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Expression of integrins αvß3 and αvß5 and their ligands in primary and secondary central nervous system neoplasms

Michel Mittelbronn[1], Arne Warth[2], Richard Meyermann[3], Simon Goodman[4] and Michael Weller[5]

[3]Institute of Brain Research, University of Tübingen, Tübingen, Germany, [1]Neurological Institute (Edinger Institute), Goethe-University, Frankfurt/Main, Germany, [2]Institute of Pathology, University of Heidelberg, Heidelberg, Germany, [4]Therapeutic Area Oncology, Merck KGaA, Darmstadt, Germany and [5]Department of Neurology, University Hospital Zurich, Zurich, Switzerland

Summary. Aims: To study the expression of integrins αvß3 and αvß5 and their ligands in tumour, stroma and endothelial cells from human glioblastoma and CNS metastases from breast, lung and skin tumours. Methods and results: Integrin and integrin ligand expression was quantified in frozen tumour surgical specimens (15 glioblastomas and breast carcinoma metastases as well as 16 lung carcinoma and melanoma metastases) using immunohistochemistry. Gene expression profiles were evaluated in glioblastomas (n=424) and in normal brain (n=11). Overall, αvß3 expression was more common than αvß5, except in tumours derived from lung. αvß3 expression was most frequent in glioblastomas and melanoma metastases. Most lung-derived tumours expressed αvß5, but expression was less frequent in other tumours; about 20% of breast-derived tumours strongly expressed αvß5. Melanoma-derived tumours did not express αvß5. Expression of integrin ligands vitronectin, fibrinogen, fibronectin and osteopontin was variable between tumours, although most tumours expressed the ligands to some extent. Marked αvß3, but not αvß5, expression was common in stroma of CNS metastases. In blood vessels, αvß3 expression was more frequent than αvß5 and more pronounced in CNS metastases than in glioblastomas. Integrin ligand expression occurred in blood vessels in most tumours. In glioblastomas, mRNA expression of αvß3, αvß5, osteopontin and fibronectin were significantly upregulated over normal brain. Conclusions: Overall, we report distinct and heterogeneous patterns of integrin expression in primary and secondary brain tumours that may be relevant to the future development of integrin-targeting therapeutic approaches to brain tumours.

Key words: Cancer, Integrin expression, Tumour microenvironment

Offprint requests to: Michel Mittelbronn, Goethe-University Frankfurt, Institute of Neurology (Edinger Institute), Heinrich-Hoffmann-Str. 7, 60528 Frankfurt/Main, Germany. e-mail: michel.mittelbronn@kgu.de

Introduction

Integrins are important in promoting tumour development and metastasis. The interaction of integrins with the extracellular matrix influences the assembly and organisation of the intracellular cytoskeleton, enhances cell survival and reduces the likelihood of apoptosis in a variety of tumour types (Janes and Watt, 2006; Le Tourneau et al., 2007; Moschos et al., 2007; Streuli, 2009). The centres of growing solid tumours can become hypoxic, which prompts the generation of new blood supply for the tumour through angiogenesis, in which integrins expressed by the growing endothelia influence migration to and entry into the tumour (Nikolopoulos et al., 2004; Varner and Cheresh, 1996).

Activation of integrins αvß3 and αvß5 has been implicated in these cellular processes (Enns et al., 2005; Hodivala-Dilke, 2008; Beauvais et al., 2009; Somanath et al., 2009). In mice with tumours derived from intracranially injected glioblastoma cells, the tumours had lower vessel density in αvß3-deficient (knockout) than in wild-type mice (Kanamori et al., 2006). Similarly, inhibition of integrins, including αvß3, suppressed tumour growth in a nude rat model (Mikkelsen et al., 2009). Conversely, increased activation of αvß3 increased the ability of cultured cells to migrate (Verbisck et al., 2009). A further experimental study showed that selective inhibition of αvß3 and αvß5

integrins with an Arg-Gly-Asp (RGD) peptide induced endothelial cell death via anoikis (cell death associated specifically with detachment from the extracellular matrix) (Maubant et al., 2006). In human glioblastoma, the expression of αvß3 appears to be more prominent in higher-grade tumours (glioblastomas World Health Organization [WHO] grade IV relative to low-grade astrocytomas), but integrin inhibition induces only detachment, not anoikis (Schnell et al., 2008; Maurer et al., 2009). As the growth of a tumour is influenced strongly by its environment (Kanamori et al., 2006), mechanisms related to the expression of αvß3 and αvß5 are likely to be relevant to both primary central nervous system (CNS) tumours and metastases from other tumour types. Here we have studied the expression profile of αvß3 and αvß5 and their principal ligands (vitronectin, fibrinogen, fibronectin, osteopontin) in primary human CNS neoplasms (WHO grade IV glioblastomas) and secondary neoplasms in the brain originating from primary tumours in the lung, breast and skin (melanoma) to elucidate potential differences in the expression profile of primary and secondary malignant CNS neoplasms. The expression pattern of these proteins was studied in tumour parenchyma, stromal cells, and in blood vessels. Gene expression profiles of the integrins and their ligands derived from open-source databases were also evaluated in brain tumours and compared with their expression in normal CNS tissues.

Materials and methods

mRNA expression of integrins and integrin ligands in primary tumour tissues and normal tissues

Differences in the gene expression profile in primary glioblastomas (n=424) versus normal CNS tissues (n=11) were assessed from two publicly available platforms at the US National Institute of Health: The Cancer Genome Atlas (TCGA) Data Portal (http://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp) and the REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) (https://caintegrator.nci.nih.gov/rembrandt/). Both platforms contain clinical information, genomic characterisation data, and high-throughput sequencing analysis of tumour genomes and provide researchers with the ability to perform ad hoc querying across multiple data domains. Since for secondary brain tumours (breast and lung) only *z*-scores, but no expression ratios (mRNA expression vs normal tissue), were available, these data were not included in the present study.

Preparation of tumour samples and immunohistochemical staining

Tumour tissue samples were immediately frozen in liquid nitrogen (15 glioblastomas and breast carcinoma metastases as well as 16 lung carcinoma and melanoma metastases). Tumour diagnoses were confirmed by at least two neuropathologists. Sections of 10 μm thickness were cut using a microtome and mounted on Super Frost Plus slides (Microm International, Walldorf, Germany). Sections were dried, and postfixed for 10 min in ethanol at 4°C, followed by acetone for 1 min and Tris-buffered saline for 5 min at room temperature. For automatic immunohistochemical tissue labelling, the Benchmark immunohistochemistry system (Ventana, Strasbourg, France) was used. Endogenous peroxidase of the tissue sections was blocked with 3% H_2O_2 in methanol for 14 min. A cell-conditioning pretreatment was performed. Primary antibodies (Table 1) were applied at a working concentration of 10 μg ml^{-1} for 30 min. An avidin and a biotin blocker were applied to the samples for 4 min, respectively, followed by an 8-min incubation with one drop of I-View-Biotin Ig (Ventana). For diaminobenzidine (DAB) visualisation, the sections were incubated with one drop of I-View streptavidin-horseradish peroxidase for 8 min and then with DAB/H_2O_2 for an additional 8 min. Sections were finally incubated with a copper enhancer (Ventana) for 4 min, then washed, counterstained with haematoxylin and mounted. Negative and isotype controls were conducted for all tumours.

Quantification of expression in tissue sections

For all tumours, only sections where staining could be unequivocally attributed to blood vessels, tumour cells, or stromal cells were evaluated. In CNS metastases, stromal cells were defined as non-neoplastic tissue intermingled between tumour cell clusters, blood vessels and/or CNS tissue. Astrocytic tumours do not develop stroma, and consist of diffusely infiltrating tumour cells which invade preexisting CNS parenchyma, thus, expression on stromal cells could not be assessed in these tumours. M. Mittelbronn and D. Capper quantified staining intensity and frequency separately for blood vessels, tumour and stromal cells (see above), using an Olympus Bx50 microscope. Staining frequency for tumour and stromal cells was categorised as: 0, no staining; 1, single cells positive (focal pattern); 2, single cells positive (diffuse pattern); 3, up to 20% positive cells; 4, 20-50% positive cells; or 5, >50% positive cells. Staining frequency for blood vessels was categorised as: 0, all vessels negative; 1, moderate number of positive blood vessels; or 2, most blood vessels positive. Staining

Table 1. Antibodies[a], clones, and providers.

Antibody	Clone	Provider
Fibrinogen	Polyclonal	Dako Cytomation, Carpinteria, CA, USA
Fibronectin	Polyclonal	Dako Cytomation, Carpinteria, CA, USA
αvß3	Lm609 (IgG1)	Millipore, Schwalbach, Germany
Osteopontin	Polyclonal	NeoMarkers, Lab Vision Corporation, CA, USA
αvß5	P1F6 (IgG1)	Millipore, Schwalbach, Germany
Vitronectin	153	Dr D. Seiffert, Scripps, La Jolla, CA, USA

[a]: Rabbit polyclonal antibodies were used. IgG1, immunoglobulin 1.

intensity was categorised as: 1, weak; 2, moderate; or 3, strong. Statistical analysis was performed by a contingency table analysis using χ^2 tests.

Results

Expression of αvβ3 and αvβ5 integrins in tumour cells of primary and secondary CNS tumours

Fig. 1 summarises the expression of αvß3 and αvß5 integrins in tumour cells of primary and secondary CNS tumours and Fig. 2 shows representative immunohistochemical stainings. αvß3 was more commonly expressed than αvß5 in tumour cells, except for lung carcinomas, in which the tumour cells showed a slightly higher expression frequency for αvß5 than for αvß3. Glioblastomas and melanoma-derived brain metastases were most likely to express αvß3 in a moderate or high proportion of tumour cells (20-50% or >50%). Although αvß3 expression was detected in most tumour samples, about one-third of lung- and breast-derived brain metastases had no αvß3 expression. The intensity of staining in all samples expressing αvß3 was generally weak (score 1, data not shown). In contrast, six out of seven lung-derived brain tumours showed αvß5-expressing tumour cells. Expression of this integrin was less frequent in the other tumour types: there was no expression of αvß5 in melanoma-derived metastases. Most glioblastomas or breast-derived brain metastases also did not express αvß5. Nevertheless, approximately 20% of breast-derived brain tumours strongly expressed αvß5. In general, in glioblastomas, integrin αvß3 was

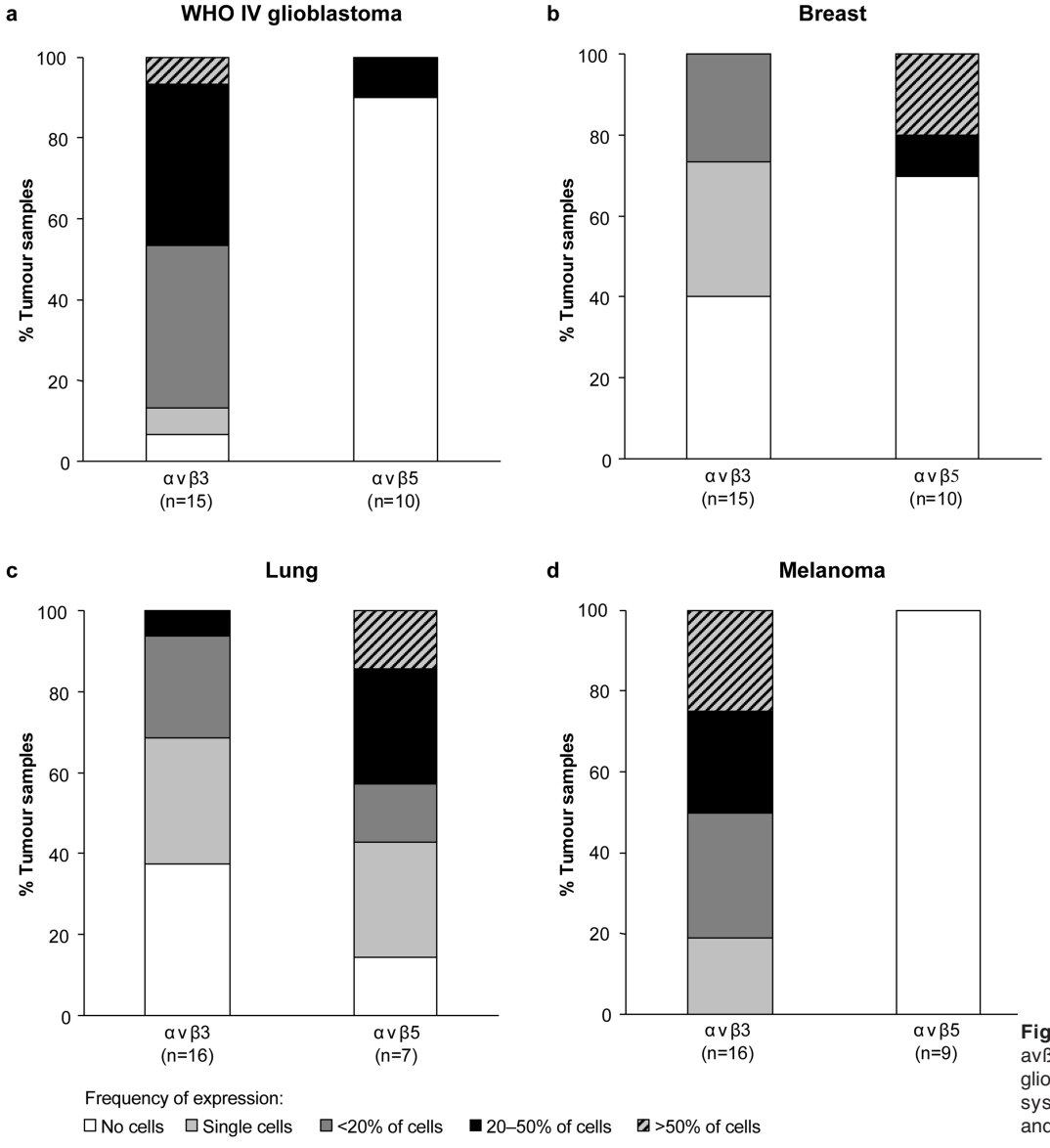

Fig. 1. Expression of avß3 and avß5 integrins in tumour cells of glioblastomas and central nervous system metastases from breast and lung cancer or melanoma.

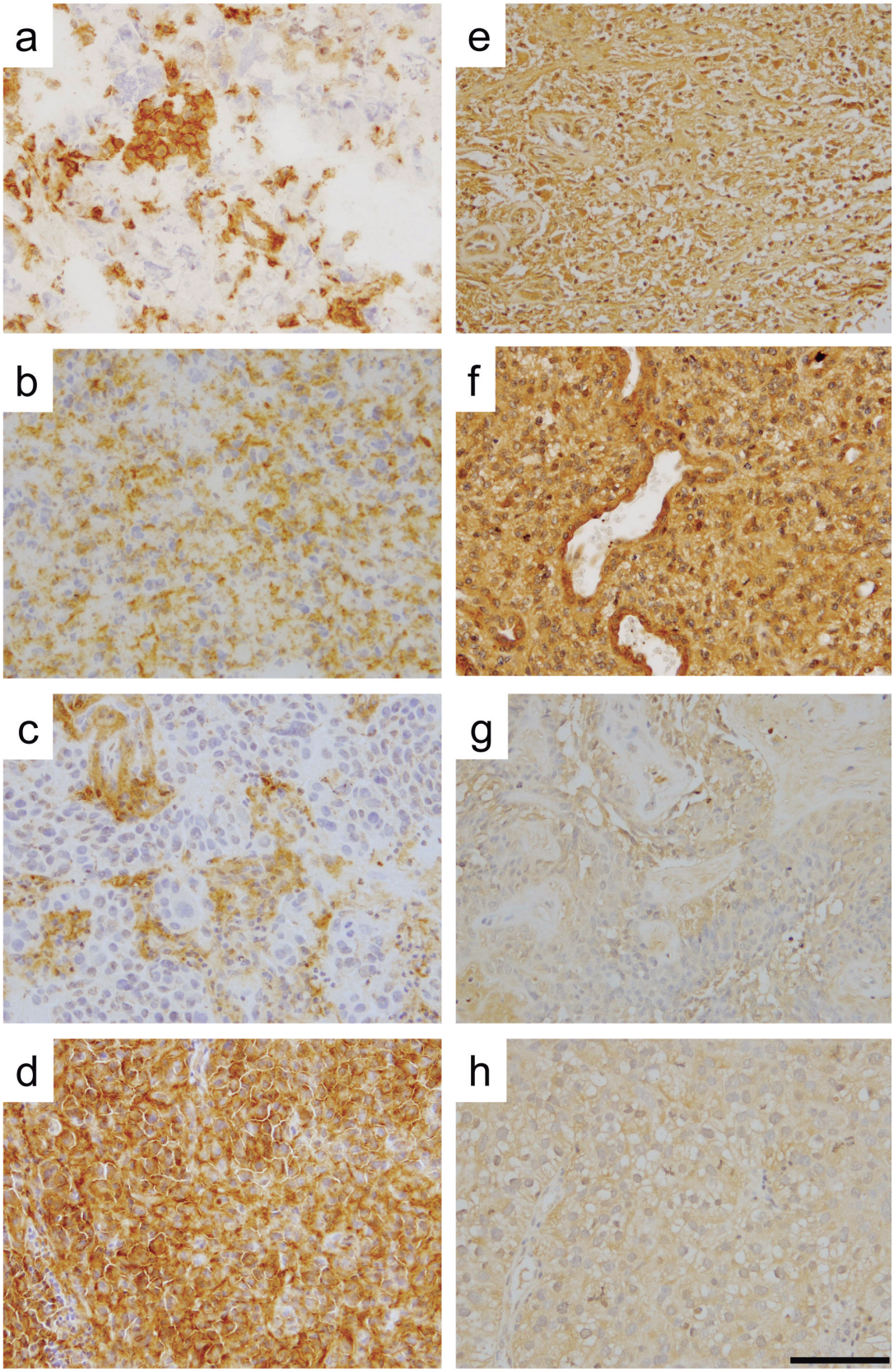

Fig. 2. Immunohistochemical analysis of αvß3 **(a-d)** and αvß5 **(e-f)** integrins in glioblastomas (**a, b and e, f)** and central nervous system metastases from breast **(c)** and lung **(g, h)** cancer or melanoma **(d)**. Scale bar: 100 μm.

much more strongly expressed as compared to αvß5. Expression intensity of αvß3 was generally weak in secondary brain tumours, with only one sample scoring higher than 1 (a single breast carcinoma which scored 2 [moderate]).

Expression of integrin ligands in tumour cells of primary and secondary CNS tumours

The frequency of expression of integrin ligands is summarised in Fig. 3 and representative immunohistochemical stainings are shown in Fig. 4. About half of the glioblastomas and breast-derived brain metastases expressed fibrinogen, although the frequency of expression was mostly in <50% of cells. Stronger expression of fibrinogen was observed in tumour cells derived from melanoma or especially lung carcinoma metastases. Staining intensities for fibronectin in tumour cells were mostly weak, with moderate staining intensity observed only in a single glioblastoma sample and in two lung-derived tumours. Between one-third and two-thirds of samples from each tumour type did not express fibronectin, and no primary or secondary CNS neoplasm expressed fibronectin in >50% of all tumour cells.

Table 2. Numbers of frozen samples analysed (glioblastoma and central nervous system metastases of different origin).

Antibody:	αvß3	αvß5	Fibrinogen	Fibronectin	Osteopontin	Vitronectin
Glioblastoma	15	10	15	15	14	1
Breast	15	10	10	10	9	14
Lung	16	7	11	10	11	15
Melanoma	16	9	9	11	11	14

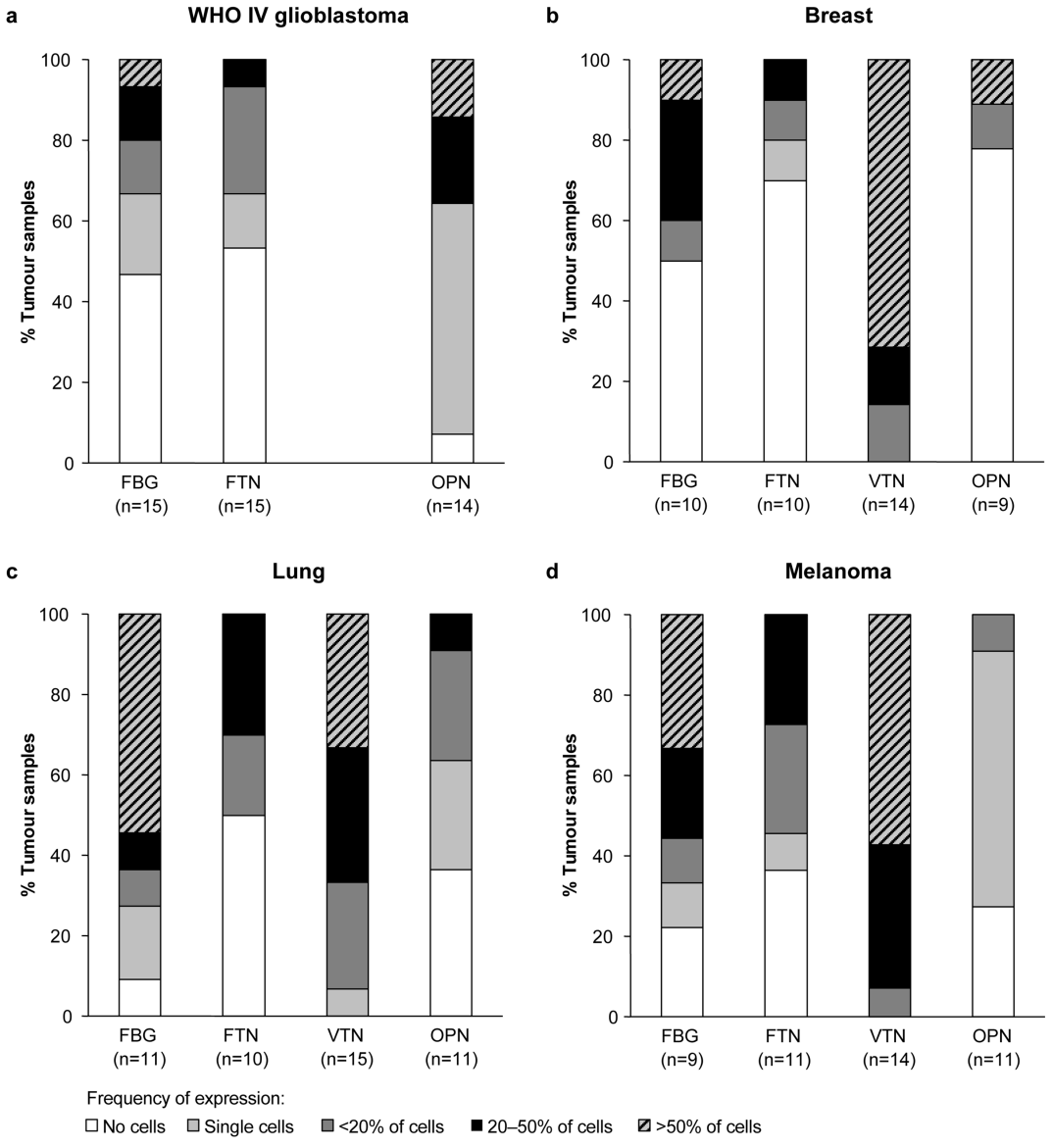

Fig. 3. Expression of integrin ligands in tumour cells of glioblastomas and central nervous system metastases from breast and lung cancer or melanoma. FBG, fibrinogen; FTN, fibronectin; OPN, osteopontin; VTN, vitronectin. Vitronectin expression was not analysed in glioblastomas as only one sample was available.

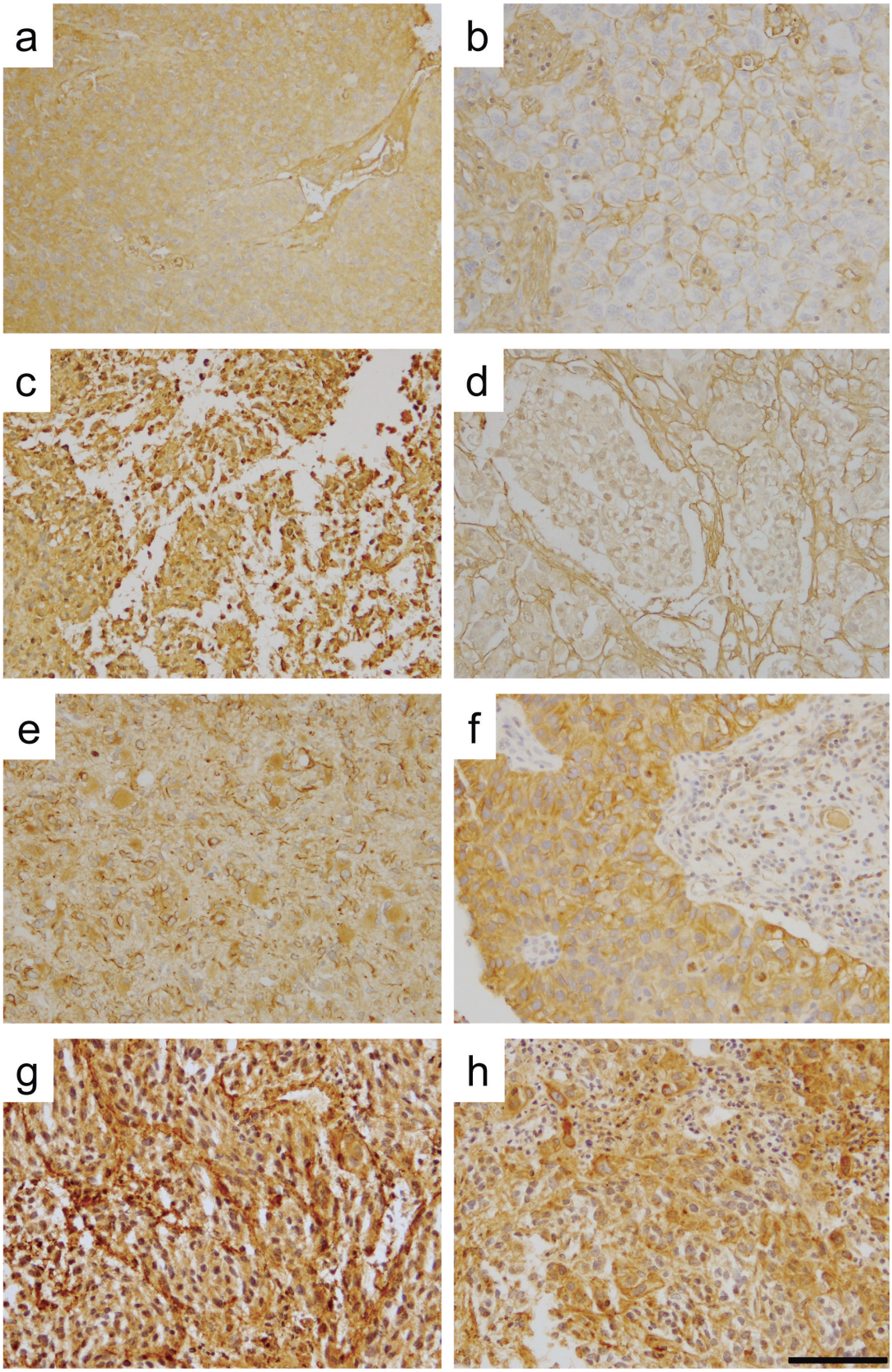

Fig. 4. Immunhistochemical analysis of fibrinogen **(a, b),** fibronectin **(c, d)**, osteopontin **(e, f)** and vitronectin **(g, h)** in glioblastomas **(a, c, e)** and central nervous system metastases from breast **(d, h)** and lung **(b, f)** cancer or melanoma **(g)**. Scale bar: 100 μm.

Vitronectin was moderately or strongly expressed in all secondary brain tumours studied. In contrast, in glioblastoma, most samples did not show a reproducible staining result; therefore, these stainings were not included in the evaluation. Osteopontin was expressed by tumour cells of most glioblastomas and the majority of lung- and melanoma-derived tumours, whereas expression of this ligand was uncommon in breast-derived brain tumours. Intensity scores for osteopontin in glioblastoma cells were mostly moderate (9/14), with four samples demonstrating weak and one sample strong intensity.

Expression of αvβ3 and αvβ5 integrins and their ligands in stromal cells of secondary CNS neoplasms

Intrinsic brain malignancies do not usually develop stromal elements, so analysis of stromal expression was restricted to metastatic brain tumours (Table 3). Marked expression of αvß3 was observed in tumour-associated stromal cells of the majority of lung- and melanoma-derived tumours, and in 40% of breast tumours. αvß5 expression was uncommon in breast tumour stroma and was not studied in lung- or melanoma-derived tumours

Table 3. Data on expression of integrins and integrin ligands in stromal cells: numbers of tumours demonstrating minor/marked expression.

Tumour origin[a]	Breast	Lung	Melanoma
αvß3	9/6	3/6	2/4
αvß5	4/1	-[b]	-[b]
Vitronectin	2/8	0/10	0/6
Fibronectin	2/6	0/7	0/3
Osteopontin	6/1	5/1	1/1
Fibrinogen	1/7	0/9[c]	1/3

Each table entry shows the number of tumours with minor expression (expression frequency scores 0-3)/numbers of tumours with marked expression (expression frequency scores 4-5). χ^2 analysis indicated no significant differences within the dataset. [a]: Tumours of central nervous system origin do not display stromal cells and this analysis was therefore not conducted for glioblastoma samples. [b]: Insufficient number of samples for analysis. [c]: All samples had frequency score 5 (expression in >50% of cells).

Table 4. Data on expression of integrins and integrin ligands in tumour-associated blood vessels.

Tumour origin	Glioblastoma	Breast	Lung	Melanoma
αvß3	9/4/2	4/1/9	1/3/12	5/5/6
αvß5	5/4/0	6/2/2	3/4/0	3/6/0
Vitronectin	-[a]	2/1/10	0/1/12	0/3/11
Fibronectin	0/0/15	0/2/8	1/1/8	3/1/7
Osteopontin	0/4/10	0/4/5	1/3/4	1/3/4
Fibrinogen	3/3/8	0/5/5	0/3/8	1/4/5

Data shown refer to sections with blood vessel frequency score=0 (no expression in blood vessels [lowest category])/blood vessel frequency score=1 (moderate number of positive blood vessels/blood vessel frequency score=3 (expression in most blood vessels). [a]: Insufficient number of samples for analysis.

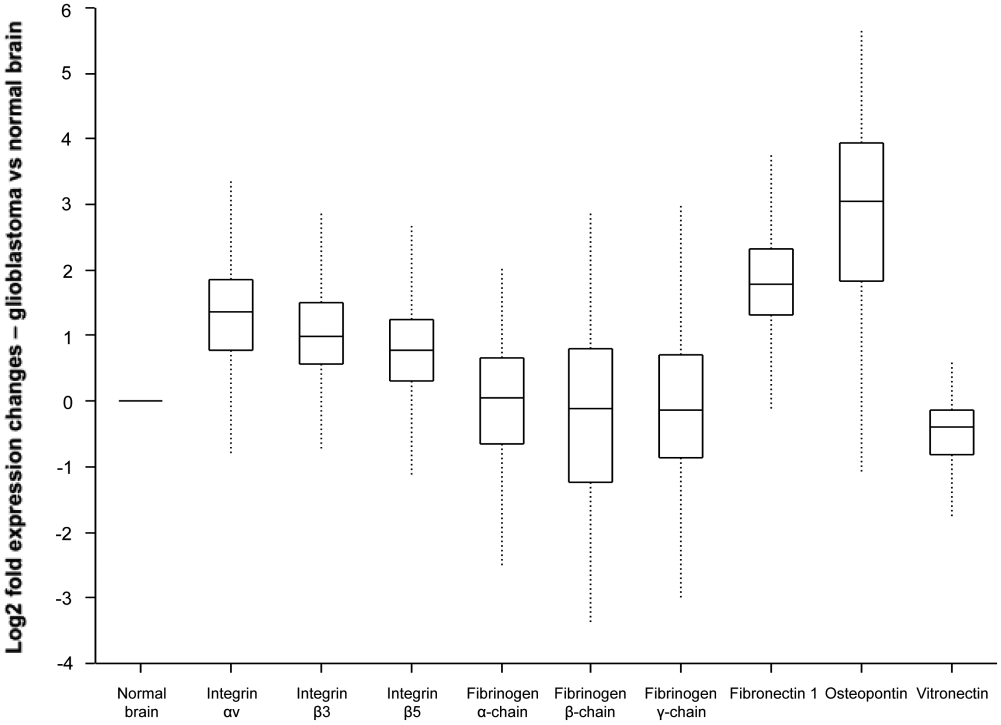

Fig. 5. mRNA expression of integrins and their ligands in glioblastomas (n=424) relative to normal central nervous system tissues (n=11). The data derive from The Cancer Genome Atlas (TCGA) Data Portal (https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp). For this study, the TCGA Portal has been assessed in November 2011. Log2-fold mRNA expression changes are depicted.

since the stromal cells were not unequivocally assessable for αvß5 in these samples. The majority of the metastatic brain tumours showed marked stromal expression of fibrinogen, vitronectin and fibronectin (Table 3). In contrast, strong expression of osteopontin was only seen in single cases.

Expression of αvß3 and avß5 integrins and their ligands on blood vessels of primary and secondary CNS neoplasms

Most tissue specimens of breast-, lung- and melanoma-derived tumours showed expression of αvß3 in most tumour-associated blood vessels, whereas only a minority of glioblastoma-associated vessels expressed αvß3. αvß5 in blood vessels was generally absent or only moderately frequent in both primary and secondary CNS neoplasms. Most tumours expressed the integrin ligands in blood vessels at moderate or high frequency (Table 4).

mRNA expression of integrins and integrin ligands in glioblastomas compared to normal CNS tissues

Fig. 5 illustrates integrin and integrin ligand mRNA expression in glioblastomas relative to the normal CNS tissues. cDNA expression of αvß3 and αvß5 was significantly upregulated in glioblastomas (n=424) as compared to normal brain (n=11) (P=0.0003 and P=0.0072, respectively). Regarding the expression of integrin ligands, osteopontin demonstrated the strongest upregulation compared with normal brain tissue (P<0.0001). Fibronectin was also significantly upregulated (P<0.0001), whereas vitronectin and fibrinogen expression in glioblastomas did not significantly differ from those in normal brain tissue.

Discussion

We have studied the expression of αv integrins and their ligands in primary and metastatic tumours in human brain specimens. Our primary findings are that integrin αvß3 was expressed by most glioblastomas and in brain metastases from breast and lung cancers and melanomas, often at high frequencies. Integrin αvß5 was expressed at low frequency in all tumours except in those from lung, where expression frequency was higher than that for αvß3. Our data from glioblastoma samples show that glioblastomas express αvß3 and αvß5 integrins, both on neoplastic astrocytes and on invasive blood vessels (Gladson and Cheresh, 1991; Paulus et al., 1993; Gladson, 1996; Bello et al., 2001; Kanamori et al., 2004). In contrast to the constantly high αvß3 expression on blood vessels in CNS metastases, αvß3 expression was considerably lower in primary CNS glioblastomas. In contrast to CNS metastases, glioma vascularisation occurs in a cooptive manner for a long time (Fischer et al., 2005). In CNS metastases, activated blood vessels have to actively invade the cohesive tumour cell clusters. Glioma cells often migrate along preexisting blood vessels, thereby gaining access to oxygen and nutrients. This might be at least partly responsible for a later and more moderate activation of glioma-associated blood vessels in contrast to a generally stronger expression of integrins in blood vessels associated with CNS metastases. Potentially αvß3 is just overexpressed in a distinct temporo-regional manner in case of insufficient supply of nutrients and oxygen by vascular cooption alone. In general, our data on integrin expression in secondary brain tumours are consistent with reports of αvß3 and αvß5 expression in primary breast cancer (Meyer et al., 1998; Silvestri et al., 2002; Havaki et al., 2007; Vellon et al., 2007; Beer et al., 2008), lung cancer (Chen et al., 2005; Cui et al., 2007; Fong et al., 2009), and melanoma (Neto et al., 2007; Tzukert et al,. 2010), where the expression of αvß3 was suggested to be linked to the invasive potential of the tumour cells. Our study also defines the expression patterns of αvß3 and αvß5 in tumour stroma and endothelial cells within the different tumour types. Results in breast- and lung-derived brain metastases are consistent with earlier reports of αvß3 expression in capillaries of primary breast and lung carcinomas (Max et al., 1997).

Expression of integrin ligands was variable in tumour tissues. For all secondary brain tumours, vitronectin was the most commonly expressed integrin ligand evaluated. The abundant expression of this ligand has been previously reported in primary breast cancer (Aaboe et al., 2003), but not, to our knowledge, in the other tumours we describe here. The strong expression of vitronectin is all the more of interest since emerging evidence especially presents the vitronectin/αvß3 axis as an important pathway affecting intracellular signalling in cancer progression (Reuning, 2011). αvß3 was found in more than 50% of all tumour entities, at least to some extent, in our cohort, while αvß3 was absent in all melanomas and approximately 70% of breast carcinomas. These findings suggest that the inhibition of the vitronectin/αvß3 pathway could be an especially promising target for secondary brain tumours. Concerning vitronectin expression in glioblastomas, other studies showed that most human astrocytic tumours are completely negative for vitronectin. Interestingly, only “loose focal vitronectin deposits“ were described in single cases of glioblastoma in one study (Zámecník et al., 2004). This is quite in line with our findings, although we did not feel comfortable with the staining results and were not convinced that such “loose focal vitronectin deposits“ really represent specific staining signals. Furthermore, the fact that vitronectin seems slightly downregulated in glioblastomas as compared to normal CNS tissue (see Fig. 5) might be an additional indicator for our interpretation that the vitronectin staining signals in glioblastomas are not reliable. In general, about half of the tumours expressed the other ligands, with a low frequency of expression of fibronectin and osteopontin in breast-derived metastases, and a higher expression of

fibrinogen in lung-derived brain metastases. While a high expression of fibrinogen has been demonstrated in primary lung cancer tissue (Sierko et al., 2012), our data regarding low osteopontin expression in breast-derived brain metastases diverge from its reported high expression in primary breast carcinoma (Kim et al., 1998). The expression of integrin ligands did not clearly follow expression of individual integrins, despite vitronectin being described as a monospecific ligand for αvß5, while fibrinogen and osteopontin preferentially target αvß3 (Hynes, 2002). Osteopontin has been described as increasing the migratory potential of cancer cells via activation of αvß3 (Fong et al., 2009). The majority of tumours expressed osteopontin. The high levels of osteopontin expression in glioblastomas could be experimentally linked to an increased glioma cell invasion and proliferation (Jan et al., 2010) correlate with the extent of angiogenesis *in vivo* (Matusan-Ilijas et al., 2008) and could meanwhile be established as a serum biomarker for worse patient prognosis in human gliomas (Sreekanthreddy et al., 2010). Concerning the expression of integrins and their corresponding integrin ligands in primary tumours or cells, a similar heterogeneity to our study was described (Marshall et al., 1991; Taherian et al., 2011).

Overall, some tumours demonstrated high-frequency expression of integrins and their ligands. In addition, in glioblastomas, cDNA expression of αvß3, αvß5, osteopontin and fibronectin were significantly upregulated compared with normal CNS tissues. The role of αvß3 and αvß,5 integrins in tumour development and metastasis is being exploited clinically. Cilengitide, a cyclised Arg-Gly-Asp (RGD)-containing peptide (Cyclo-[Asp-D-Phe-N-MeVal-Arg-Gly]), is a selective inhibitor of these integrins, and is currently being studied in the clinics in various tumour settings, including glioblastoma (Reardon et al., 2008; Stupp et al., 2010). Further studies are needed to reveal whether the expression of αvß3 vs αvß5 we have measured in brain tumours is related to hypoxia or ischaemia, which can upregulate αvß3 (but not αvß5) (del Zoppo and Milner, 2006), or if this expression profile is a phenomenon intrinsic to the tumour itself.

Conclusions

Integrins αvß3 and αvß5 in human glioblastoma and in CNS metastases from lung and breast cancer and melanoma are expressed in a distinct and heterogeneous pattern. The differential expression of integrins and integrin ligands may help to develop targeted integrin-antagonistic therapies for human brain cancers.

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