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Identification of genes differentially expressed in glioblastoma versus pilocytic astrocytoma using Suppression Subtractive Hybridization

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Glioblastoma (GBM) is a highly malignant glioma, which has the propensity to infiltrate throughout the brain in contrast to pilocytic astrocytoma (PA) of the posterior fossa, which does not spread and can be cured by surgery. We have used Suppression Subtractive Hybridization to define markers that better delineate the molecular basis of brain invasion and distinguish these tumor groups. We have identified 106 genes expressed in PA versus GBM and 80 genes expressed in GBM versus PA. Subsequent analysis identified a subset of 20 transcripts showing a common differential expression pattern for the two groups. GBM differs from PA by the expression of five genes involved in invasion and angiogenesis: fibronectin, osteopontin, chitinase-3-like-1 (YKL-40), keratoepithelin and fibromodulin. PA differs from GBM by the expression of genes related to metabolism (apolipoprotein D), proteolysis (protease-serine-11), receptor and signal transduction (PLEKHB1 for Pleckstrin-Homology-domain-containing-protein-family-B-member-1), transcription/translation (eukaryotic-translation-elongation-factor-1-α1) processes and cell adhesion (SPOCK1 for SPARC/Osteonectin-CWCV-kazal-like-domains-proteoglycan). The expression of these genes was confirmed by real-time quantitative RT-PCR and immunohistochemistry. This study highlights the crucial role of brain invasion in GBM and identifies specific molecules involved in this process. In addition, it offers a restricted list of markers that accurately distinguish PA from GBM.
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Introduction

Gliomas are a heterogeneous group of intracranial neoplasias of glial origin. They are the most common type of primary tumor (40%) in the central nervous system. Different subtypes can be distinguished, among them pilocytic astrocytomas (PA), oligodendrogliomas, oligoastrocytomas and glioblastomas (GBM) (Kleihues and Cavenee, 2000).

GBM (WHO grade IV) is the most frequent and malignant primary neoplasm of the human central nervous system. GBM have the propensity to infiltrate throughout the brain. They carry complex genetic aberrations resulting in the inactivation of various tumor suppressor genes, as well as the amplification of different protooncogenes (Kleihues and Cavenee, 2000). Despite progress in surgery and adjuvant therapy, patients with GBM have a very poor prognosis. In contrast, PA (WHO grade I), the most common glioma in children, has a favorable prognosis. Its slow and circumscribed growth pattern with almost no infiltrative behavior enables it to be cured, in most cases, by total surgical excision.

The heterogeneity of astrocytic tumors has made their pathological classification rather difficult. Although several critical genes have been identified as being associated with tumorigenesis and anaplastic progression of gliomas (Caskey *et al*., 2000), their contribution to the molecular classification of astrocytic tumors has been limited.

New genomic technologies such as DNA microarrays may facilitate the molecular profiling of human tumors. Some studies have used microarrays to compare the expression pattern of high-grade tumor with low-grade tumor or nontumoral brain tissue (Sallinen *et al*., 2000; Rickman *et al*., 2001; Gutmann *et al*., 2002; Khatua *et al*., 2003; van den Boom *et al*., 2003; Mischel *et al*., 2004; Oncomine database). They identified cell cycle and growth regulators, invasion and growth factors and cytokines that probably play a role in astrocytoma progression.

We chose to compare GBM and PA in order to find a restricted list of genes involved in tumor brain invasion.

Owing to their putative common origin (astrocytic cells or glial precursor cells), but their radically different behavior, we found more judicious to compare these two types of gliomas rather than comparing GBM with normal brain. We chose to use Suppression Subtractive Hybridization (SSH) (Diatchenko *et al*., 1996) to identify genes expressed differentially between these two types of tumors. We were able to identify a number of mostly novel potential candidate genes. Our data indicate that GBM differ from PA by the expression of subset of genes involved in invasion and angiogenesis, whereas PA are characterized by the expression of genes related to metabolism, proteolysis, receptor and signal transduction, transcription/translation processes and cell adhesion.

Results

Identification of differentially expressed genes by SSH

To identify differentially expressed genes in the two groups of tumors, SSH was performed. Two subtractive cDNA libraries were generated and a total of 1296 clones (648 for GBM and 648 for PA) were further subjected to dot blot screening in order to eliminate false positives. In all, 186 selected clones (80 for GBM and 106 for PA) were sequenced and found to represent, respectively, 36 different known genes for PA (plus eight unknown and 20 unexploitable) and 29 for GBM (plus three unknown and 10 unexploitable) (Tables 1 and 2). They were functionally categorized on the basis of known or inferred biological function of their protein

Table 1 Results of genes expressed in pilocytic astrocytomas after screening by SSH against glioblastomas

Gene name	*NCBI number*	*Locus*	*Frequency*	*Putative function from NCBI database*
Cell adhesion and invasion				
Testican-1 precursor, SPOCK1	NM004598	5q31	7/106	Role in cell–cell and cell–matrix interactions. May contribute to various neuronal mechanisms in the CNS
Integrin alpha-3 chain, ITGA3	M59911	17q21.33	1/106	Integrin receptor for fibronectin, laminin, collagen and thrombospondin
Proteolytic processes/Protein degradation regulation				
Protease serine 11, HTRA1	D87258	10q25.3-26.2	6/106	Specific protease for IGF binding proteins. Increased in osteoarthritis cartilage
Glia-derived-nexin 1, PN1A, SERPIN 2	M17783	2q33-q35	3/106	Protease, regulator of a-thrombin in tissues. Belongs to the serpine superfamily. Highly expressed and developmentally regulated in the NS.
Serine or cysteine proteinase inhibitor, SERPIN A3	NM001085	14q32.1	3/106	Anti-trypsin, anti-chymotrypsin
Transcription/translation processes				
Eukaryotic translation elongation factor 1 α1, EEF1A	NM001402	6q14	7/106	GTP binding activity. Involved in translation elongation
Receptors and signal transduction/ Developmental processes				
PLEKHB1	NM021200	11q13.5	5/106	Pleckstrin homology domain containing protein, family B, number 1. May be involved in cell differentiation
Delta-Notch-like EGF related receptor, DNER	NM139072	2q37	3/106	Strongly expressed in developing and mature neurons. Excluded from axons
Reticulon 3 neuroendocrine protein, RTN3	NM006054	11q13	1/106	Membrane protein, highly expressed in brain
Scrapie-responsive gene 1, SCRG1	NM007281	4q31-32	1/106	Overexpressed in brains of patients with Creutz-feldt–Jacob disease
Transmembrane 4 superfamily member 2, TSPAN7	NM004615	Xp11.4	1/106	Mediates signal transduction events that play a role in the regulation of cell development, activation, growth and motility
G protein coupled receptor, GPRC5B	NM016235	16p12	1/106	May mediate the cellular effects of retinoic acid on the G protein signal transduction cascade
Protein tyrosine kinase 3	U18934	15q15	1/106	TYRO 3 or DTK. High expression in fetal and adult brain
Neuroligin 2, NLGN2	NM020795	17p13.2	1/106	Expressed in all brain regions, lung, ovary and kidney. Functions as ligand for the neurexin family of cell surface receptors
Dickkopf homolog 3, DKK3	NM013253	11p15.2	1/106	Involved in Wnt signaling inhibition
Olig 2	NM005806	21q 22	1/106	bHLH transcription factor. Precocious factor of glial lineage
Sox 10	NM006941	22q12.3	1/106	SRY-related HMG box transcription factor. Expressed in glial cells and oligodendrocytes. Important for early development of neural crest
Sox 8	NM014587	16p13.3	1/106	SRY-related HMG box transcription factor. Strongly expressed in many tissues including nervous system. May be involved in mental retardation
Structural				
GFAP	BC041765	17q21	4/106	Intermediate filament protein highly specific of cells of astroglial lineage

Table 1 (*continued*)

Gene name	*NCBI number*	*Locus*	*Frequency*	*Putative function from NCBI database*
Metabolic				
Apolipoprotein D, APOD	NM001647	3q26.2-ter	5/106	Member of the lipocalin family. Involved in the transport of small hydrophobic molecules
Aldolase C, fructose biphosphate, ALDOC	NM005165	17q11.2	3/106	Brain specific. Involved in fructose metabolism
Phospholipase A2 group IIA, PLA2G2A	NM000300	1p35	2/106	Involved in metabolism of phospholipids as well as the production of precursors for inflammatory reactions
Na/K transporting α2 polypeptide, ATP1A2	NM000702	1q21-q23	2/106	ATPase
Glutamine synthase, GLUL	NM002065	1q31	2/106	Neurotransmitters conversion
Transferrin, TF	M12530	3q21	1/106	Iron binding transport protein. Expressed by liver and brain and secreted in plasma
Ferritin, heavy polypeptide 1, FTH1	NM002032	11q13.1	1/106	Iron storage protein. Expressed by liver and brain
Stearoyl-CoA desaturase, SCD	NM005063	10q23	1/106	Desaturation of fatty acids
Carboxypeptidase E, CPE	NM001873	4q32.3	1/106	Involved in neuropeptide processing
Superoxyde dismutase 2, SOD2	NM000636	6q25.3	1/106	Manganese SOD. Intramitochondrial free radical scavenging enzyme
UDP-N-acetyl-αD-galactosamine, GALNT13	NM052917	2q24.1	1/106	Glycosyltransferase. Expressed in neuroblastoma cells and primary cultured neurons but not in glioblastoma cells and primary cultured astrocytes
Lactate deshydrogenase B			1/106	
Others				
PDZK3 protein, KIAA0300	AB002298	5p13.3	4/106	PDZ domain containing protein 3
Angiopoietin-like 2, ANGPTL2	NM012098	9q34	1/106	Induces sprouting of endothelial cells through an autocrine and paracrine action
TNF Receptor, member 21, Death receptor 6	NM014452	6p21.1	1/106	May activate NF-kappa-B and JNK and promote apoptosis
Ras association domain family 2 protein, KIAA0168	NM014737	20p13	1/106	
HLA-A2 class I antigen	NM002116	6p21.3	1/106	
Unknown			8/106	
Unexploitable			20/106	

product. In PA versus GBM, the 15 genes recorded at least twice and representing 64/106 of the clones sequenced encoded proteins that were related to metabolism (apolipoprotein D: five clones), proteolysis (protease-serine-11: six clones), receptor and signal transduction (PLEKHB1: five clones; DNER: three clones), transcription/translation processes (eukaryotic-translation-elongation-factor-1-α1: seven clones), and cell adhesion and invasion (SPOCK1: seven clones). Interestingly, in GBM versus PA, the five genes recorded at least twice and representing 43/80 (>50%) of the clones sequenced encoded proteins that were all related to cell adhesion and invasion. They included fibronectin (FN1: 34 clones), chitinase-3-like-1 (three clones), osteopontin (OPN), keratoepithelin and fibromodulin (two clones each). Four additional genes, including TIMP3, were recorded once.

Validation using QPCR: genes expressed in PA versus GBM

Quantification of PLEKHB1 and DNER mRNA transcripts revealed higher mRNA levels in PA compared with GBM samples ($P=0.0009$ and $P=0.004$, respectively). The mean level of PLEKHB1 mRNA was 192.71 ± 107.21 in PA versus 67.13 ± 76.65 in GBM samples and the mean level of DNER was 2.19 ± 1.04 in PA versus 0.70 ± 0.86 in GBM samples. SPOCK1 mRNA levels were on average 5.7 times higher in PA (mean $=3.76\pm2.35$) than in GBM samples (mean $=0.65\pm0.77$) ($P=0.0017$) (Figure 1a).

Validation using QPCR: genes expressed in GBM versus PA

The mean level of DEC1 mRNA expression was 1.42 ± 1.05 in GBM versus 0.43 ± 0.19 in PA samples ($P=0.0032$). Quantification of OPN mRNA confirmed higher OPN mRNA levels in GBM compared with PA samples (Figure 1b). The mean level of OPN mRNA expression was 14.77 ± 16.14 in GBM, versus 1.61 ± 1.55 in PA samples ($P=0.0002$). Interestingly, we observed that a ratio of OPN/SPOCK1 > 4.64 was strongly associated with the diagnosis of GBM ($P<0.0001$), with specificity of 100% and sensitivity of 93.3% (Figure 1c and d). Quantification of FN1 mRNA levels also confirmed the data obtained with the SSH technique. The mean level of FN1 expression was 2.64 ± 7.12 in GBM versus 0.039 ± 0.021 in PA samples ($P=0.0002$) (Figure 1e). Finally, the role of TIMP3 in GBM is unclear as this metalloproteinase inhibitor is a well known tumor suppressor and apoptosis inducing protein (Lam *et al*., 2005). We did not find any significant difference between GBM and PA regarding to the level of TIMP3 mRNA ($P=0.85$) (data not shown). However, TIMP3/SPOCK1 > 2.083 ratio could be used to distinguish GBM from PA ($P=0.0032$), with specificity of 93.3% and sensitivity of 80% (Figure 1f).

Validation at protein level using immunohistochemistry

FN1, OPN and TIMP3 protein expression was analysed by immunohistochemistry in series of formalin-fixed and paraffin-embedded GBM (Figure 2a) and PA (Figure 2b). FN1 showed strong staining in the 20 GBM

Table 2 Results of genes expressed in glioblastomas after screening by SSH against pilocytic astrocytomas

Gene name	*NCBI number*	*Locus*	*Frequency*	*Putative function from NCBI database*
Cell adhesion and invasion				
Fibronectin 1, FN1	NM002026	2q34-q36	34/80	Major cell surface glycoprotein involved in the adhesion of cells to collagen (ECM)
Chitinase-3-like-1, CHI3L1	NM001276	1q32.1	3/80	Cartilage glycoprotein
Osteopontin, OPN, SPP1	NM000582	4q21-q25	2/80	Important to cell–matrix interaction. Promotes cell migration
Keratoepithelin, TGFBI	NM000358	5q31.1	2/80	Structural protein. Important role in cell–collagen interactions
Fibromodulin, FMOD	NM002023	1q32.1	2/80	Small collagen-binding proteoglycan constituent of the extra-cellular matrix
Tissue inhibitor of metalloproteinase 3, TIMP3	U14394	22q12.1-q13.2	1/80	Plays pivotal roles in ECM regulation. Inhibits activity of MMP
Annexin II, ANXA2	NM004039	15q21	1/80	Calcium-dependent phospholipid binding protein.
Collagen type IV α3, COL4A3	NM031366	2q36-q37	1/80	Functions as an endothelial cell-specific inhibitor of proteins synthesis. Inhibits angiogenesis and tumor growth
NMB transmembrane glycoprotein, GPNMB	NM002510	7p15	1/80	Involved in growth delay and reduction of metastatic potential
Proteolytic processes/Protein degradation regulation				
α2 macroglobuline, A2M	NM000014	12p13.3	1/80	Inhibits many proteases, including trypsin, thrombin and collagenase
Transcription/translation processes				
RNA Polymerase II, subunit C 33 kD, POLR2C	NM032940	16q13-q21	1/80	DNA-dependent RNA polymerase, catalyses the transcription of DNA into RNA
SNAP C3	NM003084	9p22.2	1/80	Small nuclear RNA activating complex, polypeptide 3. Required for transcription of small nuclear RNA genes
SLC30A9	AY594282	4p12-p13	1/80	Cation transporter activity. Involved in transcriptional regulation
Receptors and signal transduction/ Developmental processes				
Transcriptional Intermediary factor 1α, TIF1	BC028689	7q32-q34	1/80	Interacts selectively with different nuclear receptors. Growth suppressor required for the growth-inhibitory activity of retinoic acid
Beta-catenin, CTNNB 1	NM001904	3p22	1/80	Involved in the regulation of cell adhesion and in signal transduction through the Wnt pathway
Casein kinase 1, CSNK1A 1	NM001892	5q31-q32	1/80	Downregulated in lung cancer. Participates in Wnt signalling by phosphorylating CTNNB1 on Ser-45
Paired related Homeobox, 1PRRX1	NM006902	1q24	1/80	Transcriptional regulator of developmental processes. Expressed in a mesodermally restricted pattern in embryos
BHLHB2 or DEC 1	AB004066	3p25.3-p26	1/80	cAMP inducible bHLH transcription factor involved in embryo chondrocytes and neurons proliferation and/or differentiation
Dynein, DNCI1	NM004411	7q21.3	1/80	Cytoplasmic, intermediate chain 1. Microtubule-activated ATPase. Upregulated during early development (forebrain)
Adipose differentiation-related protein, ADFP	NM001122	9p22	1/80	ADRP mRNA induced rapidly during adipose differentiation. Membrane-associated protein
AWP1	NM019006	15q24	1/80	Specifically associated with PRK1. AWP1 may play a critical role in cellular regulation, such as protein phosphorylation and apoptosis
Endothelin receptor type B, EDNRB	NM000115	13q22	1/80	Nonspecific receptor for endothelin 1, 2 and 3. Mediates its action by association with G-proteins
Epidermal growth factor receptor, EGFR	NM005228	7p12	1/80	Activating phosphatidylinositol and Ras pathways. Involved in the control of cell growth and differentiation. Amplified in GBM
Structural				
γS Crystallin, CRYGS	NM017541	3q25-q28	1/80	Dominant structural component of the vertebrate eye lens. Possible role in cataract formation
Metabolic				
Thioredoxin reductase 1	NM003330	12q23.3	1/80	Key enzyme in the regulation of the intracellular redox environment
Transferrin receptor, TFRC	NM003234	3q29	1/80	Involved in iron homeostasis. Important for development of erythrocytes and nervous system
Phosphoglycerate kinase 1, PGK1	NM000291	Xq13.3	1/80	Glycolitic enzyme. Key enzyme for ATP generation. Acts also as a polymerase α cofactor protein
Epididymal secretory protein, NPC2	NM006432	14q24.3	1/80	Niemann–Pick disease type 2 protein (NPC2) : cause abnormally high cholesterol accumulation in cells
Ubiquitin-binding protein p62 Sequestosome 1	NM003900	5q35	1/80	Catabolism of proteins. Highly expressed in brain
Others				
Unknown			3/80	
Unexploitable			10/80	

analysed. In all specimens, microvascular proliferation and some tumor cells located near the neoplastic vessels were deeply stained (Figure 2c). In contrast, neither vessels nor tumor cells were immunolabelled in PA samples (Figure 2d) except for three of them exhibiting weak immunostaining in less than 5% of tumor cells (data not shown). Anti-OPN immunoreactivity was recorded in all GBM cases. The percentage of immunoreactive tumor cells ranged from 10 to 50%. The staining had a dot-like appearance within tumor cell cytoplasm (Figure 2e). In most cases, areas with numerous immunoreactive cells contrasted with others devoid of immunostaining. No staining was recorded in PA samples (Figure 2f) except in one case. Finally, TIMP3 expression was observed in 13/20 and 3/21 in GBM and PA samples, respectively (Figure 2g and h). The immunostaining was intracytoplasmic, weak and ranged from 10 to 50% of cells.

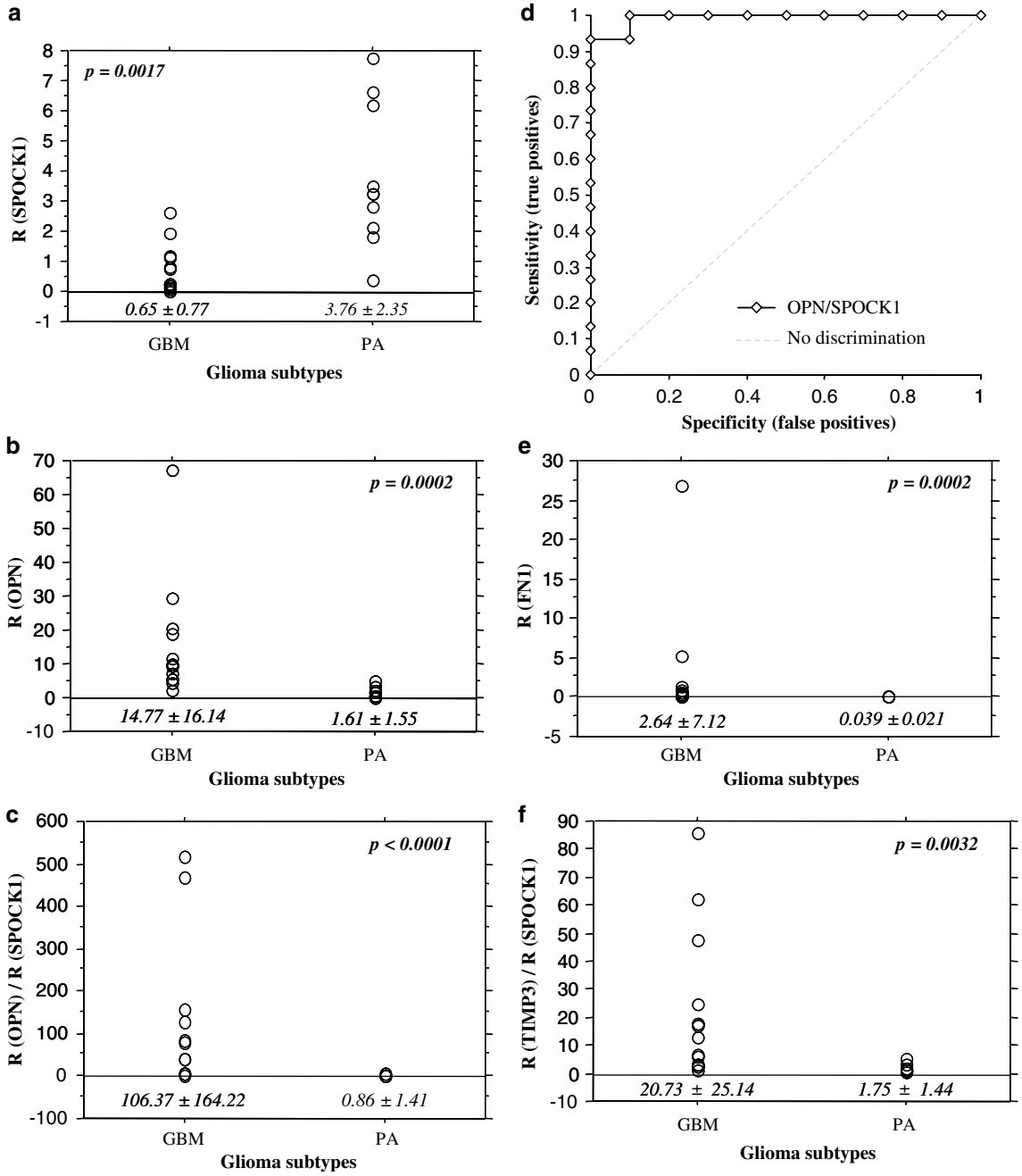

Figure 1 Scatter plots for the relative expression (R) of SPOCK1 (**a**), OPN (**b**), OPN/SPOCK1 (**c**), FN1 (**e**), and TIMP3/SPOCK1 (**f**) in GBM and PA. ROC (receiver operator characteristic) curve for differentiating GBM from PA by the OPN/SPOCK1 ratio (**d**). The cutoff point was set at 4.64 and the OPN/SPOCK1 ratio showed good agreement with the pathological diagnosis (specificity of 100% and sensitivity of 93.3%).

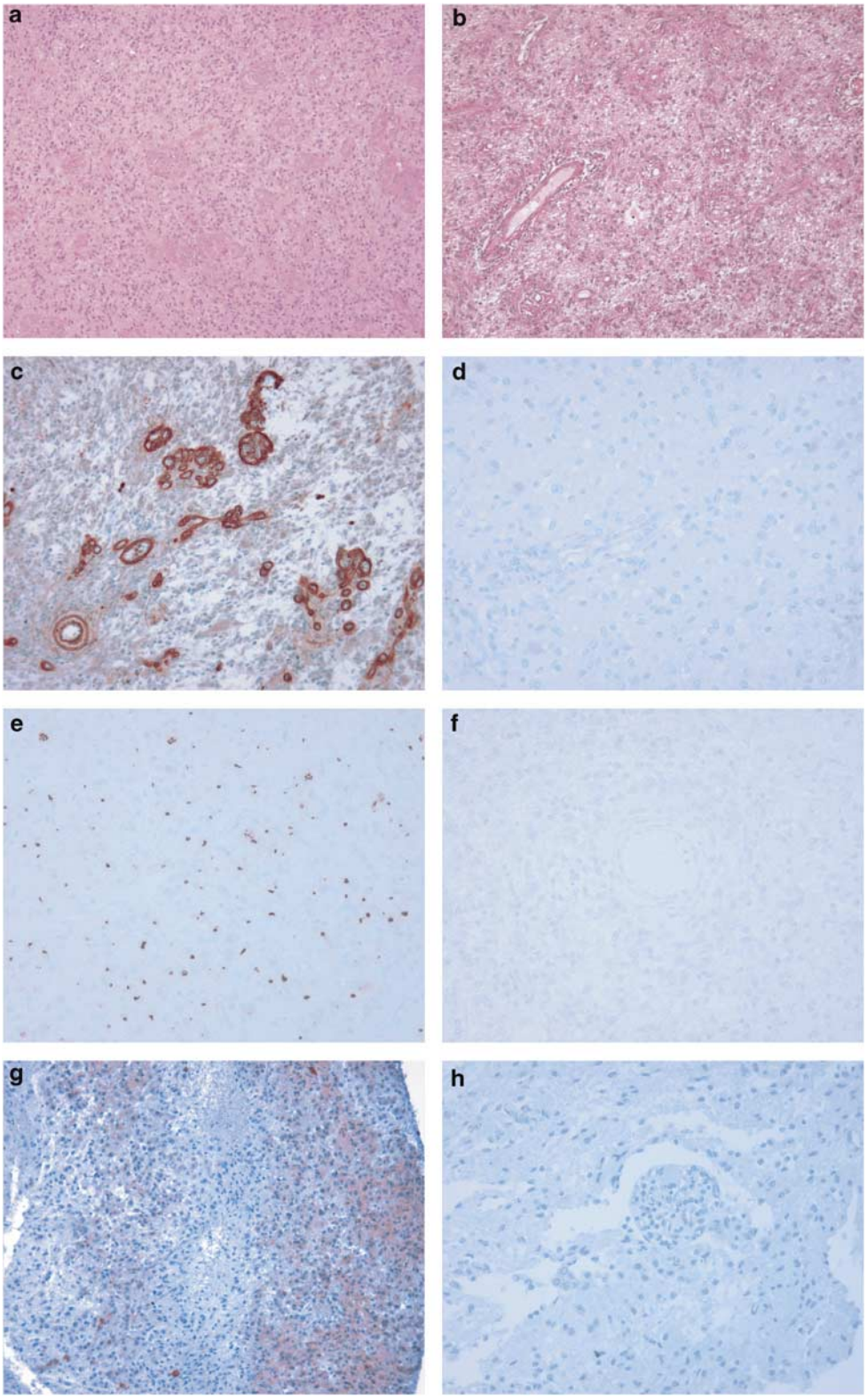

Figure 2 Immunohistochemical analysis of the expression of FN1, OPN and TIMP3 in GBM and PA. Microphotographs of immunohistochemical stained tumor sections for hematoxylin and eosin (**a** and **b**). FN1 immunostaining was observed in microvascular proliferation and in tumor cells usually located near the neoplastic vessels (**c**). Neither vessels nor tumor cells were immunolabeled in PA (**d**). The OPN immunostaining had a dot-like appearance, within the cytoplasm of GBM (**e**) but no staining could be detected in PA (**f**). Finally, TIMP3 immunostaining was intracytoplasmic and weak in GBM (**g**) and majority of PA studied did not express TIMP3 (**h**). (**a-c**, **g**) 50X; (**d-f**, **h**) 150X.

Discussion

Using SSH approach, we identified 66 genes differentially expressed in PA and GBM tumors. They were functionally categorized in six groups (see Tables 1 and 2). Our results are in agreement with two previous microarray analyses using cDNA or oligonucleotide chips, identifying candidate genes expressed in PA but not in infiltrating anaplastic astrocytoma (Hunter *et al*., 2002) and in PA but not in GBM (Rickman *et al*., 2001). These studies identified apolipoprotein D, SPOCK1, protease-serine-11, glia-derived-nexin-1 and protein-tyrosine-kinase-3 as strong markers of PA versus anaplastic astrocytoma or GBM. We also confirmed the expression of a large number of genes involved in brain development in PA (Gutmann *et al*., 2002; Wong *et al*., 2005), suggesting that these tumors share gene expression profile with fetal brain and oligodendroglial precursors. Moreover, we reported in PA and confirmed by QPCR, strong expression of two novel genes, PLEKHB1 and DNER, which might be of diagnostic value. On the other hand, the expression of DEC1, a cAMP inducible bHLH transcription factor involved in embryo chondrocyte and neuron proliferation and/or differentiation, characterizes GBM (Shen *et al*., 1997).

As invasive behavior is a hallmark of GBM, we have focused our study on genes involved in this process. GBM spread throughout the brain as single cells, but usually, they do not metastasize outside the brain (Kleihues and Cavenee, 2000). Our study shows that GBM differ from PA by their expression of FN1, OPN, keratoepithelin, fibromodulin, chitinase-3-like-1 (YKL-40) and to a lesser extent TIMP3, suggesting that these proteins generate a neoplastic extracellular matrix (ECM) providing a permissive environment critical for tumor progression and brain invasion.

FN1, a glycoprotein of about 220 kDa which forms ECM when secreted, has also been identified in GBM by microarray strategy using cDNA or oligonucleotide chips (Sallinen *et al*., 2000; Tanwar *et al*., 2002; Freije *et al*., 2004). The FN1 mRNA has three alternative splicing sites termed EDA, EDB (B-FN) and IIICS in human (Kumazaki *et al*., 1999). B-FN is a marker of angiogenesis that accumulates in blood vessels in high-grade astrocytomas but not in PA (Castellani *et al*., 2002). Therefore, GBM cells known to express at least three different FN1 receptors $\alpha5\beta1$, $\alpha v\beta3$ or CD44 (Bellail *et al*., 2004) also secrete FN1, providing both a permissive environment for tumor progression and angiogenesis.

OPN glycoprotein is expressed and secreted by numerous human cancers including malignant astrocytomas (Saitoh *et al*., 1995; Coppola *et al*., 2004). The OPN protein contains important functional domains including a central region that binds to seven different integrins (including $\alpha5\beta1$, $\alpha v\beta3$ and $\alpha v\beta5$), a calcium-binding domain and an heparan-binding domain which mediates CD44V3 binding (Wai and Kuo, 2004). OPN increases invasiveness by inducing proteinases, particularly uPA (Tuck *et al*., 1999) and acts in concert with several growth factors including EGF (Tuck *et al*., 2003). OPN is a high-affinity ligand for the $\alpha v\beta3$ integrin and has also been implicated in angiogenesis (Rittling and Chambers, 2004). Therefore, it is likely that OPN increased GBM tumorigenicity by different mechanisms: degradation of ECM, angiogenesis and cell spreading on enriched fibronectin ECM.

GBM expressed YKL-40. It is a growth factor for connective tissue cells and stimulates migration of endothelial cells. YKL-40 has been shown to be strongly elevated in serum and biopsy material from GBM patients by ELISA assay, Western blot and oligonucleotide array analysis (Lal *et al*., 1999; Markert *et al*., 2001; Tanwar *et al*., 2002). These independent methods of determining gene expression levels additionally validate our findings. High YKL-40 mRNA expression was significantly associated with poorer survival; moreover, they demonstrated that YKL-40-transfected immortalized human astrocytes consistently showed increased resistance to radiation and a fivefold induction in invasive potential as compared with control cells (Nigro *et al*., 2005).

In addition, SPOCK1, another gene specifically involved in brain cell adhesion and invasion (Bonnet *et al*., 1992; Charbonnier *et al*., 1998), was highly expressed in PA. The purified gene product has been shown to inhibit cell attachment and neurite extension in culture and to inhibit membrane type 1 and 2 matrix metalloproteinases (Edgell *et al*., 2004). It remains to be determined whether SPOCK1 expressed by PA contributes to their noninvasive behavior. Interestingly, we observed that a ratio of OPN/SPOCK1 > 4.64 within a tumor was highly predictive of GBM.

In conclusion, our study provides cues to the identity of molecules likely to play a crucial role in GBM brain invasion. Moreover, it offers a restricted list of markers that accurately distinguish GBM from PA. Diagnosis of PA might be difficult in some cases (more than 60% interobserver discordance according to Mittler *et al*. (1996)) and finding reliable markers is critical because of the marked differences in the therapy and prognosis of these two tumors.

Materials and methods

Human tissues

The present study was undertaken after informed consent was obtained from each patient or relatives. Brain tumors were classified according to WHO CNS tumor classification (Kleihues and Cavenee, 2000). SSH was performed using four PA of the posterior fossa (one male and three females, mean age 5 years) and four grade IV GBM (three males and one female, mean age 67 years). PA were cured by total surgical excision and no recurrence was observed 5 years later. In contrast, patients with GBM died within 1 year. Samples were fixed in 10% formalin for routine histology and immunohistochemistry and snap frozen and stored at $-80^{\circ}C$ for transcriptome analysis. Before RNA isolation, a cryostat section was taken from each block of tissue in order to confirm that it was representative of the tumor and did not contain normal parenchyma or more than 20% of necrosis. For QPCR, the same specimens and additional specimens of

six PA of the posterior fossa and 11 GBM were obtained and processed in the same way as above. Immunohistochemistry was carried out on formalin-fixed paraffin-embedded specimens of 5/6 PA and 4/11 GBM. In total, 16 additional PA of the posterior fossa and 16 additional GBM were also studied by immunohistochemistry.

RNA preparation
Total RNA extraction was performed using the acid guanidinium isothiocyanate/phenol/chloroform procedure (Chomczynski and Sacchi, 1987), and analysed on both spectrophotometer and Agilent 2100 bioanalyzer (Agilent Technologies, France). Only samples with 28S/18S ratio of >1.5 and no evidence of ribosomal degradation were included. Before use, RNA samples were treated with 1 U ribonuclease-free deoxyribonuclease (Promega, France) at 37°C for 1 h.

Suppression Subtractive Hybridization
Poly(A)+ mRNA were isolated from PA and GBM total RNA using an Oligotex mRNA isolation kit (Qiagen, France) and comparison of gene expression between these two RNA population was carried out by SSH using a PCR-Select cDNA subtraction kit (Clontech, Heidelberg, Germany).

Cloning and analysis of subtracted clones
Products from the final PCR amplification were cloned into a pGEM-T vector (Promega) and electro-transferred into XL1BlueMRF. Differential screening was performed to eliminate false positives. Hybridizations were performed in duplicate following classic procedures. Specific clones were prepared using a Qiagen plasmid mini-kit and sequenced (MWG-Biotech, Ebersberg, Germany). Nucleic acid homology searches were carried out using the BLAST program at the NCBI, USA.

Real-time quantitative RT-PCR
cDNA synthesis, real-time quantitative RT-PCR (QPCR) conditions and data analysis were as previously described (Rocchi *et al*., 2004). Forward and reverse primers for each gene are listed below: PLEKHB1: 5′-GCTGACTGTG AACCTACGGGA-3′, 5′-TCCAGCAGTGCTGTCTTCCA-3′; DNER: 5′-CCTGGTATCCTTTGAAGTGCCA-3′, 5′-TCCA GAGCAAAATCAGTGAGG-3′; DEC1: 5′-CCAAGTGTAC AAGTCAAGACGGG-3′, 5′-GGTGCGGCAATTTGTAGG TC-3′; SPOCK1: 5′-GGACAAGTACTGGAACCGCTTT-3′, 5′-GGTAGTCCTGGGTCACACACACT-3′; OPN: 5′-GCCG AGGTGATAGTGTGGTTTA-3′, 5′-TGATGTCCTCGTCTG TAGCATC-3′; TIMP3: 5′-AAGATGCCCCATGTGCAGT-3′, 5′-CCCACCTCTCCACGAAGTT-3′. PCR conditions using the Light Cycler Fast Start DNA Master Sybr Green I kit (Roche Applied Science) were as follow: 5 min at 94°C, followed by 40 cycles of 10 s at 94°C, 10 s at 65, 66, 67, 64, 65, and 61°C for PLEKHB1, DNER, DEC1, SPOCK1, OPN, and TIMP3, respectively, and 15 s at 72°C to generate a 89-bp PCR-product for PLEKHB1, 82-bp for DNER, 72-bp for DEC1, 148-bp for SPOCK1, 95-bp for OPN, and 150-bp for TIMP3. Efficiencies (E) were calculated from the given slopes in the Light Cycler software using dilutions of total RNA prepared from U87 cell line and shown to be 1.82 for PLEKHB1 assay, 1.81 for DNER assay, 1.96 for DEC1 assay, 1.87 for SPOCK1 and TIMP3 assays, and 1.91 for OPN assay. The QPCR conditions for FN and 18S RNA levels have been previously described (Eikmans *et al*., 2003; Rocchi *et al*., 2004). The relative expression ratio (R) of the target mRNA and reference rRNA (18S) was calculated using QPCR efficiencies (E) and the crossing point (Cp) deviation of a tumor sample versus the U87 RNA cell line used as control as previously described (Pfaffl, 2001).

Immunohistochemistry
After steam-heat-induced antigen retrieval, 5 μm sections of formalin-fixed paraffin-embedded samples of 20 GBM and 21 PA were tested for the presence of fibronectin (FN1, A0245, 1:1000 dilution, Dako, France), OPN (1B20, 1:50 dilution, Assay Designs Inc., Ann Arbor, USA) and tissue inhibitor of metalloproteinase 3 (TIMP3, RB 9253, 1:50 dilution, Lab Vision corporation, USA). An avidin-biotine enzyme complex kit (Zymed) was used for the detection of OPN whereas the detection of FN1 and TIMP3 was performed using a Ventana automate (Ventana Medical Systems SA, Illkirch, France). Positive staining was defined as staining of more than 5% of tumor cells.

Statistical analysis
The association of the results of QPCR with the diagnosis (PA versus GBM) was assessed by the Kolmogorov–Smirnov test performed using the Statview™ software. Threshold values of OPN/SPOCK1 and TIMP3/SPOCK1 ratios were determined by ROC (Receiver Operator Characteristic) curves using the Analyse-It software (Excel™, Microsoft). Sensitivity and specificity of the tests were deduced.

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