

Identification of Potential Serum Biomarkers of Glioblastoma: Serum Osteopontin Levels Correlate with Poor Prognosis

Peddagangannagari Sreekanthreddy¹, Harish Srinivasan², Durairaj Mohan Kumar¹, Mamatha Bangalore Nijaguna¹, Sambandam Sridevi², Marigowda Vrinda³, Arimappamagan Arivazhagan⁴, Anandh Balasubramaniam⁴, Alangar Sathyananjandas Hegde⁵, Bangalore A. Chandramouli⁴, Vani Santosh⁴, Manchanahalli R.S. Rao⁶, Paturu Kondaiah², and Kumaravel Somasundaram¹

Abstract

Background: The aim of this study is to identify serum biomarkers with classification and prognosis utility for astrocytoma, in particular glioblastoma (GBM).

Methods: Our previous glioma microarray database was mined to identify genes that encode secreted or membrane-localized proteins. Subsequent analysis was done using significant analysis of microarrays, followed by reverse transcription-quantitative PCR (RT-qPCR) and immunohistochemical validation in tumor tissues, ELISA and Western blot validation in sera, and correlation with survival of GBM patients.

Results: Significant analysis of microarrays identified 31 upregulated and 3 downregulated genes specifically in GBMs. RT-qPCR validation on an independent set of samples confirmed the GBM-specific differential expression of several genes, including three upregulated (*CALU*, *CXCL9*, and *TIMP1*) and two downregulated (*GPX3* and *TIMP3*) novel genes. With respect to osteopontin (OPN), we show the GBM-specific upregulation by RT-qPCR and immunohistochemical staining of tumor tissues. Elevated serum OPN levels in GBM patients were also shown by ELISA and Western blot. GBM patients with high serum OPN levels had poorer survival than those with low serum OPN levels (median survival 9 versus 22 months respectively; $P = 0.0001$). Further, we also show high serum TIMP1 levels in GBM patients compared with grade II/III patients by ELISA and downregulation of serum GPX3 and TIMP3 proteins in GBMs compared with normal control by Western blot analysis.

Conclusions: Several novel potential serum biomarkers of GBM are identified and validated. High serum OPN level is found as a poor prognostic indicator in GBMs.

Impact: Identified serum biomarkers may have potential utility in astrocytoma classification and GBM prognosis. *Cancer Epidemiol Biomarkers Prev*; 19(6); 1409–22. ©2010 AACR.

Introduction

An immediate need in oncology is biomarkers, which can be identified through simple noninvasive or less invasive methods with the potential to identify cancer risk, promote early diagnosis, grade accurately, and monitor treatment response (1, 2). Serum seems to be a good choice for this purpose because it can be easily obtained from

patients and evaluated for expression of biomarkers. Blood components are also highly dynamic that it can reflect the normal or abnormal status of an individual (1). Genomic and proteomic approaches using high-throughput techniques have resulted in identification of several novel genes and their products associated with cancer. Secreted proteins can be used as circulating tumor markers for diagnosis and monitoring patient response to treatment.

Gliomas are the most common primary central nervous system tumors of the brain. They include astrocytomas, oligodendrogliomas, and oligoastrocytomas based on type of cells they originate from, with the latter having components of both cell types (3). Astrocytomas are further divided into pilocytic astrocytoma (grade I), diffuse astrocytoma (DA; grade II), anaplastic astrocytoma (AA; grade III), and glioblastoma (GBM; grade IV), whereas oligodendrogliomas are classified as diffuse oligodendroglioma (DO; grade II) and anaplastic oligodendroglioma (AO; grade III). Pilocytic tumors are slow growing, non-infiltrating, and usually cured by surgery. Although malignant gliomas are represented by AA, AO, and

Authors' Affiliations: Departments of ¹Microbiology and Cell Biology and ²Molecular Reproduction, Development and Genetics, Indian Institute of Science; Departments of ³Neuropathology and ⁴Neurosurgery, National Institute of Mental Health and Neuro Sciences; ⁵Sri Satya Sai Institute of Higher Medical Sciences; and ⁶Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India

Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (<http://cebp.aacrjournals.org/>).

Corresponding Author: Kumaravel Somasundaram, Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560012, India. Phone: 91-80-23607171; Fax: 91-80-23602697. E-mail: skumar@mcbliisc.ernet.in

doi: 10.1158/1055-9965.EPI-09-1077

©2010 American Association for Cancer Research.

GBMs, patients with GBM have the worst prognosis with a median survival <12 months despite advances in surgery, radiation therapy, and chemotherapy (4). The situation is further complicated by existing histopathology-based classification, as it is likely to be subjective and also suffers from considerable interobserver variation (5). Accurate classification seems to be important as the treatment modalities are substantially different between grade III and IV patients (6, 7). Moreover, the existence of subclass with varying responses to treatment within similar histologic grades of glioma has been shown (8). It is believed that classification based on gene signatures has a good chance of achieving clinically relevant subgroups. This warrants a need for identification of more reliable biomarkers with extensive validation.

A number of serum biomarkers with multiple utility for glioma have been reported (2). However, none of these markers are approved for routine use for patients. With an aim to identify new serum biomarkers that could classify glioma grades more precisely and provide prognostic value among GBM patients, we carried out data mining of our glioma microarray database (9) and identified genes that are located in the membrane or secreted based on protein localization information obtained from the SOURCE database as potential serum biomarkers. Subsequent analysis by significant analysis of microarrays (SAM), followed by reverse transcription-quantitative PCR (RT-qPCR) validation, identified several new GBM-specific serum markers. For selected genes, we carried out additional validations. For one gene, that is, *osteopontin* (OPN), the validation was also carried out by immunohistochemical analysis using tissue sections and ELISA using serum samples. Serum OPN level was also correlated with patient survival. With this, we have identified several secreted biomarkers, some of which are potential serum markers of GBM.

Materials and Methods

Tumor samples

Tumor samples were collected from patients who were operated at National Institute of Mental Health and Neurosciences (NIMHANS) and Sri Satya Sai Institute of Higher Medical Sciences (SSIHMS), Bangalore, India. Normal brain tissue samples (anterior temporal lobe) obtained during surgery for intractable epilepsy were used as control samples. Informed consent from each patient was obtained per IEC guidelines and approval. A total of 118 samples, which included different grades of astrocytomas: 8 DA (grade II), 26 AA (grade III), 44 GBM (grade IV), oligodendroglioma including 7 DO (grade II) and 26 AO (grade III), and 7 normal brain samples were used for real-time qPCR analysis in this study. Tissues were bisected and one-half was snap-frozen in liquid nitrogen and stored at -80°C until RNA isolation. The other half was fixed in formalin and processed for paraffin sections, and these tissues were used for histopathologic characterization and immunohistochemistry.

Microarray data and SAM analyses, RNA isolation, and RT-qPCR and Western blot analyses

The details, as previously described, are given in the supplementary section (9-12).

Histopathology and immunohistochemistry

Histologic sections of normal brain and tumor tissues were examined by light microscopy using hematoxylin and eosin preparation. Tumor sections of diffusely infiltrating astrocytomas were graded using the WHO grading scheme (13). A total of 82 cases (78 cases of diffusely infiltrating astrocytoma and 4 control brain tissues) were analyzed by immunohistochemistry. Paraffin sections (4 μm) from the tumor tissue and control samples were collected on silane-coated slides, and immunohistochemistry was done using an antibody against OPN (polyclonal: ab8448, Abcam, UK, diluted to 1:200). Antigen retrieval was not required for OPN staining. After the initial processing steps, sections were incubated with the primary antibody, overnight at 4°C . This was followed by incubation with Super Sensitive poly-horseradish peroxidase detection system (QD440-XAK, Biogenex). 3,3'-Diaminobenzidine (Sigma) was used as the chromogenic substrate. The tumor sample, which showed significant upregulation of OPN by qPCR, served as the positive control. A negative control slide in which the primary antibody was excluded was incorporated with each batch of slides. Staining was scored on a scale of 0 to 2 (0, no staining; 1+, mild to moderate intensity; 2+, strong staining). Only 2+ staining was considered for interpretation.

Serum collection, OPN, and TIMP1 ELISA

Serum collection and storage conditions are described previously (12). OPN levels were determined in serum samples using the human OPN immunoassay kit from R&D Systems according to the manufacturer's protocol. Human TIMP1 ELISA quantitation kit from R&D Systems was used to measure serum TIMP1 levels per the manufacturer's instructions. More details can be seen in the supplementary section.

Survival correlation with serum OPN levels

Newly diagnosed GBM patients ($n = 30$) who underwent surgery in the two clinical centers (NIMHANS/SSIHMS) were prospectively included. Approval by the ethics committee and patient consent were obtained before initiation of the study. The patients were recruited based on the following inclusion criteria: (a) adult patients (age between 18 and 65 years) with a supratentorial lobar tumor; (b) patients who underwent maximal safe resection of the tumor with minimal residue noted on postoperative magnetic resonance imaging scan; and (c) patients with postoperative Karnofsky's performance score ≥ 70 . The demographic data and clinical features of these patients were noted. Blood was collected from all patients preoperatively, and serum OPN levels were assessed by the methods described above.

Table 1. GBM-specific potential serum biomarkers

SI no.	Symbol	Gene name	Fold change
I. Genes overexpressed in grade 4 (GBM) compared with grades II and III (lower-grade astrocytomas)			
1	<i>SPP1</i>	Secreted phosphoprotein 1 (OPN, bone sialoprotein I, early T-lymphocyte activation 1)	5.73
2	<i>CHI3L1</i>	Chitinase 3-like 1 (cartilage glycoprotein-39)	3.51
3	<i>TIMP1</i>	Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)	2.69
4	<i>IGFBP2</i>	Insulin-like growth factor binding protein 2, 36 kDa	2.49
5	<i>FSTL1</i>	Follistatin-like 1	1.70
6	<i>CCL2</i>	Chemokine (C-C motif) ligand 2	1.64
7	<i>SILV</i>	Silver homologue (mouse)	1.60
8	<i>PSG9</i>	Pregnancy specific β -1-glycoprotein 9	1.60
9	<i>PRL</i>	Prolactin	1.57
10	<i>CYR61</i>	Cysteine-rich, angiogenic inducer, 61	1.54
11	<i>F11</i>	Coagulation factor XI (plasma thromboplastin antecedent)	1.53
12	<i>VEGF</i>	Vascular endothelial growth factor	1.52
13	<i>CXCL14</i>	Chemokine (C-X-C motif) ligand 14	1.51
14	<i>PLAT</i>	Plasminogen activator, tissue	1.51
15	<i>IGF2</i>	Insulin-like growth factor 2 (somatomedin A)	1.50
16	<i>GNRH1</i>	Gonadotropin-releasing hormone 1 (leutinizing-releasing hormone)	1.49
17	<i>SERPINE1</i>	Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	1.47
18	<i>LAMP2</i>	Lysosomal-associated membrane protein 2	1.47
19	<i>SERPINC1</i>	Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1 (angioedema, hereditary)	1.46
20	<i>BDNF</i>	Brain-derived neurotrophic factor	1.44
21	<i>APOC3</i>	Apolipoprotein C-III	1.43
22	<i>CXCL9</i>	Chemokine (C-X-C motif) ligand 9	1.42
23	<i>SERPINC1</i>	Serine (or cysteine) proteinase inhibitor, clade C (antithrombin), member 1	1.41
24	<i>CALU</i>	Calumenin	1.40
25	<i>SPOCK2</i>	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2	1.38
26	<i>TNC</i>	Tenascin C (hexabrachion)	1.38
27	<i>IL16</i>	Interleukin 16 (lymphocyte chemoattractant factor)	1.34
28	<i>LAD1</i>	Ladinin 1	1.31
29	<i>AP2A2</i>	Adaptor-related protein complex 2, α 2 subunit	1.30
30	<i>IL6</i>	Interleukin 6 (interferon, β 2)	1.29
31	<i>MAPT</i>	Microtubule-associated protein τ	1.25
II. Genes overexpressed in grades II and III (lower-grade astrocytomas) compared with grade IV (GBM)			
1	<i>SPARCL1</i>	SPARC-like 1 (mast9, hevin)	3.27
2	<i>TIMP3</i>	Tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy,pseudoinflammatory)	2.27
3	<i>GPX3</i>	Glutathione peroxidase 3 (plasma)	2.02

All patients were treated uniformly with adjuvant radiotherapy and chemotherapy. Radiotherapy was administered with a total dose of 59.4 Gy, given in 33 fractions along with concomitant chemotherapy with temozolamide, administered at the dose of 100 mg/d, which was continued daily for 45 days. Subsequently, five cycles of cyclical chemotherapy with temozolamide at the

dose of 150 mg/m² body surface area for 5 days every 28 days was administered.

Patients were followed up clinically and with magnetic resonance imaging. Re-surgery was offered for those who developed symptomatic recurrence. The maximum follow-up period was 34 months. At the end of study period, 23 patients had expired and 7 were on follow up.

The median survival of patients in the whole group was 15 months.

Results

Analysis of secreted or plasma membrane-associated genes in astrocytoma microarray expression profile database

With the idea that genes that encode protein located in the plasma membrane or secreted are likely to be potential serum biomarkers, we analyzed our recently published microarray data (9). Using protein localization information from the SOURCE database, we identified 226 such genes from a total of 18,981 genes that were spotted in the microarray. In the next step, we used SAM to find genes that are differentially expressed between GBM and lower-grade astrocytomas (grade II/III). Of the total of 34 genes that are differentially expressed, 31 were upregulated in GBMs as against lower-grade astrocytomas (grade II/III) and 3 were downregulated in GBM as against lower-grade astrocytomas (grade II/III) were identified (Table 1). To get a visual appreciation as well as to see the substructure of the data containing expression

values of 34 genes, we carried out hierarchical cluster analysis using the TMEV software (Fig. 1). Besides many novel genes, the list included genes such as *VEGF*, *IGFBP2*, and *CHI3L1*, which were previously reported as GBM serum markers.

Validation of GBM-specific secreted biomarkers

Among 34 potential secretory biomarkers that can discriminate GBM from lower-grade astrocytomas (AA and DA), some of the genes have already been reported, whereas many were novel and relatively less studied. Some of the genes that were previously reported, such as *CHI3L1*, *IGFBP2*, *VEGF*, *TNC*, and *FSTL1*, were validated in the present study (data not shown). We have recently reported *FSTL1* as a GBM-specific upregulated marker (9). The novel genes that were validated in this study are *CALU*, *CXCL9*, *TIMP1*, *GPX3*, and *TIMP3* (Table 2); other important genes that we have validated are *SERPINE1*, *PLAT*, *IL6*, and *OPN* (Table 2).

A novel gene upregulated in GBM is *calumenin* (*CALU*), which is a calcium-binding protein located in the endoplasmic reticulum and involved in protein folding and sorting (14). We found the transcript levels of

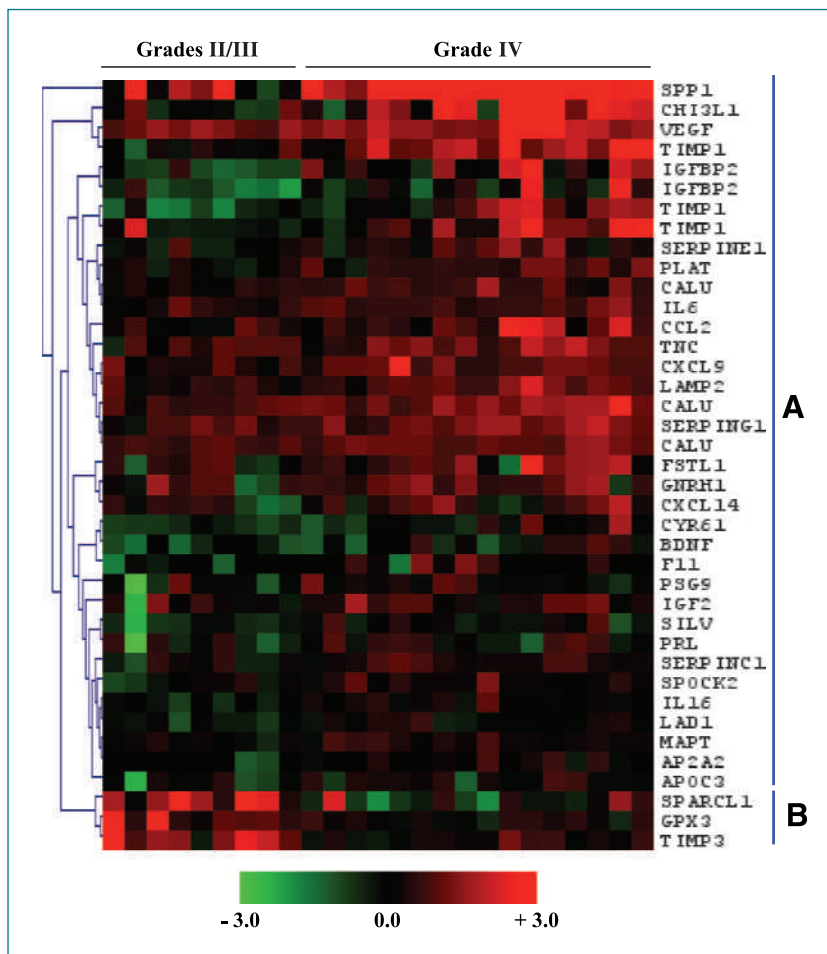


Figure 1. One-way hierarchical cluster analysis of SAM-identified genes using the TMEV software to see the data substructure. Normalized, log₂-transformed expression ratios of SAM-identified differentially regulated genes between GBMs (grade IV) and lower-grade astrocytomas (grades II and III) were subjected to one-way hierarchical clustering using the TMEV software. A dual-color code was used, with red and green indicating upregulation and downregulation, respectively, in a particular glioma sample compared with normal brain tissue. Subclusters A and B represent genes that are upregulated and downregulated respectively in GBMs compared with lower-grade astrocytoma (grades II and III).

CALU to be significantly upregulated to very high levels in GBMs (mean log 2 ratio 1.26 ± 1.07) compared with DA, AA, DO, AO, and normal brain samples (mean log 2 ratios -0.06 ± 1.01 , -1.20 ± 0.71 , -1.40 ± 0.78 , -0.98 ± 0.88 , and 0.00 ± 1.44 , respectively; Fig. 2A; Table 2). Another interesting gene found upregulated in a majority of GBMs was *CXCL9*, a member of the chemokine family, which is a group of small structurally related molecules that regulates cell trafficking of various types of leukocytes through interactions with a subset of seven-transmembrane, G protein-coupled receptors. *CXCL9* transcripts were found upregulated significantly in GBMs (mean log 2 ratio 3.74 ± 1.94) compared with AA, DO, AO, and normal brain samples (mean log 2 ratios 2.10 ± 2.30 , 0.53 ± 2.64 , 1.28 ± 2.14 , and 0.00 ± 1.91 , respectively; Fig. 2C; Table 2).

Two novel genes that were found to be downregulated in GBMs are *GPX3* and *TIMP3*. *GPX3* is a secreted member of glutathione peroxidase family involved in reduction of hydrogen peroxide, thereby protecting cells from oxidative damage. *GPX3* transcripts were found downregulated significantly in GBMs (mean log 2 ratio 2.20 ± 1.59) compared with AA and normal brain samples (mean log 2 ratios 4.02 ± 1.70 and 0.00 ± 1.63 , respectively; Fig. 2G; Table 2). However, oligodendroglioma grade II/DO (mean log 2 ratio of 0.99 ± 2.33) and grade III/AO (mean log 2 ratio of 2.27 ± 1.36) also had an expression pattern similar to GBM (Fig. 2G; Table 2). We also analyzed serum *GPX3* protein levels by Western blot (Fig. 3A). Statistical analysis using Mann-Whitney test revealed that the serum *GPX3* protein level, as measured by intensity, in GBM sera (mean intensity 0.91 ± 0.24) was significantly ($P = 0.048$) less compared with that of normal sera (mean intensity 1.11 ± 0.21).

The transcript levels of tissue inhibitor of metalloproteinase 3 (*TIMP3*) was found to be downregulated significantly in GBMs (mean log 2 ratio -1.53 ± 1.04) compared with DA, AA, and normal brain samples (mean log 2 ratios -0.31 ± 0.50 , -0.60 ± 0.74 , and 0.00 ± 1.57 , respectively; Fig. 2H; Table 2). However, oligodendroglioma grade 2/DO (mean log 2 ratio of -1.22 ± 0.72) and grade 3/AO (mean log 2 ratio of -1.19 ± 1.05) also had an expression pattern similar to GBM (Fig. 2H; Table 2). We also analyzed the serum *TIMP3* protein levels by Western blot (Fig. 3B). Statistical analysis using Mann-Whitney test revealed that the serum *TIMP3* protein level, as measured by intensity, in GBM sera (mean intensity 0.67 ± 0.28) was less compared with that of normal sera (mean intensity 1.00 ± 0.48), which showed a tendency toward statistical significance ($P = 0.068$). Thus, these two genes, *GPX3* and *TIMP3*, seem to discriminate GBM from lower-grade astrocytomas (AA and DA) but not oligodendrogliomas (AO and DO).

We have also confirmed GBM-specific expression of four more relatively less studied genes. The first gene is *serine proteinase inhibitor, clade E, member 1* (*SERPINE1*), also known as *plasminogen activator inhibitor, type 1* (*PAI-1*). *SERPINE1* was found to be upregulated significantly in GBMs compared with DA, AA, DO, and AO (Fig. 2B;

Table 2). The second gene, *TIMP1*, is overexpressed significantly in GBMs compared with DA, AA, DO, AO, and normal samples (Fig. 2D; Table 2). We have also validated GBM-specific upregulation of *TIMP1* at the level of serum *TIMP1* protein by ELISA (Fig. 3C). Comparisons of serum *TIMP1* levels were made between different grades of astrocytoma and normal sera. Kruskal-Wallis ANOVA followed by post hoc Tukey's Honest Significant Difference (HSD) test showed that the difference between GBM and DA and AA to be significant ($P < 0.0001$ and $P < 0.0001$, respectively). The mean serum *TIMP1* level in the GBM sera was 159.96 ± 55.40 compared with 61.58 ± 26.41 in AA and 63.14 ± 25.45 in DA. However, the serum *TIMP1* levels in normal individuals (mean 173.33 ± 55.08) was not different from that of GBM patients. Because the GBM samples used for this analysis ($n = 52$) underwent uniform treatment protocol and were prospectively followed up, we analyzed the survival utility of *TIMP1*. The median survival among GBM patients in the group with serum *TIMP1* levels >195 ng/mL was less than the group with *TIMP1* level less than 195 ng/mL (11 months versus 17 months, respectively). However, these data were found to be statistically not significant (data not shown).

The third gene found to be upregulated in GBMs is *plasminogen activator, tissue type* (*PLAT; t-PA*). *PLAT* transcripts were found to be significantly upregulated in GBMs compared with lower-grade astrocytomas (DA and AA) as well as oligodendrogliomas (DO and AO; Fig. 2F; Table 2). *Interleukin 6* (*IL6*) was the fourth gene found to be significantly upregulated in GBMs as against DA, AA, DO, AO, and normal samples (Fig. 2E; Table 2).

We have also measured the transcript levels of these eight genes in glioma-derived cell lines. In principle, all the eight genes were found to show similar GBM-specific differential regulation, like GBM tissue samples, compared with normal brain samples in most glioma cell lines (Supplementary Fig. S1).

OPN is a GBM-specific serum biomarker with prognostic value

Although there were few reports of OPN upregulation in GBM, we thought that a thorough investigation of OPN regulation in different grades of glioma is needed. We carried out detailed analysis of OPN at RNA and protein levels in tumor tissue and serum OPN levels in different grades of astrocytoma as well as its effect on patient survival. OPN transcript exists in three isoforms, a, b, and c (corresponding to transcript variants 1, 2, and 3, respectively), which arises due to differential splicing. All the three isoforms were found to be upregulated significantly in GBMs compared with DA, AA, DO, AO, and normal samples, except DA versus GBM in the case of OPN tv1 (Fig. 4A-C; Table 2). Interestingly, when glioma-derived cell lines were tested for OPN transcript levels, we found the OPN transcript variant 2 alone to be upregulated in most glioma cell lines, whereas the transcript levels of variants 1 and 3 were actually

Table 2. Statistical analysis of regulation of potential GBM-specific serum biomarkers in different glioma grades

Sl no.	Gene	Cutoff log 2 ratios	Normal		DO II		DA II		AO III		AA III		GBM		Post hoc P*	Kruskal-Wallis test	
			Percent-age	Mean log 2 ratios \pm S.D	Percent-age	Mean log 2 ratios \pm S.D	Percent-age	Mean log 2 ratios \pm S.D	Percent-age	Mean log 2 ratios \pm S.D	Percent-age	Mean log 2 ratios \pm S.D	Percent-age	Mean log 2 ratios \pm S.D		χ^2	P
I. GBM upregulated genes																	
1	<i>CXCL9</i>	2.50	14.28% (1/7)	0.00 \pm 1.91	14.28% (1/7)	0.53 \pm 2.64	50.00% (4/8)	2.78 \pm 2.35	26.92% (7/26)	1.28 \pm 2.14	34.61% (9/26)	2.10 \pm 2.30	84.09% (37/44)	3.74 \pm 1.94	$\epsilon = 0.0007$ $\omega = 0.006$ $\phi = 0.0002$ $\pi = 0.0365$	29.93	<0.0001
2	<i>TIMP1</i>	2.00	14.20% (1/7)	0.00 \pm 1.84	0% (0/7)	-2.93 \pm 1.35	0% (0/8)	-1.95 \pm 1.61	11.50% (3/26)	-1.54 \pm 2.24	0% (0/26)	-2.30 \pm 1.28	68.10% (30/44)	2.36 \pm 1.79	$\alpha = 0.03$ $\delta = 0.028$ $\epsilon = 0.018$ $\omega < 0.0001$ $\lambda < 0.0001$ $\phi < 0.0001$ $\pi < 0.0001$	69.38	<0.0001
3	<i>SERPINE1</i>	1.00	42.80% (3/7)	0.00 \pm 2.47	0% (0/7)	-3.25 \pm 1.41	0% (0/8)	-1.50 \pm 1.39	23.10% (6/26)	-1.29 \pm 2.88	11.50% (3/26)	-1.45 \pm 1.69	68.10% (30/44)	2.09 \pm 2.55	$\omega < 0.0001$ $\lambda = 0.0017$ $\phi < 0.0001$ $\pi < 0.0001$	52.69	<0.0001
4	<i>IL6</i>	1.00	42.80% (3/7)	0.00 \pm 2.21	0% (0/7)	-1.57 \pm 1.33	0% (0/8)	-0.96 \pm 1.16	7.60% (2/26)	-1.82 \pm 1.48	7.60% (2/26)	-1.05 \pm 1.63	65.90% (29/44)	2.10 \pm 2.00	$\epsilon = 0.0427$ $\omega < 0.0001$ $\lambda = 0.0002$ $\phi < 0.0001$ $\pi < 0.0001$	59.62	<0.0001
5	<i>CALU</i>	0.56	42.85% (3/7)	0.00 \pm 1.44	0% (0/7)	-1.40 \pm 0.78	25.00% (2/8)	-0.06 \pm 1.01	7.69% (2/26)	-0.98 \pm 0.88	0% (0/26)	-1.20 \pm 0.71	79.54% (35/44)	1.26 \pm 1.07	$\delta = 0.0437$ $\epsilon = 0.0206$ $\omega < 0.0001$ $\mu = 0.0432$ $\lambda = 0.0069$ $\phi < 0.0001$ $\pi < 0.0001$	70.04	<0.0001
6	<i>PLAT</i>	0.56	28.57% (2/7)	0.00 \pm 1.73	0% (0/7)	-3.02 \pm 0.82	12.50% (1/8)	-2.25 \pm 1.86	3.84% (1/26)	-2.20 \pm 1.67	3.84% (1/26)	-2.40 \pm 1.46	72.72% (32/44)	0.90 \pm 1.28	$\alpha = 0.0027$ $\beta = 0.0426$ $\gamma = 0.0079$ $\delta = 0.0027$	64.66	<0.0001

(Continued on the following page)

Table 2. Statistical analysis of regulation of potential GBM-specific serum biomarkers in different glioma grades (Cont'd)

SI no.	Gene	Cutoff log 2 ratios	Normal		DO II		DA II		AO III		AA III		GBM		Post hoc <i>P</i> *	Kruskal- Wallis test	
			Percent- age	Mean log 2 ratios ± S.D	Percent- age	Mean log 2 ratios ± S.D	Percent- age	Mean log 2 ratios ± S.D	Percent- age	Mean log 2 ratios ± S.D	Percent- age	Mean log 2 ratios ± S.D	Percent- age	Mean log 2 ratios ± S.D		χ^2	<i>P</i>
															$\omega < 0.0001$		
															$\lambda < 0.0001$		
															$\phi < 0.0001$		
															$\pi < 0.0001$		
7A	<i>OPN tv 1</i>	2.00	0% (0/4)	0.00 ± 1.07	0% (0/7)	-0.86 ± 0.86	25.00% (2/8)	1.43 ± 0.83	19.20% (5/26)	0.53 ± 1.87	7.60% (2/26)	0.19 ± 1.27	77.20% (34/44)	2.97 ± 1.62	$\epsilon = 0.0041$	54.48	<0.0001
															$\omega < 0.0001$		
															$\phi < 0.0001$		
															$\pi < 0.0001$		
7B	<i>OPN tv 2</i>	4.00	0% (0/4)	0.00 ± 1.08	0% (0/7)	1.67 ± 1.07	12.50% (1/8)	1.59 ± 2.01	26.90% (7/26)	2.94 ± 1.75	7.60% (2/26)	2.19 ± 1.24	97.70% (43/44)	6.34 ± 1.36	$\mu = 0.0039$	78.25	<0.0001
															$\epsilon < 0.0001$		
															$\omega < 0.0001$		
															$\lambda < 0.0001$		
															$\phi < 0.0001$		
															$\pi < 0.0001$		
7C	<i>OPN tv 3</i>	1.00	25.00% (1/4)	0.00 ± 1.11	0% (0/7)	-1.94 ± 1.42	0% (0/8)	-0.15 ± 0.90	15.30% (4/26)	-0.30 ± 1.64	23.10% (6/26)	-0.44 ± 1.84	90.90% (40/44)	3.32 ± 1.66	$\epsilon = 0.0024$	67.38	<0.0001
															$\omega < 0.0001$		
															$\lambda < 0.0001$		
															$\phi < 0.0001$		
															$\pi < 0.0001$		
II. GBM downregulated genes																	
8	<i>GPX3</i>	3.00	100% (7/7)	0.00 ± 1.63	71.42% (5/7)	0.99 ± 2.33	37.50% (3/8)	3.74 ± 1.61	80.00% (21/26)	2.27 ± 1.36	23.07% (6/26)	4.02 ± 1.70	75.0% (33/44)	2.20 ± 1.59	$\beta = 0.0003$	34.00	<0.0001
															$\gamma = 0.0160$		
															$\delta < 0.0001$		
															$\epsilon = 0.0145$		
															$\eta = 0.0170$		
															$\Phi = 0.0004$		
															$\zeta = 0.0024$		
															$\pi = 0.0002$		
9	<i>TIMP3</i>	-0.56	57.14% (4/7)	0.00 ± 1.57	71.42% (5/7)	-1.22 ± 0.72	12.50% (1/8)	-0.31 ± 0.50	57.69% (15/26)	-1.19 ± 1.05	38.46% (10/26)	-0.60 ± 0.74	79.54% (35/44)	-1.53 ± 1.04	$\epsilon = 0.0027$	22.56	0.0004
															$\lambda = 0.0188$		
															$\pi = 0.0073$		

*Tukey's post hoc test showing a significant *P* value: α , normal vs DO; β , normal vs DA; γ , normal vs AO; δ , normal vs AA; ϵ , normal vs GBM; η , DO vs DA; Ψ , DO vs AO; Φ , DO vs AO; ω , DO vs GBM; Ω , DA vs AO; μ , DA vs AA; λ , DA vs GBM; ζ , AO vs AA; ϕ , AO vs GBM; π , AA vs GBM.

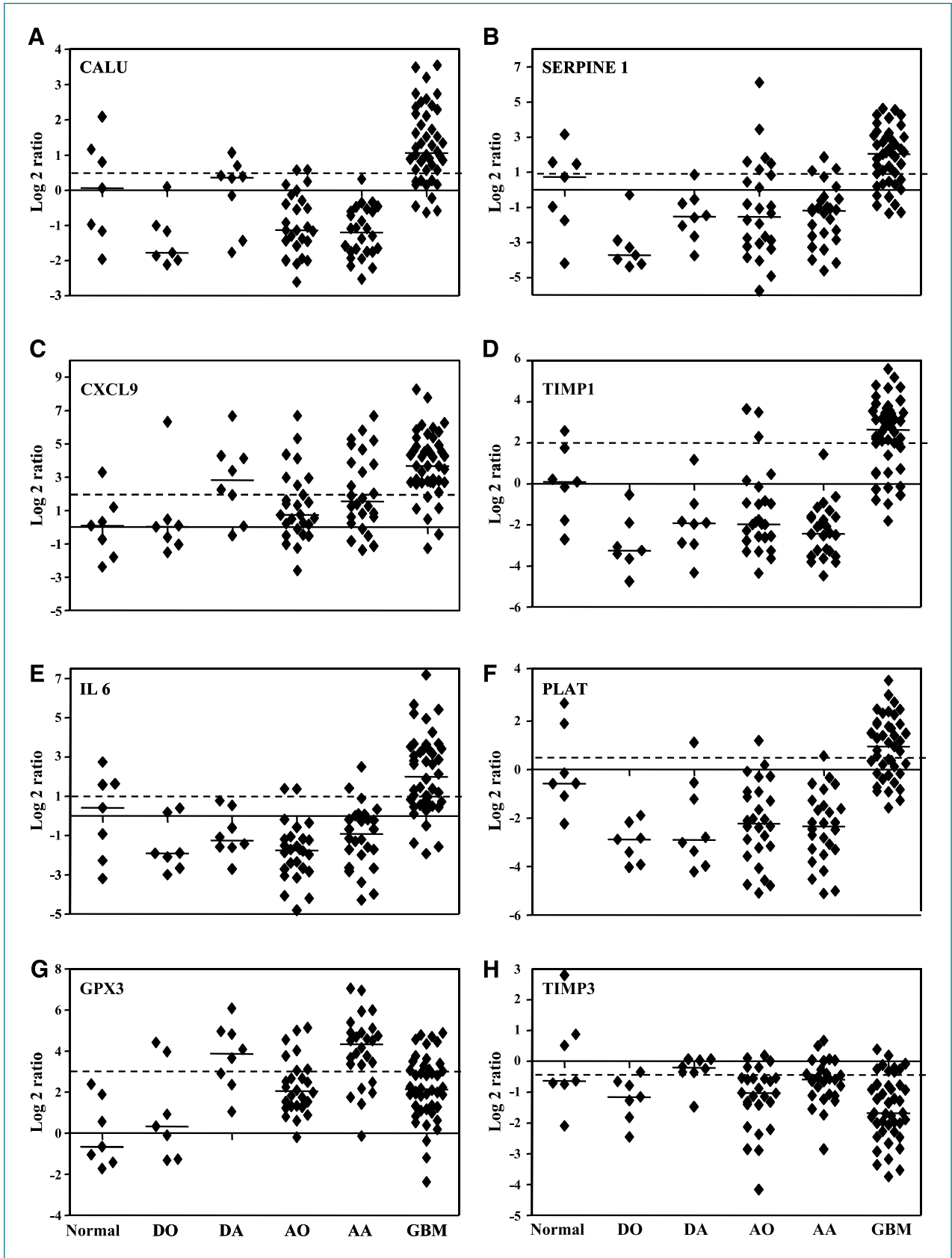
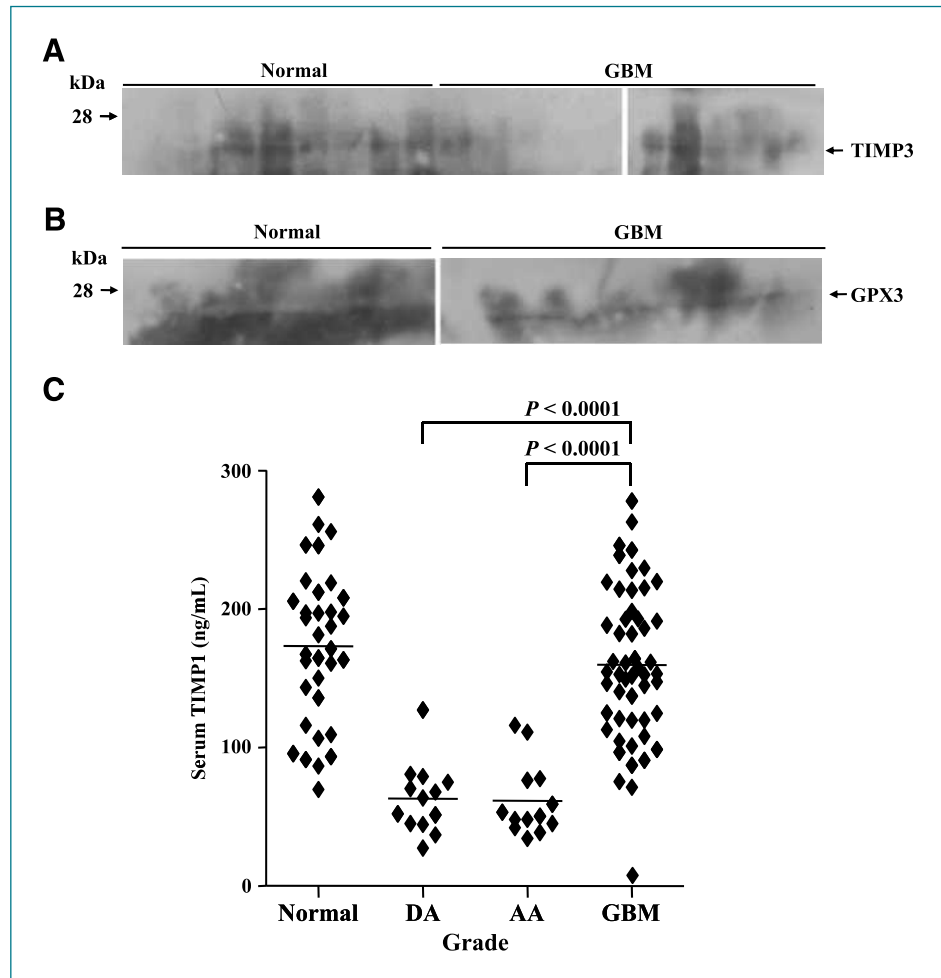


Figure 3. Analysis of serum TIMP3, GPX3, and TIMP1. A, total serum protein (5 μ L) from normal healthy controls and GBM patients was subjected to Western blot analysis for TIMP3. B, albumin/IgG depleted serum protein (50 μ g) from normal healthy controls and GBM patients was subjected to Western blot analysis for GPX3. C, serum levels of TIMP1 (ng/mL) in normal controls ($n = 34$), DAs ($n = 13$), AAs ($n = 13$), and GBMs ($n = 52$) are plotted. Horizontal line in each group designates the mean value. Statistical significance was tested by Kruskal-Wallis one-way ANOVA using SPSS 15.0 for Windows software.



downregulated in glioma cell lines compared with normal brain controls (Fig. 4D-F).

We also analyzed the OPN protein levels by immunohistochemistry in astrocytoma tissue sections. We found OPN protein staining to be cytoplasmic and accentuated at the surface of the tumor cells (Fig. 5). The macrophages infiltrating the tumor were also stained. In addition to the tumor cells and macrophages, the interstitial stroma stained prominently in several examined cases. OPN expression was pronounced around vessels and at the border of necrosis. Upon semiquantitative analysis of tumor cell positivity of OPN, we found that a majority of GBM (70%; 33 of 44) had immunopositive cells compared with AA (23.0%; 6 of 26) and DA (0.0%; 0 of 8). However, staining of the extracellular matrix was extensive and

seen in 95.5% (42 of 44) of GBM and 50% cases of DA and AA. It was observed that the extent of staining of the extracellular matrix was diffuse and more extensive in GBM tumors compared with cases of DA or AA (Fig. 5). In the control samples, there was no staining of neurons, astrocytes, oligodendrocytes, neuropil, and stroma as well as the parenchymal vessels.

The levels of OPN protein in serum samples belonging to different grades of astrocytoma and normal controls were measured by ELISA (Fig. 6A). Comparisons of serum OPN levels were made between different grades of astrocytoma and normal controls as well as between different grades. Kruskal-Wallis ANOVA followed by post hoc Tukey's HSD test showed the difference between GBM and normal controls ($P = 0.001$), GBM and DA

Figure 2. Scatter plots of the transcript levels of novel secretory biomarkers, which can potentially discriminate GBM from astrocytoma grades II/III and oligodendroglioma grades II/III. Log 2-transformed gene expression ratios obtained from real-time RT-qPCR analysis of RNA derived from tumor tissue samples (as indicated) are plotted for *CALU* (A), *SERPINE1* (B), *CXCL9* (C), *TIMP1* (D), *IL6* (E), *PLAT* (F), *GPX3* (G), and *TIMP3* (H). Each dot represents a data derived from one sample. In each sample, fold change in gene expression is calculated over its mean expression in normal brain samples. An increase or decrease in gene expression at a particular cutoff (horizontal dotted line) based on the expression pattern of a specific gene across different grades of tumors was considered significant. Horizontal line in each group designates the median value.

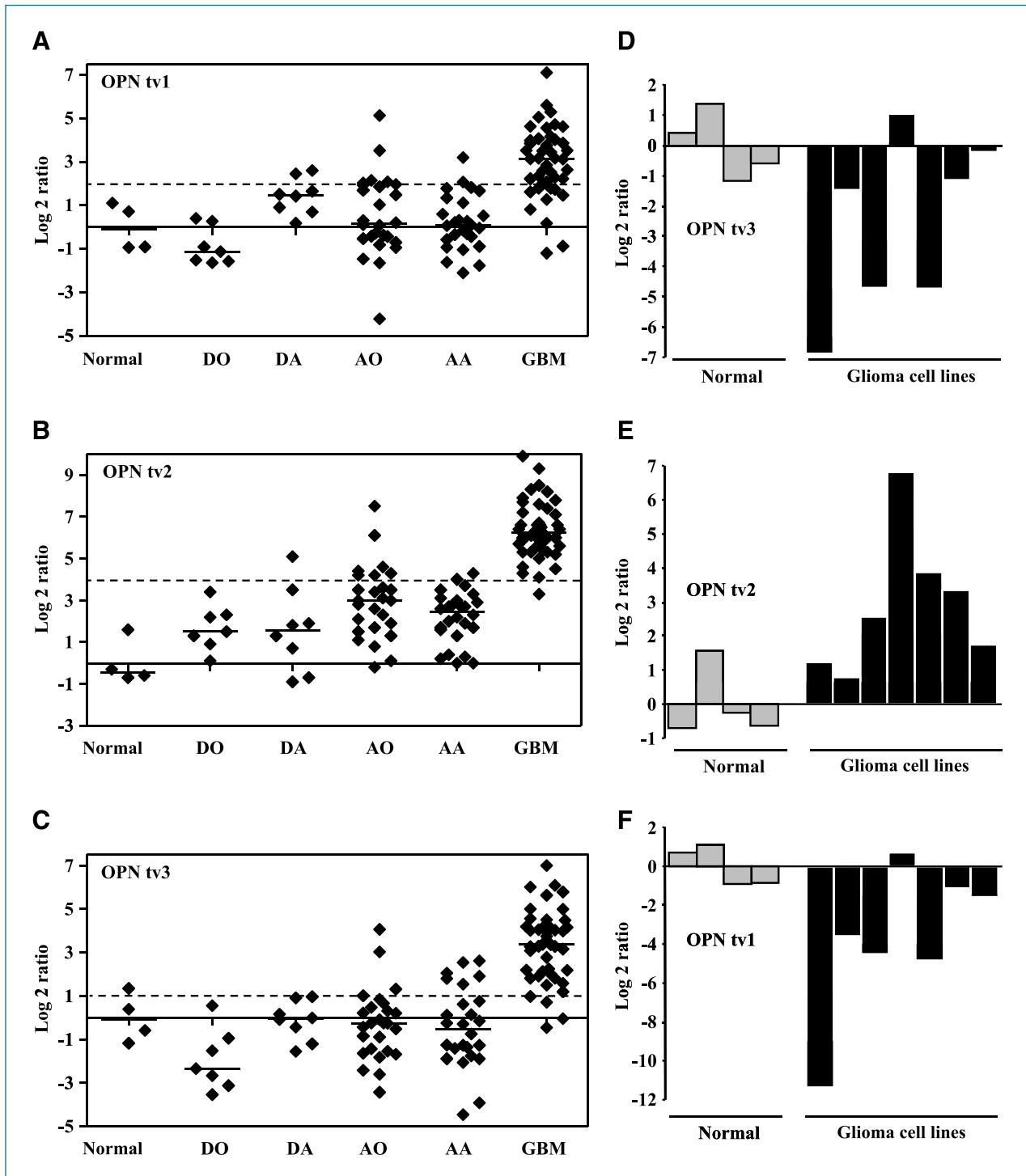


Figure 4. Scatter plot of transcript levels of OPN in different grades of astrocytoma and glioma cell lines. A to C, log₂-transformed gene expression ratios obtained from real-time RT-qPCR analysis of RNA derived from tumor tissue samples (as indicated) are plotted for OPN tv1 (transcript variant 1; A), tv2 (B), and tv3 (C). Each dot represents a data derived from one sample. In each sample, fold change in gene expression is calculated over its mean expression in normal brain samples. An increase or decrease in gene expression at a particular cutoff (horizontal dotted line) based on the expression pattern of a specific gene across different grades of tumors was considered significant. Horizontal line in each group designates the median value. D to F, log₂-transformed gene expression ratios obtained from real-time RT-qPCR analysis of RNA derived from different glioma cell lines (black bars, left to right order of cell lines: LN-18, LN-229, U-138, U-251, U-343, U-373, U-87) are plotted for OPN tv1 (transcript variant 1; D), tv2 (E), and tv3 (F). The gray bars represent the normal brain control samples used in this experiment. Each bar represents a data derived from one sample. In each sample, fold change in gene expression is calculated over its mean expression in normal brain samples.

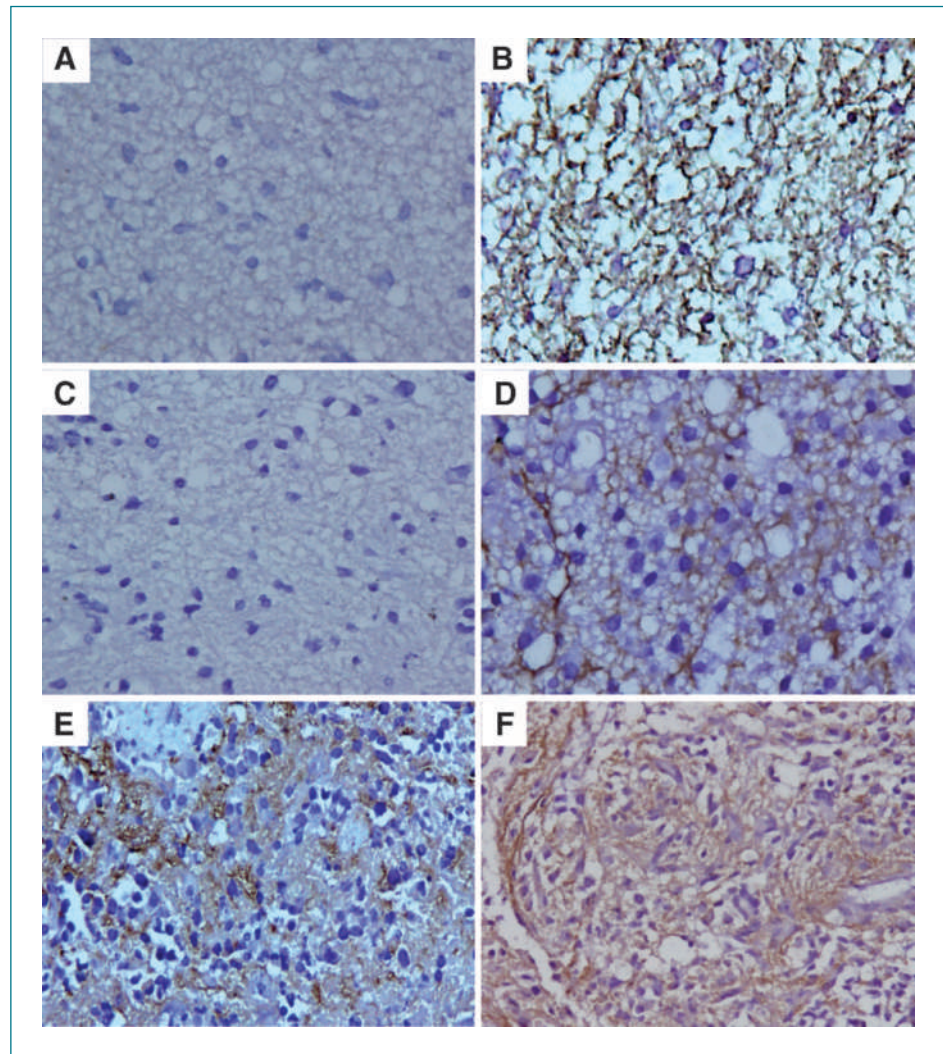


Figure 5. Immunohistochemical validation of OPN overexpression in astrocytoma. Sections from DA-negative for staining (A), DA-positive for staining (B), AA-negative for staining (C), AA-positive for staining (D), and secondary GBMs and primary GBM—positive for staining (E and F) were stained for OPN.

($P = 0.046$), and GBM and AA ($P = 0.008$) to be significant. The mean serum OPN level (ngm/mL) in GBM was 31.54 ± 28.98 compared with 17.38 ± 7.91 in AA, 13.79 ± 4.56 in DA, and 11.70 ± 7.26 in normal control. We have also validated serum OPN levels between normal healthy individuals and GBM patients by Western blot analysis (Fig. 6B). Statistical analysis using Mann-Whitney test revealed the serum OPN protein level, as measured by intensity, in GBM sera (mean intensity 1.40 ± 0.43) to be more significant compared with that of normal sera (mean intensity 1.00 ± 0.41 ; $P = 0.037$).

A subset of GBM patients ($n = 30$) who underwent uniform treatment protocol and were prospectively followed up was used for survival analysis (Fig. 6C). The serum OPN level of 20 ng/mL was found to significantly correlate with the survival. The median survival among GBM patients in the group with serum OPN levels >20 ng/mL was significantly less than in that group with ≤ 20 ng/mL (9 months versus 21 months, respectively; $P \leq 0.0001$; univariate analysis). On multivariate analysis, taking into

consideration other factors like age, cognitive dysfunction, and p53 status, a serum OPN level cutoff of 20 ng/mL was still independently significant ($P = <0.0001$; Cox regression analysis, forward Walds). This indicates that the preoperative serum OPN level is a definite prognostic indicator in GBM.

Discussion

In this study, we have used a unique approach to screen our cancer microarray database to identify potential secreted biomarkers. In the first step, we pulled out expression data of all the genes whose protein products localized either in the plasma membrane or were secreted. Using SAM, we identified GBM-specific serum markers and subsequently validated many of them. Some of the genes identified in this screen, such as *CHI3L*, *IGFBP2*, and *VEGFA*, have been previously reported as GBM-specific serum markers with prognostic value (15-18). In our study, we could also show the GBM-specific

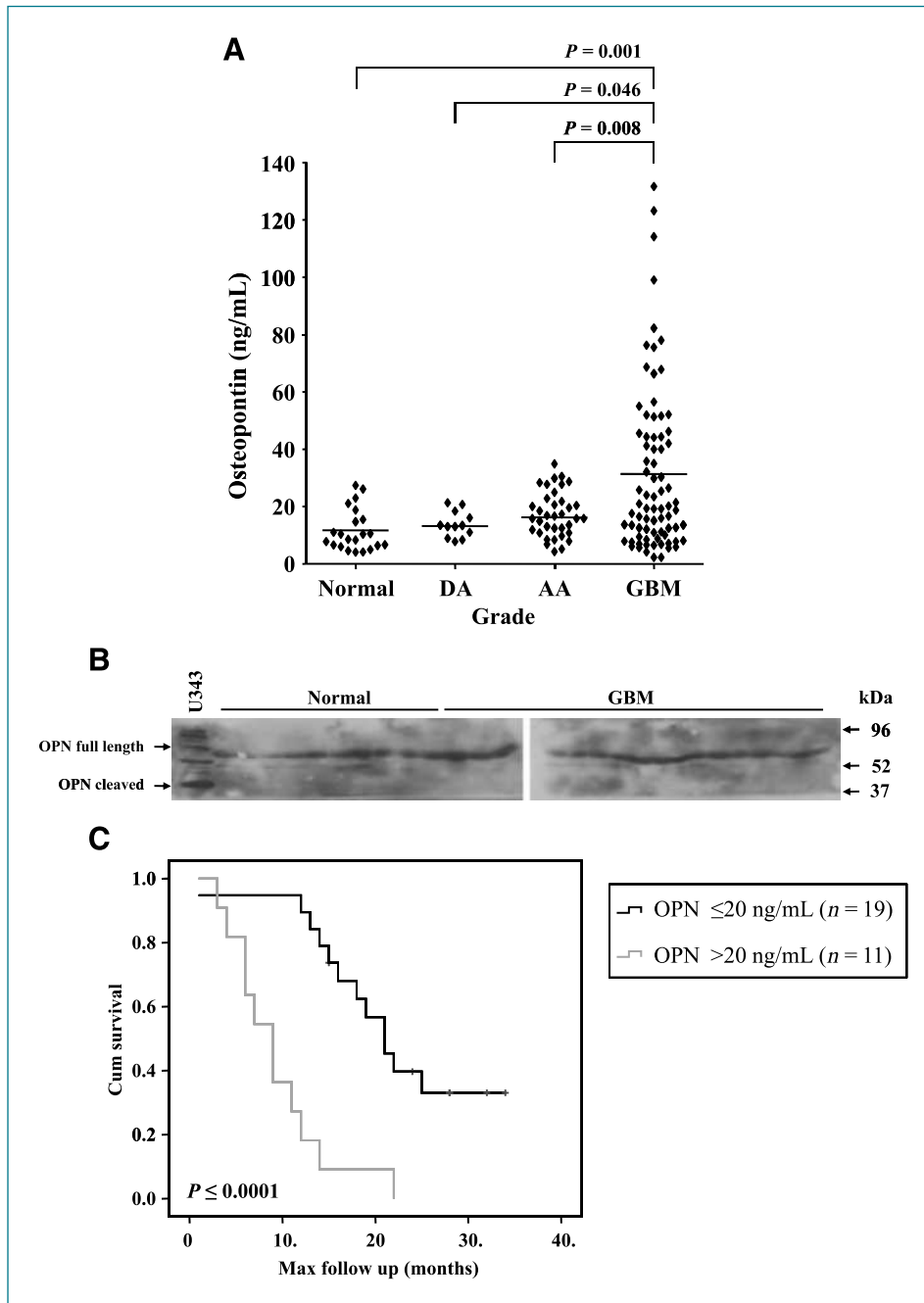


Figure 6. Scatter plot of serum OPN levels and its correlation with patient survival. A, serum levels of OPN (ng/mL) in normal controls ($n = 22$), DAs ($n = 12$), AAs ($n = 36$), and GBMs ($n = 78$). The horizontal line in each group designates the mean value. B, total serum protein (2.5 μ L) from normal healthy controls and GBM patients was subjected to Western blot analysis for OPN. C, Kaplan-Meier survival estimates for 30 GBM patients are calculated for their serum OPN levels. Survival curves for the groups positive and negative for OPN in univariate analysis are shown. Cases that were low for serum OPN levels (≤ 20 ng/mL; black line; $n = 19$) had a better survival than the cases that were high for serum OPN levels (> 20 ng/mL; gray line; $n = 11$; $P = 0.0001$).

expression of the transcripts of these genes by RT-qPCR (data not shown). These previous findings from others and the real-time RT-qPCR validation from our study provide support to our methodology of detecting serum biomarkers.

We have also identified few novel genes that are specifically regulated in GBM. *CALU* is a novel gene found upregulated specifically in GBM but not in low-grade astrocytomas and oligodendrogliomas. *CALU* belongs to a family of multiple EF-hand proteins that include

reticulocalbin, ERC-55, and Cab45, and human *CALU* has been shown to be secreted (19). However, the significance of upregulation of *CALU* in GBMs is not known. *CXCL9*, a member of chemokine family, was found in this study as an upregulated GBM-secreted biomarker. Although *CXCL9* and other members have been shown to be upregulated in other cancers, we report for the first time that *CXCL9* is a GBM-specific secretory marker (20, 21). It is interesting to note that although our microarray data had other *CXCL* genes, *CXCL10*, *CXCL12*, and

CXCL13, only *CXCL9* was identified by SAM analysis, suggesting the specific upregulation of *CXCL9* from the chemokine family in GBMs.

We also identified two genes, *GPX3* and *TIMP3*, specifically downregulated in GBMs compared with lower-grade astrocytomas (DA and AA) but not in oligodendrogliomas (DO and AO). We show our initial microarray data-based finding that these two genes are downregulated in GBM by ELISA and Western blot. *GPX3* is also called as extracellular or plasma glutathione peroxidase and is the only member of glutathione peroxidase family that is secreted. Although our microarray data had other members of the GPX family, *GPX1*, *GPX4*, and *GPX6*, only *GPX3* was identified by SAM, which showed specific upregulation in GBMs. These two markers, *GPX3* and *TIMP3*, are of value in differentiating oligodendroglioma grade 2 and 3 from astrocytoma grade 2 and 3, as these markers were overexpressed in both DO and AO (Fig. 2G and H; Table 2).

TIMP1 and *TIMP3*, members of the tissue inhibitor of metalloproteinases, were found differentially regulated in GBMs. Matrix metalloproteinases, many of which are overexpressed in malignant tumors, including GBM, are inhibited by TIMPs. It is suggested that a balance between the matrix metalloproteinases and TIMPs may contribute to malignant behavior of many cancers. Although TIMPs are known for their inhibition of tumor malignancy, a differential role for some of TIMPs has been reported. For example, *TIMP1* has been shown to stimulate proliferation of many cell types (22-25). In good correlation, our data clearly provide evidence for the GBM-specific upregulation of *TIMP1*. We have found that *TIMP1* transcripts were upregulated severalfold in GBMs compared with other grades of astrocytoma and oligodendroglioma (Fig. 2D). In addition, serum *TIMP1* levels are elevated in GBM patients compared with lower-grade astrocytoma patients (Fig. 3C). Unlike *TIMP1*, *TIMP3* has been shown to promote apoptosis possibly through stabilization of tumor necrosis factor α receptors (26-28). Accordingly, we found *TIMP3* transcripts downregulated significantly in GBMs compared with lower-grade astrocytomas (Fig. 2H).

Another interesting gene we have validated was *OPN*, which is a member of the SIBLING (small integrin-binding ligand N-linked glycoprotein) family and has been found to be overexpressed in many cancers and associated with tumor progression and metastasis (29-32). High levels of *OPN* expression correlate with tumor invasion, progres-

sion, or metastasis in multiple cancers. Studies show that *OPN* mediates the molecular mechanisms that determine metastatic spread, such as prevention of apoptosis, extracellular matrix proteolysis and remodeling, cell migration, evasion of host-immune cells, and neovascularization. Transcriptional regulation of *OPN* is complex and involves multiple pathways, including AP-1, Myc, v-Src, Runx/CBF, TGF- β /BMPs/Smad/Hox, and Wnt/ β -catenin/APC/GSK-3 β /Tcf-4 (29). *OPN* expression has been found to correlate with tumor progression in astrocytoma with high levels in GBM (33-35). *OPN* levels detected by immunohistochemistry identified that high cytoplasmic *OPN* expression correlates with poor patient survival (36). Although serum *OPN* levels have been shown to correlate with patient survival in other cancers (37), there has been no such report with respect to glioma. Our results show that *OPN* is upregulated both at the RNA and protein levels only in GBM. Further, serum *OPN* levels were high in GBM and are poor prognostic indicators. Our findings are also supported by recently published TCGA data, in which we found that seven of nine genes (*CALU*, *CXCL9*, *IL6*, *PLAT*, *TIMP1*, *OPN*, and *SERPINE1*) are similarly regulated (38). Thus, we provide evidence for analyzing large microarray data through bioinformatics methods to find potential serum/secreted biomarkers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Shailaja Chandrashekararao, Cini Samuel, K. Premkumar, and K. Chandrasekar. Infrastructural support by funding from Indian Council of Medical Research (Center for Advanced Studies in Molecular Medicine), Department of Biotechnology, Department of Science and Technology (Fund for Improvement of S&T Infrastructure), and University Grants Commission to the Department of MCB, Indian Institute of Science, is acknowledged.

Grant Support

Grant from CSIR, Government of India, under the NMITLI program. K. Somasundaram is a Wellcome Trust International Senior Research Fellow.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 10/15/2009; revised 03/18/2010; accepted 03/31/2010; published online 06/08/2010.

References

- Xue H, Lu B, Lai M. The cancer secretome: a reservoir of biomarkers. *J Transl Med* 2008;6:52.
- Somasundaram K, Nijaguna MB, Kumar DM. Serum proteomics of glioma: methods and applications. *Expert Rev Mol Diagn* 2009;9:695-707.
- Ohgaki H, Kleihues P. Genetic alterations and signaling pathways in the evolution of gliomas. *Cancer Sci*, 2009;100:2235-41.
- Berger M, Wilson C, editors. The gliomas. Philadelphia: W.B. Saunders; 1999.
- Coons SW, Johnson PC, Scheithauer BW, Yates AJ, Pearl DK. Improving diagnostic accuracy and interobserver concordance in the classification and grading of primary gliomas. *Cancer* 1997;79:1381-93.
- Stupp R, Reni M, Gatta G, Mazza E, Vecht C. Anaplastic astrocytoma in adults. *Crit Rev Oncol Hematol* 2007;63:72-80.
- Reardon DA, Wen PY. Therapeutic advances in the treatment of glioblastoma: rationale and potential role of targeted agents. *Oncologist* 2006;11:152-64.

8. Mischel PS, Nelson SF, Cloughesy TF. Molecular analysis of glioblastoma: pathway profiling and its implications for patient therapy. *Cancer Biol Ther* 2003;2:242–7.
9. Reddy SP, Britto R, Vinnakota K, et al. Novel glioblastoma markers with diagnostic and prognostic value identified through transcriptome analysis. *Clin Cancer Res* 2008;14:2978–87.
10. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A* 2001;98:5116–21.
11. Saeed AI, Sharov V, White J, et al. TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* 2003;34:374–8.
12. Reddy PS, Umesh S, Thota B, et al. PBEF1/NAmPRTase/Visfatin: a potential malignant astrocytoma/glioblastoma serum marker with prognostic value. *Cancer Biol Ther* 2008;7:663–8.
13. Louis DN, Ohgaki HO, Wiestler OD, Cavenee WK. WHO classification of tumors of the central nervous system. *Acta Neuropathol* 2007;114:97–109.
14. Yabe D, Nakamura T, Kanazawa N, Tashiro K, Honjo T. Calumenin. A Ca(2+)-binding protein retained in the endoplasmic reticulum with a novel carboxyl-terminal sequence, HDEF. *J Biol Chem* 1997;272:18232–9.
15. Hormigo A, Gu B, Karimi S, et al. YKL-40 and matrix metalloproteinase-9 as potential serum biomarkers for patients with high-grade gliomas. *Clin Cancer Res* 2006;12:5698–704.
16. McDonald KL, O'Sullivan MG, Parkinson JF, et al. IQGAP1 and IGFBP-2: valuable biomarkers for determining prognosis in glioma patients. *J Neuropathol Exp Neurol* 2007;66:405–17.
17. Chi AS, Norden AD, Wen PY. Antiangiogenic strategies for treatment of malignant gliomas. *Neurotherapeutics* 2009;6:513–26.
18. Lin Y, Jiang T, Zhou K, et al. Plasma IGFBP-2 levels predict clinical outcomes of patients with high-grade gliomas. *Neuro-Oncol* 2009;11:468–76.
19. Vorum H, Hager H, Christensen BM, Nielsen S, Honoré B. Human calumenin localizes to the secretory pathway and is secreted to the medium. *Exp Cell Res* 1999;248:473–81.
20. Hiroi M, Ohmori Y. Constitutive nuclear factor κB activity is required to elicit interferon-γ-induced expression of chemokine CXC ligand 9 (CXCL9) and CXCL10 in human tumour cell lines. *Biochem J* 2003;376:393–402.
21. Erreni M, Bianchi P, Laghi L, et al. Expression of chemokines and chemokine receptors in human colon cancer. *Methods Enzymol* 2009;460:105–21.
22. Gasson JC, Golde DW, Kaufman SE, et al. Molecular characterization and expression of the gene encoding human erythroid-potentiating activity. *Nature* 1985;315:768–71.
23. Hayakawa T, Yamashita K, Tanzawa K, Uchijima E, Iwata K. Growth-promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells. A possible new growth factor in serum. *FEBS Lett* 1992;298:29–32.
24. Wingfield PT, Sax JK, Stahl SJ, et al. Biophysical and functional characterization of full-length, recombinant human tissue inhibitor of metalloproteinases-2 (TIMP-2) produced in *Escherichia coli*. Comparison of wild type and amino-terminal alanine appended variant with implications for the mechanism of TIMP functions. *J Biol Chem* 1999;274:21362–8.
25. Groft LL, Muzik H, Rewcastle NB, et al. Differential expression and localization of TIMP-1 and TIMP-4 in human gliomas. *Br J Cancer* 2001;85:55–63.
26. Ahonen M, Baker AH, Kahari VM. Adenovirus-mediated gene delivery of tissue inhibitor of metalloproteinases-3 inhibits invasion and induces apoptosis in melanoma cells. *Cancer Res* 1998;58:2310–15.
27. Baker AH, Zaltsman AB, George SJ, Newby AC. Divergent effects of tissue inhibitor of metalloproteinase-1, -2, or -3 overexpression of rat vascular smooth muscle cell invasion, proliferation, and death *in vitro*. *J Clin Invest* 1998;101:1478–87.
28. Smith MR, Kung H, Durum SK, Colburn NH, Sun Y. TIMP-3 induces cell death by stabilizing TNF-α receptors on the surface of human colon carcinoma cells. *Cytokine* 1997;9:770–80.
29. Wai PY, Kuo PC. Osteopontin: regulation in tumor metastasis. *Cancer Metastasis Rev* 2008;27:103–18.
30. Kang Y, Seigel PM, Shu W, et al. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* 2003;3:537–49.
31. Urquidi V, Sloan D, Kawai K, et al. Contrasting expression of thrombospondin-1 and osteopontin correlates with absence or presence of metastatic phenotype in an isogenic model of spontaneous human breast cancer metastasis. *Clin Cancer Res* 2002;8:61–74.
32. Agarwal D, Chen T, Irby R, et al. Osteopontin identified as lead marker of colon cancer progression, using pooled sample expression profiling. *J Natl Cancer Inst* 2002;94:513–21.
33. Saitoh Y, Kuratsu J, Takeshima H, Yamamoto S, Ushio Y. Expression of osteopontin in human glioma. Its correlation with the malignancy. *Lab Invest* 1995;72:55–63.
34. Jang T, Savarese T, Low HP, et al. Osteopontin expression in intratumoral astrocytes marks tumor progression in gliomas induced by prenatal exposure to *N*-ethyl-*N*-nitrosourea. *Am J Pathol* 2006;168:1676–85.
35. Toy H, Yavas O, Eren O, Genc M, Yavas C. Correlation between osteopontin protein expression and histological grade of astrocytomas. *Pathol Oncol Res* 2009;15:203–7.
36. Matusan-Iljias K, Behrem S, Jonjic N, Zarkovic K, Lucin K. Osteopontin expression correlates with angiogenesis and survival in malignant astrocytoma. *Pathol Oncol Res* 2008;14:293–8.
37. Isa SI, Kawaguchi T, Teramukai S, et al. Serum osteopontin levels are highly prognostic for survival in advanced non-small cell lung cancer: results from JMTO LC 0004. *J Thorac Oncol* 2009;4:1104–10.
38. Verhaak RGW, Hoadley KA, Purdom E, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR and NF1. *Cancer Cell* 2010;17:98–110.