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Cancer Epidemiology, Biomarkers & Prevention

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Research Article

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

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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, noninfiltrating, and usually cured by surgery. Although malignant gliomas are represented by AA, AO, and

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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.

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Table 1. GBM-specific potential serum biomarkers

Sl no.	Symbol	Gene name	Fold change
I. Genes overexpressed in grade 4 (GBM) compared with grades II and III (lower-grade astrocytomas)			
1	*SPP1*	*Secreted phosphoprotein 1 (OPN, bone sialoprotein I, early T-lymphocyte activation 1)*	5.73
2	*CHI3L1*	*Chitinase 3-like 1 (cartilage glycoprotein-39)*	3.51
3	*TIMP1*	*Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)*	2.69
4	*IGFBP2*	*Insulin-like growth factor binding protein 2, 36 kDa*	2.49
5	*FSTL1*	*Follistatin-like 1*	1.70
6	*CCL2*	*Chemokine (C-C motif) ligand 2*	1.64
7	*SILV*	*Silver homologue (mouse)*	1.60
8	*PSG9*	*Pregnancy specific β-1-glycoprotein 9*	1.60
9	*PRL*	*Prolactin*	1.57
10	*CYR61*	*Cysteine-rich, angiogenic inducer, 61*	1.54
11	*F11*	*Coagulation factor XI (plasma thromboplastin antecedent)*	1.53
12	*VEGF*	*Vascular endothelial growth factor*	1.52
13	*CXCL14*	*Chemokine (C-X-C motif) ligand 14*	1.51
14	*PLAT*	*Plasminogen activator, tissue*	1.51
15	*IGF2*	*Insulin-like growth factor 2 (somatomedin A)*	1.50
16	*GNRH1*	*Gonadotropin-releasing hormone 1 (leutinizing-releasing hormone)*	1.49
17	*SERPINE1*	*Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1*	1.47
18	*LAMP2*	*Lysosomal-associated membrane protein 2*	1.47
19	*SERPING1*	*Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1 (angioedema, hereditary)*	1.46
20	*BDNF*	*Brain-derived neurotrophic factor*	1.44
21	*APOC3*	*Apolipoprotein C-III*	1.43
22	*CXCL9*	*Chemokine (C-X-C motif) ligand 9*	1.42
23	*SERPINC1*	*Serine (or cysteine) proteinase inhibitor, clade C (antithrombin), member 1*	1.41
24	*CALU*	*Calumenin*	1.40
25	*SPOCK2*	*Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2*	1.38
26	*TNC*	*Tenascin C (hexabrachion)*	1.38
27	*IL16*	*Interleukin 16 (lymphocyte chemoattractant factor)*	1.34
28	*LAD1*	*Ladinin 1*	1.31
29	*AP2A2*	*Adaptor-related protein complex 2, α2 subunit*	1.30
30	*IL6*	*Interleukin 6 (interferon, β2)*	1.29
31	*MAPT*	*Microtubule-associated protein τ*	1.25
II. Genes overexpressed in grades II and III (lower-grade astrocytomas) compared with grade IV (GBM)			
1	*SPARCL1*	*SPARC-like 1 (mast9, hevin)*	3.27
2	*TIMP3*	*Tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy,pseudoinflammatory)*	2.27
3	*GPX3*	*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/m^2 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.

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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

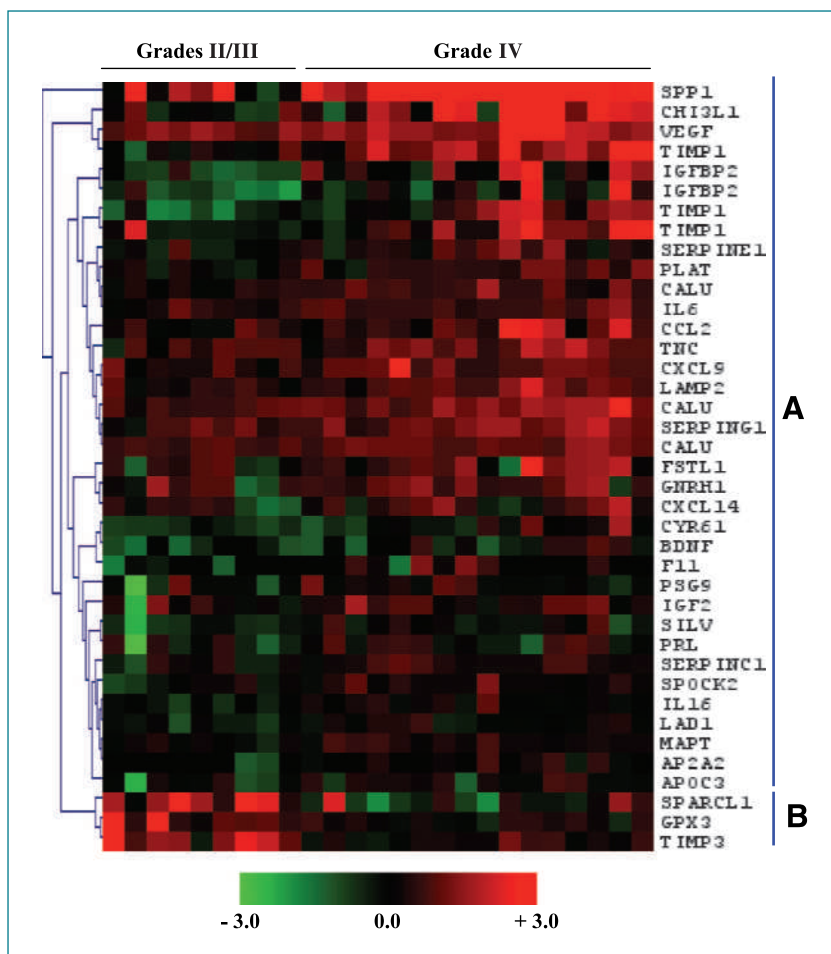

Figure 1. One-way hierarchical cluster analysis of SAM-identified genes using the TMEV software to see the data substructure. Normalized, log 2–transformed expression ratios of SAM-identified differentially regulated genes between GBMs (grade IV) and lower-grade astrocytomas (grades II and III) were 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).

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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 lesser 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

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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	
			Percentage	Mean log 2 ratios ± S.D	Percentage	Mean log 2 ratios ± S.D	Percentage	Mean log 2 ratios ± S.D	Percentage	Mean log 2 ratios ± S.D	Percentage	Mean log 2 ratios ± S.D	Percentage	Mean log 2 ratios ± S.D		χ^2	*P*
I. GBM upregulated genes																	
1	*CXCL9*	2.50	14.28% (1/7)	0.00 ± 1.91	14.28% (1/7)	0.53 ± 2.64	50.00% (4/8)	2.78 ± 2.35	26.92% (7/26)	1.28 ± 2.14	34.61% (9/26)	2.10 ± 2.30	84.09% (37/44)	3.74 ± 1.94	ε = 0.0007 ω = 0.006 ¢ = 0.0002 π = 0.0365	29.93	<0.0001
2	*TIMP1*	2.00	14.20% (1/7)	0.00 ± 1.84	0% (0/7)	−2.93 ± 1.35	0% (0/8)	−1.95 ± 1.61	11.50% (3/26)	−1.54 ± 2.24	0% (0/26)	−2.30 ± 1.28	68.10% (30/44)	2.36 ± 1.79	α = 0.03 δ = 0.028 ε = 0.018 ω < 0.0001 λ < 0.0001 ¢ < 0.0001 π < 0.0001	69.38	<0.0001
3	*SERPINE1*	1.00	42.80% (3/7)	0.00 ± 2.47	0% (0/7)	−3.25 ± 1.41	0% (0/8)	−1.50 ± 1.39	23.10% (6/26)	−1.29 ± 2.88	11.50% (3/26)	−1.45 ± 1.69	68.10% (30/44)	2.09 ± 2.55	ω < 0.0001 λ = 0.0017 ¢ < 0.0001 π < 0.0001	52.69	<0.0001
4	*IL6*	1.00	42.80% (3/7)	0.00 ± 2.21	0% (0/7)	−1.57 ± 1.33	0% (0/8)	−0.96 ± 1.16	7.60% (2/26)	−1.82 ± 1.48	7.60% (2/26)	−1.05 ± 1.63	65.90% (29/44)	2.10 ± 2.00	ε = 0.0427 ω < 0.0001 λ = 0.0002 ¢ < 0.0001 π < 0.0001	59.62	<0.0001
5	*CALU*	0.56	42.85% (3/7)	0.00 ± 1.44	0% (0/7)	−1.40 ± 0.78	25.00% (2/8)	−0.06 ± 1.01	7.69% (2/26)	−0.98 ± 0.88	0% (0/26)	−1.20 ± 0.71	79.54% (35/44)	1.26 ± 1.07	δ = 0.0437 ε = 0.0206 ω < 0.0001 μ = 0.0432 λ = 0.0069 ¢ < 0.0001 π < 0.0001	70.04	<0.0001
6	*PLAT*	0.56	28.57% (2/7)	0.00 ± 1.73	0% (0/7)	−3.02 ± 0.82	12.50% (1/8)	−2.25 ± 1.86	3.84% (1/26)	−2.20 ± 1.67	3.84% (1/26)	−2.40 ± 1.46	72.72% (32/44)	0.90 ± 1.28	α = 0.0027 β = 0.0426 γ = 0.0079 δ = 0.0027	64.66	<0.0001

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

Sl no.	Gene	Cutoff log 2 ratios	Normal		DO II		DA II		AO III		AA III		GBM		Post hoc *P**	Kruskal-Wallis test	
			Percentage	Mean log 2 ratios ± S.D	Percentage	Mean log 2 ratios ± S.D	Percentage	Mean log 2 ratios ± S.D	Percentage	Mean log 2 ratios ± S.D	Percentage	Mean log 2 ratios ± S.D	Percentage	Mean log 2 ratios ± S.D		χ^2	*P*
															ω < 0.0001 λ < 0.0001 ¢ < 0.0001 π < 0.0001		
7A	*OPN tv 1*	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	ε = 0.0041 η = 0.0488 ω < 0.0001 ¢ < 0.0001 π < 0.0001	54.48	<0.0001
7B	*OPN tv 2*	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	γ = 0.0039 ε < 0.0001 ω < 0.0001 λ < 0.0001 ¢ < 0.0001 π < 0.0001	78.25	<0.0001
7C	*OPN tv 3*	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	ε = 0.0024 ω < 0.0001 λ < 0.0001 ¢ < 0.0001 π < 0.0001	67.38	<0.0001
II. GBM downregulated genes																	
8	*GPX3*	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	β = 0.0003 γ = 0.0160 δ < 0.0001 ε = 0.0145 η = 0.0170 Φ = 0.0004 ζ = 0.0024 π = 0.0002	34.00	<0.0001
9	*TIMP3*	−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	ε = 0.0027 λ = 0.0188 π = 0.0073	22.56	0.0004

*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; ¢, AO vs GBM; π, AA vs GBM.

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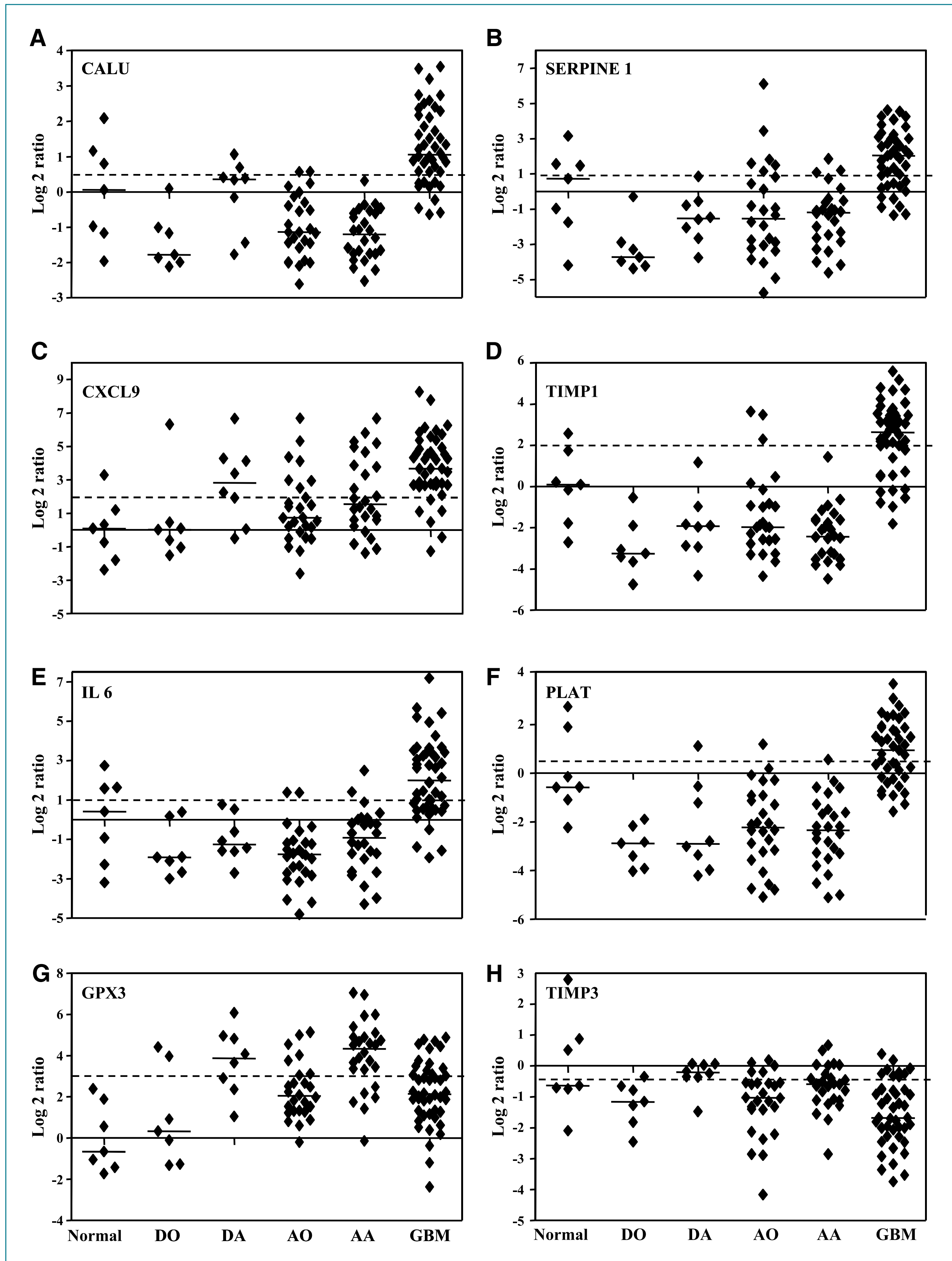

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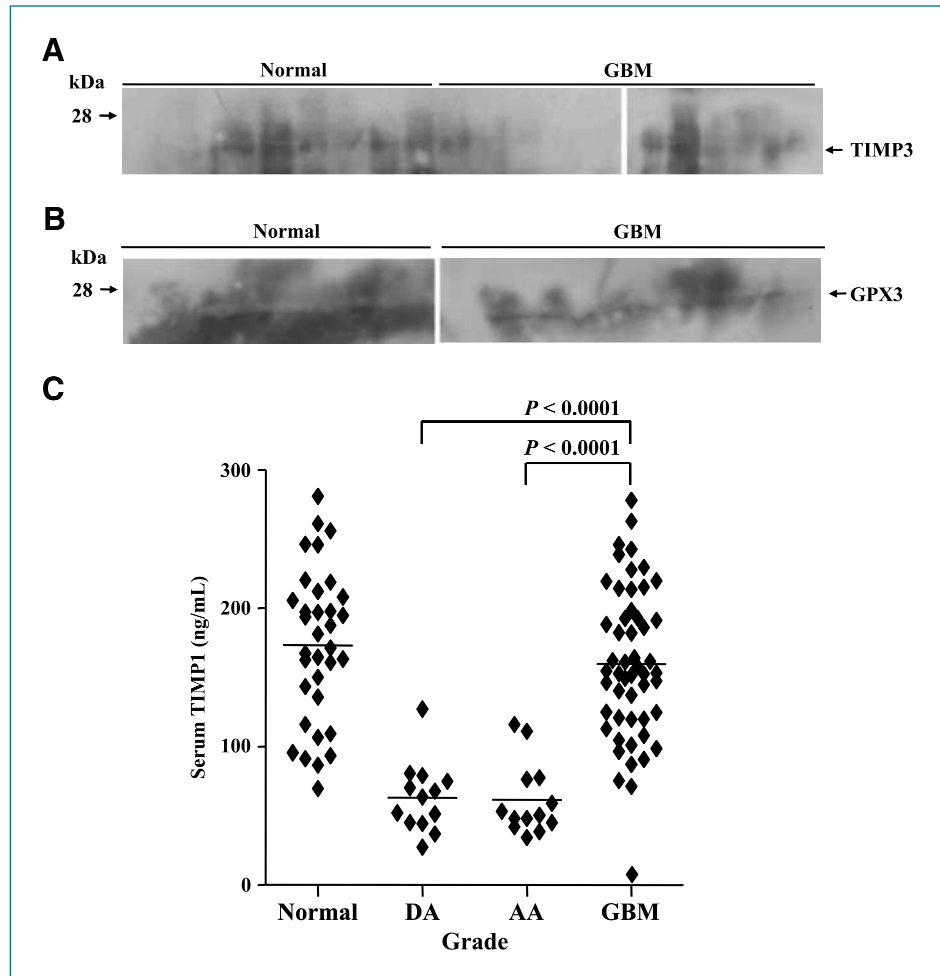

Figure 3. Analysis of serum TIMP3, GPX3, and TIMP1. A, total serum protein (5 μL) from normal healthy controls and GBM patients was subjected to Western blot analysis for TIMP3. B, albumin/IgG depleted serum protein (50 μg) from normal healthy controls and GBM patients was subjected to Western blot analysis 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.

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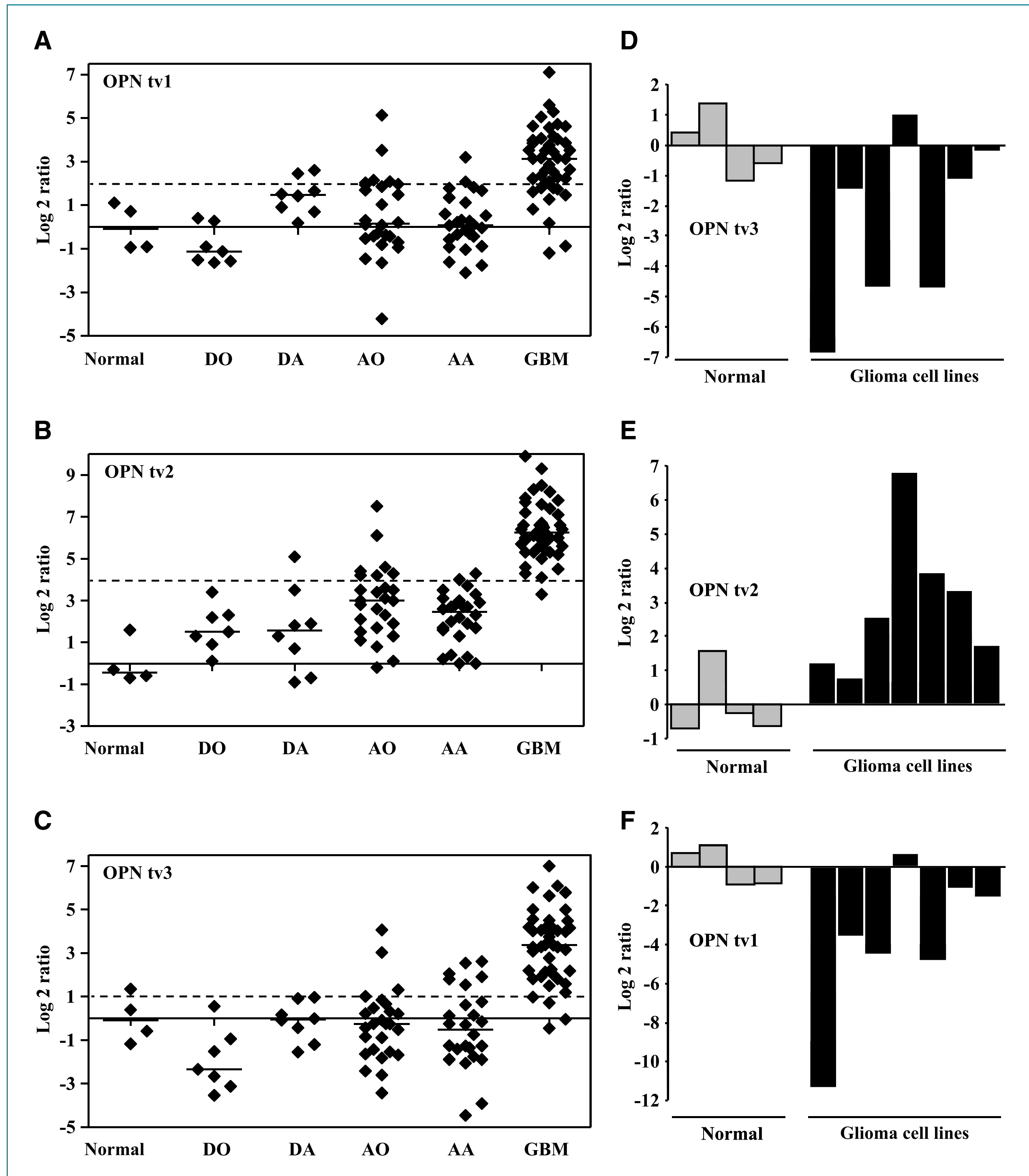

Figure 4. Scatter plot of transcript levels of OPN in different grades of astrocytoma and glioma cell lines. A to C, log 2–transformed gene expression ratios obtained from real-time RT-qPCR analysis of RNA derived from tumor tissue samples (as indicated) are plotted for OPN tv1 (transcript variant 1; 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, log 2–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.

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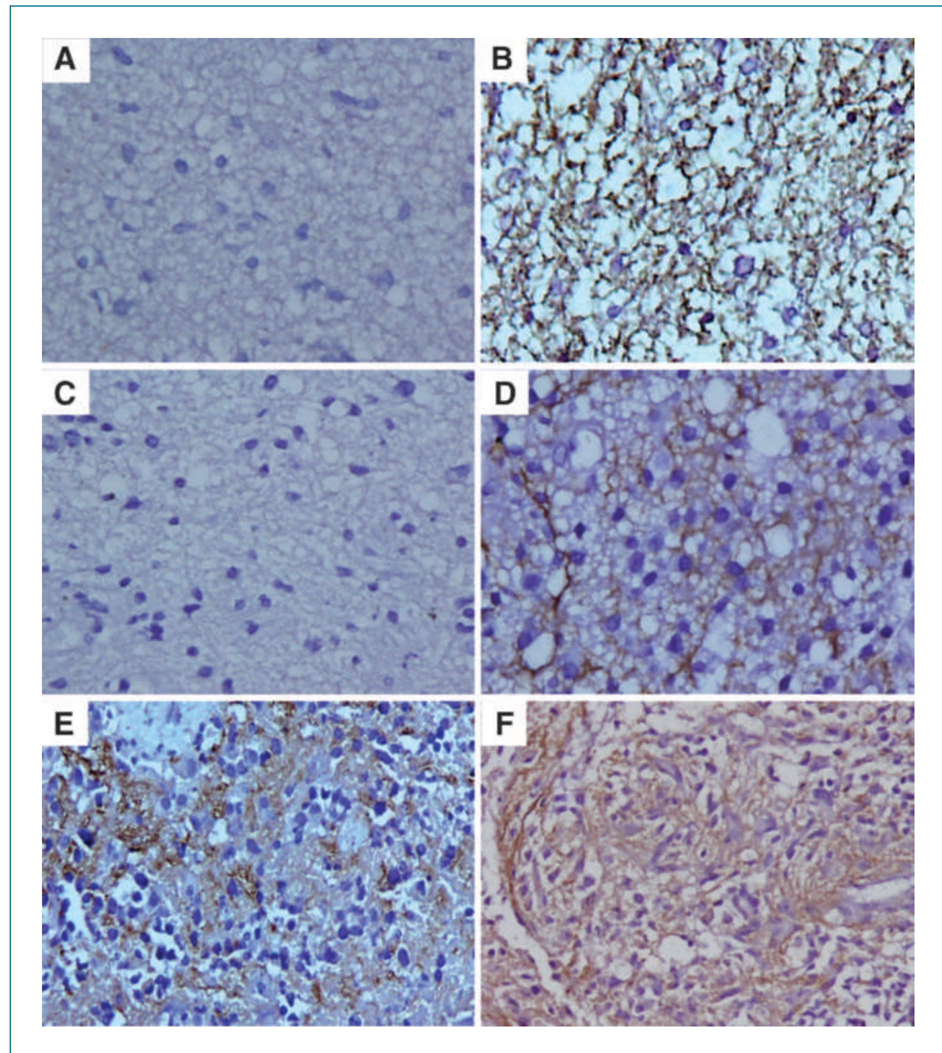

Figure 5. Immunohistochemical validation of OPN overexpression in astrocytoma. Sections from DA-negative for staining (A), DA-positive for staining (B), AA—negative for staining (C), AA—positive for staining (D), and secondary GBMs and primary GBM—positive for staining (E and F) were stained for OPN.

(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

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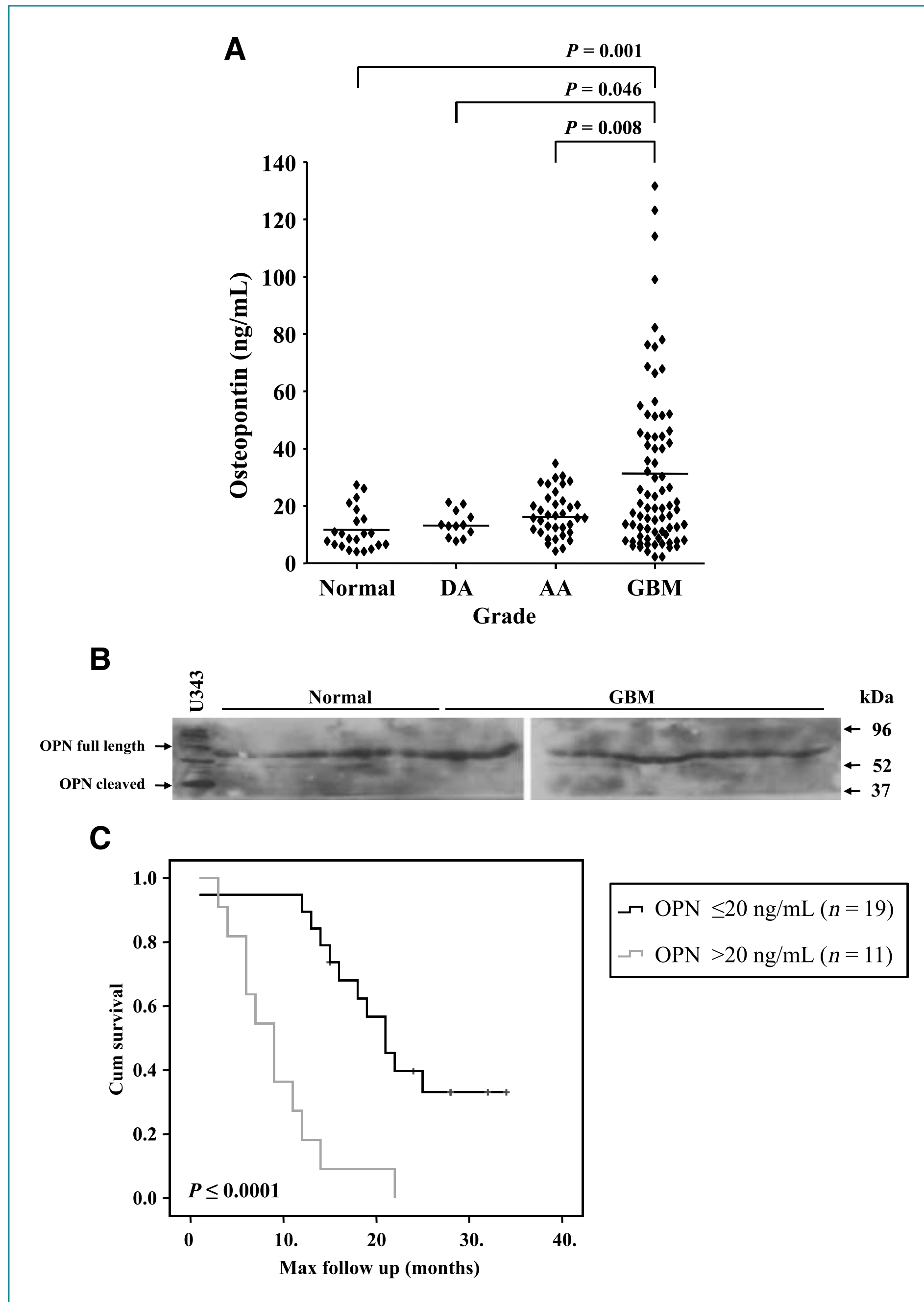

Figure 6. Scatter plot of serum OPN levels and its correlation with patient survival. A, serum levels of OPN (ng/mL) in normal controls (n = 22), DAs (n = 12), AAs (n = 36), and GBMs (n = 78). The horizontal line in each group designates the mean value. B, total serum protein (2.5 μL) from normal healthy 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

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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 metalloproteases, 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, progression, 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-3β/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.

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