scale bars = 200 µm. (C) Immunohistochemical evaluation of stromal FAP expression in brain metastases of different origin.

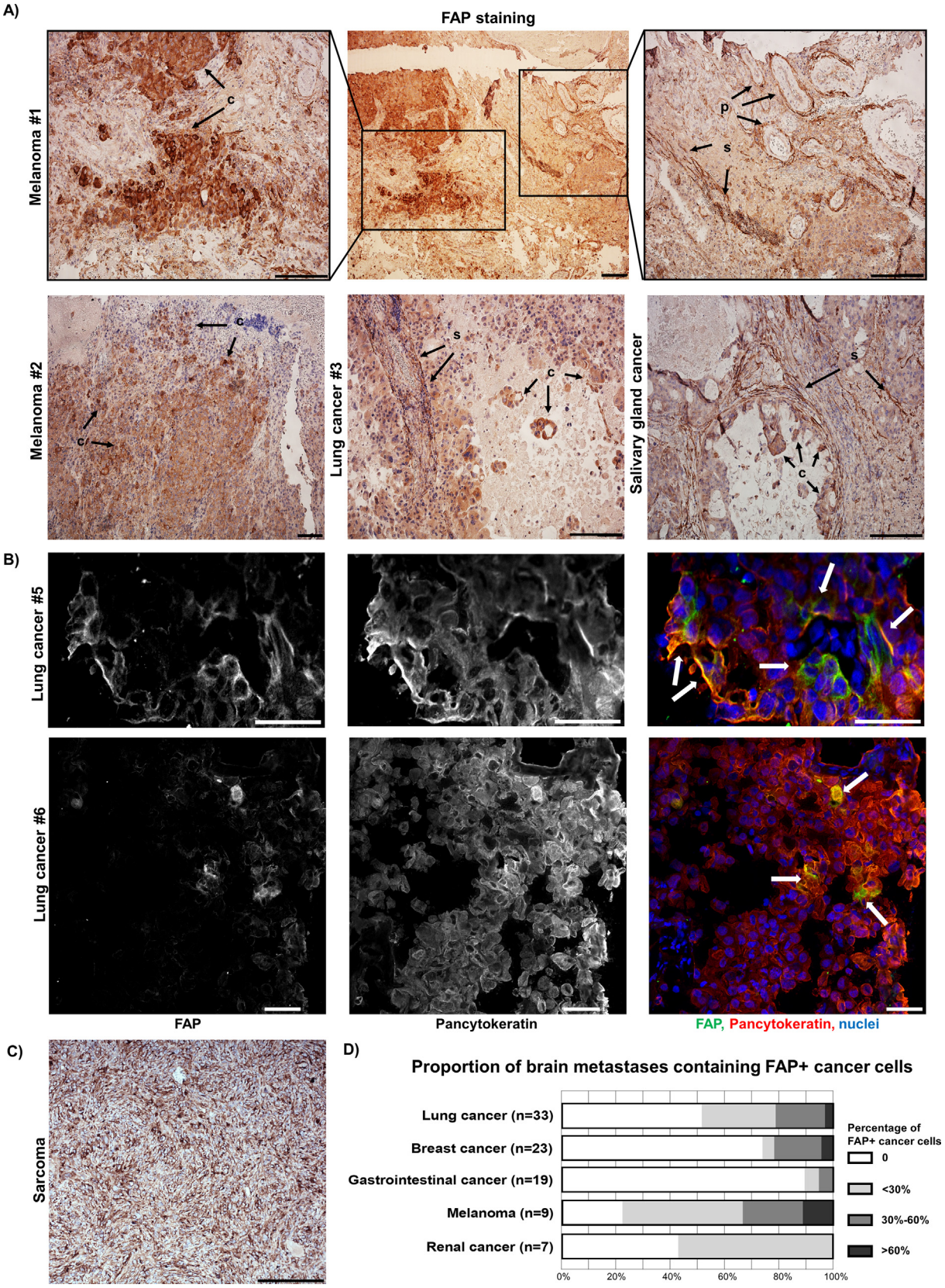

Fig. 5 FAP immunopositivity is also present in cancer cells in a portion of brain metastases. (A) FAP immunopositivity in tissues containing FAP+ cancer cells. c, cancer cells; p, perivascular regions; s, stromal/septal regions; scale bars = 200 µm. (B) Immunohistochemical double labelling of FAP and pancytokeratin; arrows = rare occurrence of double positive cells, scale bar = 50 µm. (C) FAP immunopositivity in brain metastasis of a sarcoma, scale bar = 200 µm. (D) FAP expression in cancer cells in most frequent histological types of brain metastases-summary of immunohistochemical evaluation.

Only a few brain metastases originating from the lung (2/33), breast (2/23), gastrointestinal cancer (1/19), carcinoma of unknown primary (1/11) and one case of primary peritoneal serous carcinoma showed no FAP immunopositivity in stromal or in cancer cells. We were able to evaluate FAP protein concentration in six of these tumours. Only one tumour exhibited low FAP expression similar to that observed in non-tumorous brain, whereas five tumours had substantially higher FAP expression (data not shown).

DISCUSSION

To our knowledge, we are the first to report a detailed analysis of the presence and localisation of FAP in secondary malignancies of the brain. Our data show that compared to non-tumorous brain tissue, FAP is highly expressed in brain metastases regardless of their primary origin. We have shown that FAP is enzymatically active and can be expressed both in the cells resembling CAFs and in cancer cells in the tumour microenvironment of brain metastases. Previous studies implied FAP expression in brain metastases based on case studies using FAP imaging probes[35,48] and analysis of two brain metastasis samples reported in the seminal work on FAP expression in cancer.[49]

Earlier work has shown that FAP is also overexpressed in glioblastomas, the most common and most aggressive primary brain tumours.[37,45,50] Interestingly, our data suggest that FAP expression in brain metastases is significantly higher even in comparison to glioblastomas. This may be caused by a more pronounced induction of FAP expression in brain metastases or, in our opinion more likely, due to a higher quantity of FAP-expressing cells in the microenvironment of brain metastases. Fibroblasts originating from the primary site may contribute to this accumulation of FAP+ stroma.[51]

We have confirmed the expression of FAP in brain metastases by western blotting. FAP immunopositivity was detected at 90 kDa under denaturing and reducing conditions. This corresponds to FAP monomers with a reported molecular weight of 100 kDa.[52] In addition, using native western blotting we have detected immunopositive bands with an electrophoretic mobility corresponding to 100–140 kDa and higher molecular weight, which could match FAP homodimers (reported molecular weight 170 kDa), and FAP heterodimers/oligomers, such as those with DPP-IV (220–240 kDa).[53,54] In our previous studies, we observed FAP immunopositive bands with an electrophoretic mobility ranging between 120 and 140 kDa in glioblastoma tissues.[39] These data indicate the presence of similar forms of FAP in brain metastases and glioblastomas.

We have shown that FAP-expressing cells in the microenvironment of brain metastases are located close to blood vessels and in regions with deposits of collagens, especially collagen I. We have also demonstrated that these FAP-expressing cells co-express other markers of CAFs, namely TE7,[55] αSMA[56] and PDGFRβ.[57] These data suggest that FAP-expressing stromal cells present in the brain metastases may be CAFs and/or pericytes;[50] similar FAP+ stromal mesenchymal cells were observed in glioblastomas.[45,50] In recent decades, a tremendous amount of research has been devoted to defining the role of CAFs in tumorigenesis. Studies have shown that CAFs are crucial for the deposition and remodelling of extracellular matrix and for promoting tumour invasiveness and dissemination.[58–61] In addition, CAFs are a significant source of cytokines and local mediators, such as hepatocyte growth factor, leukaemia inhibitory factor, fibroblast growth factor 5, interleukin 6, transforming growth factor beta, growth differentiation factor 15, growth arrest-specific protein 6, which can stimulate cancer cell proliferation and epithelial-mesenchymal transition, further promoting tumour spread.[62] Other works demonstrated that CAFs can enhance angiogenesis.[63,64] In the last years, CAFs have also been shown to modulate the immune response in the tumour microenvironment, influencing macrophages, cytotoxic T-cells, and regulatory T-cells, typically inducing local immunosuppression and escape from immunosurveillance.[62] Therefore, CAFs in general have been established as key tumour-promoting components of the tumour microenvironment and an attractive target for therapy, especially by targeting FAP.[65]

Literature evidence regarding CAFs in brain metastases is rather scarce and contradictory. To date, there have been very few works describing the presence of CAF-like stroma in the microenvironment of brain metastases. Duda *et al.* have shown that human brain metastases of lung cancer contain cells that resemble fibroblasts. In addition, they provided evidence that CAFs from the primary site disseminate together with the cancer cells and facilitate the establishment of brain metastases.[51] Chung *et al.* have shown that CAFs from brain metastases of breast cancer can have a chemo-attractive effect on cancer cells *in vitro*, most likely mediated by secretion of the chemokines CXCL16 and CXCL12.[66] In contrast, tumour-inhibitory properties of CAF-like cells from brain metastases were proposed by Tew *et al.*[67] These rather conflicting results may be caused by heterogeneity of the CAF-like cell population in the brain metastases. Indeed, we detected αSMA and PDGFRβ positivity only in a fraction of FAP-expressing cells, which could hint that FAP-expressing CAFs in brain metastases comprise several distinct subpopulations. This conclusion is in line with several studies reporting phenotypically and functionally different subpopulations of CAFs in the microenvironment of solid tumours, such as breast,[68] colorectal[69] and pancreatic cancers.[70]

In our patient cohort, we have also detected FAP immunopositivity in cancer cells in a portion of brain metastases of melanoma, lung, breast, renal, gastrointestinal, ovarian cancer, sarcoma, three cases of carcinoma of unknown primary and one case of salivary gland cancer. Several studies have already shown that cancer cells can also express FAP (reviewed by Busek *et al.*,[9] as well as Pure and Blomberg[71]), for example in lung adenocarcinoma[72] or in melanoma cell lines.[53,73] FAP expression in cancer cells in the lung, breast, colorectal, ovarian carcinoma, melanoma, and fibrosarcoma has been associated with increased proliferation, invasiveness, and independence on growth factors, increasing their tumorigenic potential.[9] Based on that, some authors consider FAP to be a potential oncogene. On the other hand, the work by Wesley

et al. has demonstrated that increased FAP expression can promote apoptosis and cell cycle block in melanoma and non-small cell lung cancer cells.[74,75] Ramirez-Montagut *et al.* have also shown that increased FAP expression in mouse melanoma cells can lead to attenuation of their tumorigenic potential by restoring contact inhibition and higher susceptibility to apoptosis.[76] Therefore, further studies are needed to determine whether FAP in cancer cells in brain metastases acts as a promotor or suppressor of tumorigenicity.

Although immunohistochemistry in FFPE sections revealed FAP+ cancer cells in 38% (42/112 tissues) of analysed brain metastases originating from epithelial tumours, FAP immunopositivity was very rare in cells expressing pancytokeratin or EpCAM. This could probably be due to epithelial-mesenchymal transition, in which cancer cells have been shown to lose their original epithelial phenotype in favour of a fibroblast-like phenotype that may promote their invasive and metastatic properties.[77–79] Some works even suggest that cancer cells could be one of the sources of CAFs in the tumour microenvironment, also through epithelial-mesenchymal transition.[80]

FAP was not detectable by immunohistochemistry in approximately 6% (7/112) of patients in our cohort. However, in the majority of these tissues we did observe substantially higher FAP protein concentration measured by ELISA compared to non-tumorous brain. Therefore, we can speculate that this discrepancy could be caused by lower sensitivity of immunohistochemical analyses in comparison to ELISA. Another explanation could be a possible intra-tumour heterogeneity of the respective brain metastases,[81] similar to that observed in glioblastomas.[82]

Nevertheless, when using FAP as a theranostic target in brain metastases, the presence of a small fraction of brain metastases expressing FAP at levels comparable to non-tumorous brain tissue must be taken into account.

In summary, we have shown that the microenvironment of brain metastases contains a significant amount of FAP-expressing cells. FAP expression was mostly detected in stromal cells with characteristics of CAFs. Approximately a third of our patient cohort also exhibited FAP expression in cancer cells, typically brain metastases originating from melanoma, lung, breast, and renal cancer and sarcoma. The expression of enzymatically active FAP and accumulation of FAP+ stroma was similar in brain metastases irrespective of their origin. This suggests that the presence of FAP+ stroma is a general feature in brain metastases. Moreover, FAP expression in cancer cells further argues for the potential of theranostic approaches based on conjugates with highly specific FAP inhibitors for the detection and treatment of brain metastases.

Ethics statement: This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the institutional Ethics Committee of Na Homolce Hospital, and the Military University Hospital, both in Prague, Czech Republic (study approval numbers 7.11.2018/22, 9.6.2021/21, 9.6.2021/24 and 108-39/4-2014-UVN, approved on 12 November 2018, 14 June 2021, 13 May 2022 and 21 July 2014, respectively).

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pathol.2023.05.003.

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