

Genomic and Expression Profiling of Glioblastoma Stem Cell–Like Spheroid Cultures Identifies Novel Tumor-Relevant Genes Associated with Survival

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Abstract Purpose: Glioblastoma spheroid cultures are enriched in tumor stem-like cells and therefore may be more representative of the respective primary tumors than conventional monolayer cultures. We exploited the glioma spheroid culture model to find novel tumor-relevant genes.

Experimental Design: We carried out array-based comparative genomic hybridization of spheroid cultures derived from 20 glioblastomas. Microarray-based gene expression analysis was applied to determine genes with differential expression compared with normal brain tissue and to nonneoplastic brain spheroids in glioma spheroid cultures. The protein expression levels of three candidates were determined by immunohistochemistry on tissue microarrays and correlated with clinical outcome. Functional analysis of *PDPN* was done.

Results: Genomic changes in spheroid cultures closely resembled those detected in primary tumors of the corresponding patients. In contrast, genomic changes in serum-grown monolayer cultures established from the same patients did not match well with the respective primary tumors. Microarray-based gene expression analysis of glioblastoma spheroid cultures identified a set of novel candidate genes being upregulated or downregulated relative to normal brain. Quantitative real-time PCR analyses of 8 selected candidate genes in 20 clinical glioblastoma samples validated the microarray findings. Immunohistochemistry on tissue microarrays revealed that expression of *AJAP1*, *EMP3*, and *PDPN* was significantly associated with overall survival of astrocytic glioma patients. Invasive capacity and RhoA activity were decreased in *PDPN*-silenced spheroids.

Conclusion: We identified a set of novel candidate genes that likely play a role in glioblastoma pathogenesis and implicate *AJAP1*, *EMP3*, and *PDPN* as molecular markers associated with the clinical outcome of glioma patients. (Clin Cancer Res 2009;15(21):6541–50)

Glioblastoma is the most common and most malignant primary brain tumor and has one of the worst survival rates among all human cancers. Despite aggressive multimodal treatment, the median survival time after diag-

nosis has improved only marginally and is still <1 year in population-based studies (1). A better understanding of the complex molecular and cellular mechanisms leading to glioblastoma is an important prerequisite to

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Translational Relevance

Glioma-derived primary cultures and established cell lines grown in serum-containing medium are commonly used as *in vitro* models. However, findings obtained in primary cultures and established cell lines should be interpreted with caution because genetic and transcriptional profiles in these cells often do not correspond well to those in the respective primary tumors. In this study, glioblastoma spheroid cultures were found to retain molecular profiles of human glioblastoma at the mRNA expression and DNA copy number levels. Therefore, spheroid cultures offer a promising model for *in vitro* studies of glioblastoma-associated molecular pathomechanisms. Furthermore, we show here that genomic and mRNA profiling of glioblastoma spheroid cultures identified *AJAP1*, *EMP3*, and *PDPN* as aberrantly expressed candidate genes whose protein expression levels, when immunohistochemically assessed in primary glioma tissue samples, turned out as molecular markers that are associated with the overall survival of glioma patients. Silencing of *PDPN* led to decreased invasive capacity.

the identification of novel and more effective therapeutic strategies.

Many of the most commonly used glioma cell lines are characterized by genomic aberration profiles that are distinct from those typically seen in the respective primary tumors (2). In contrast, stem cell-enriched spheroids culture-grown in serum-free medium were hypothesized to be more representative of the original tumor's genotype than are the established cell lines (3, 4).

Glioblastoma-derived stem cell-like spheroid cultures are increasingly used to study stem cell-like behavior and tumor biology. Spheroid-forming cells can be cultured from human glioblastoma using conditions that enrich for neural stem cells. Such glioma stem cell-like cells display increased tumorigenicity and resistance to radiation treatment both in cell culture and in the brains of immunocompromised mice (5).

Glioblastoma spheroid cultures positive for the stem cell marker CD133 have been molecularly characterized by expression profiling in small numbers of patients (refs. 3, 6, 7; $n \leq 4$ in each instance). These studies showed that individual glioblastoma specimens give rise to different phenotypes of long-term cultures, in spite of being established under identical conditions. In culture, growth and differentiation properties vary considerably between individual tumors. This diversity leads to the necessity of analyzing a larger number of tumors.

Given their growing importance as a model, it is of fundamental importance to unravel the degree to which glioblastoma spheroid cultures resemble primary tumors and thereby may allow for the discovery of new tumor-relevant genes. Therefore, we investigated DNA copy number abnormalities by array-based comparative genomic hybridization (array-CGH; matrix-CGH) in 20 tumor stem cell-enriched spheroid cultures (19 derived from primary glioblastoma and 1 derived from secondary glioblastoma), 13 glioma monolayer cultures, and 7 pri-

mary glioblastoma tissue samples. In addition, we did genome-wide gene expression analysis of spheroid cultures. From the genomic and gene expression analyses, we identified a set of candidate genes whose aberrant expression in glioblastoma tissues was validated at the mRNA and protein levels. Furthermore, we showed that the protein expression levels of three validated candidates, namely, *AJAP1*, *EMP3*, and *PDPN*, were associated with overall survival of glioma patients. Finally, we did a functional analysis of *PDPN*.

Materials and Methods

Cell culture conditions. Glioblastoma samples were obtained from patients undergoing surgery. Informed consent was obtained according to the research proposals approved by the Institutional Review Board at the Medical Faculty Heidelberg. Tissues were dissociated and resuspended in stem-cell medium (8)⁹ made of DMEM/F-12 containing 20% bovine serum albumin, insulin and transferrin (BIT)-serum-free supplement, and basic fibroblast and epidermal growth factors (Provitro, 20 ng/mL each), or in DMEM containing 10% serum (9). Spheroids were cultured on uncoated culture dishes (Sarstedt) and analyzed between passage 1 and 17. Serum-grown cultures were analyzed a first time between passage 1 and 8. They were analyzed a second, third, and fourth time (up to passage 39) if no tumor profile was seen in the first array-CGH analysis. Glioma cell lines A172, CCF-SSTG1, T98G, U373MG, U178MG, TP365MG, U118MG, and U251MG were obtained from the American Type Culture Collection repository and cultured in DMEM containing 10% serum.

Nucleic acid isolation. Extraction of DNA and RNA from frozen tumor samples was done via cesium chloride ultracentrifugation. Genomic DNA from blood of healthy donors and from cultured cells was isolated using the Blood and Cell Culture Kit (Qiagen). RNA from cultured cells was isolated using the RNeasy kit (Qiagen). RNA quality was assessed with the Agilent 2100 bioanalyzer (Agilent).

Array-based CGH. Array-CGH was carried out as previously described (10). Data were filtered according to signal/background ratio (>3.0), mean/median spot intensity (<0.3), replicate SD (<0.25), and normalized by print-tip loss. To identify regions of similar genomic status, we applied the segmentation software GLAD (11). Imbalances with \log_2 ratios of <-1.0 were scored as putative homozygous deletions, because this threshold corresponds to an average copy number <1 , suggesting the presence of at least one subpopulation of cells with homozygous deletions. Gains with \log_2 ratios >1.0 were scored as amplifications. Chromosomal mapping was based on Ensembl (v49) and position of candidate genes was verified using Ensembl v52.

Gene expression analysis. Sample amplification was done using 100 ng of total RNA by the method of Van Gelder et al. (12). RNA was amplified using the TotalPrep RNA Amplification kit (Illumina) following the manufacturer's instructions. Labeling was achieved by incorporation of biotin-16-UTP. Labeled material was hybridized to the Illumina Human whole genome Sentrix-6 V2 BeadChip array according to the manufacturer's instructions (Illumina). Microarray scanning was done using a Beadstation array scanner. Data extraction was done using the bead array R package (svn release 1.7.0) from bioconductor.org. Bead outliers were removed when their expression value dropped below a threshold: the imaging system background, nonspecific binding, and cross-hybridization signal. Individual bead types were flagged as filtered when their bead replicate count dropped below 17. We excluded a bead type when its filter flag was set across all samples. Data analysis was done by variance stabilizing and spline normalizing the signals using the algorithms from the lumi R package (release 1.1.0) from bioconductor.org.

⁹ B. Campos, F. Wan, M. Reza Farhadi, et al. Differentiation Therapy Exerts Anti-Tumor Effects on Stem-Like Glioma Cells, submitted for publication.

Statistical analysis. Correlations between genomic profiles were expressed as Pearson's correlation coefficients. Progression-free survival (PFS) was calculated from the date of surgery until the date of tumor recurrence or further growth or until the date of death. For patients who had not experienced recurrence or death at the time of last follow-up, PFS and overall survival (OS) were censored at the date of last follow-up. The association between PFS or OS and gene expression was calculated using log-rank tests and presented as Kaplan-Meier plots. Multivariate analysis was done using Cox proportional hazards regression. Patients with >30% of missing values were excluded. For patients with <30% of missing values, the missing values were multiple-imputed

using additive regression bootstrapping and predictive mean matching (13). Backward selection applying a stopping rule based on the Akaike information criterion was used to exclude redundant or unnecessary variables. Hazard ratios and 95% confidence intervals were computed to provide quantitative information about the relevance of results. All calculations were done using the statistical software R, version 2.4.1.4. Statistical significance was set at level of $P < 0.05$.

Tumor samples and preparation of tissue microarrays. Two custom tissue microarrays were constructed. Eligibility criteria included written informed consent from the patient and availability of follow-up data. Clinical information was obtained by reviewing the medical

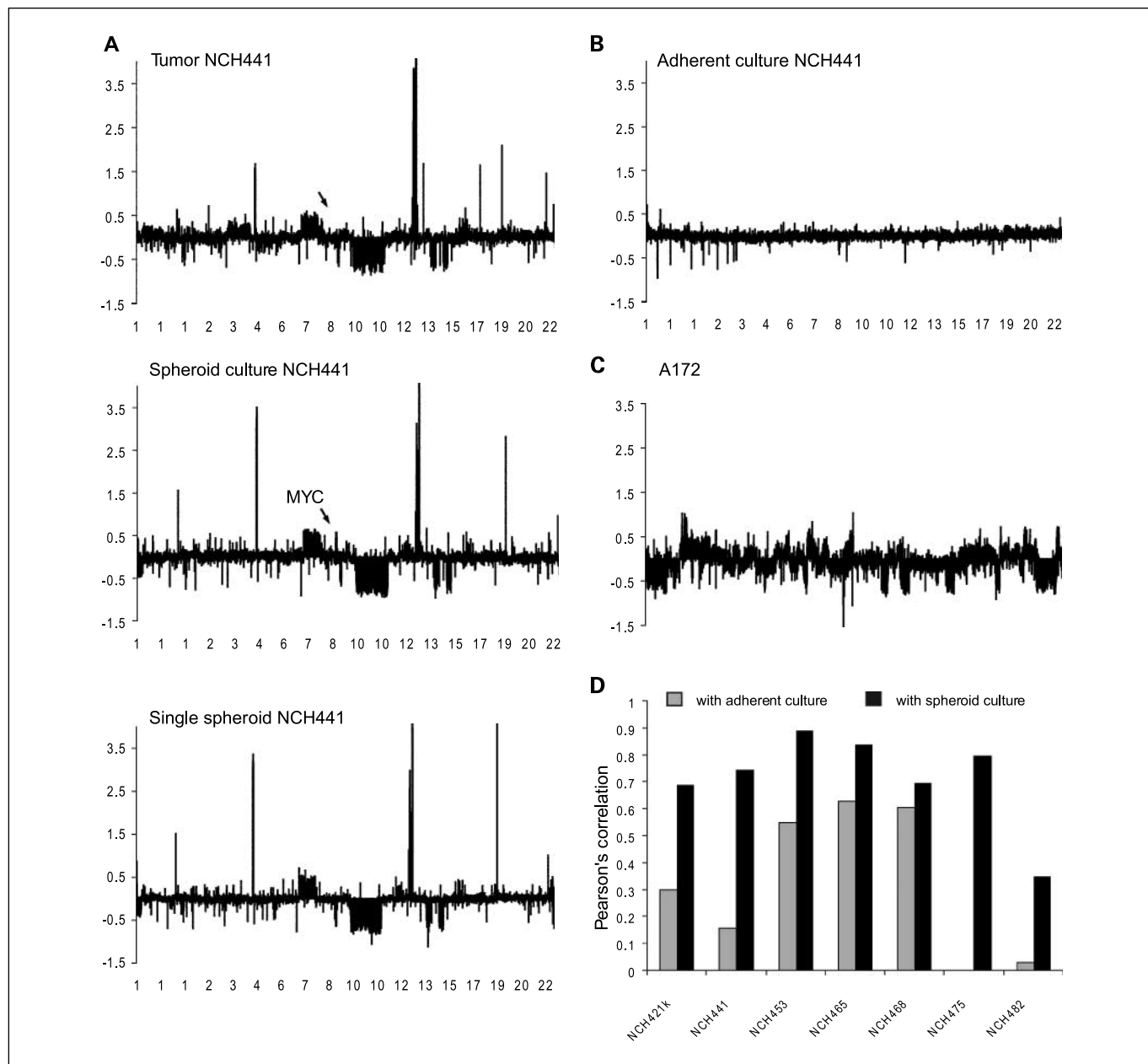


Fig. 1. The genotype of spheroid cultures closely resembles the genotype of the respective original tumor. *A*, similarity between array-CGH profiles of original tumor, spheroid culture and single neurosphere picked from the spheroid culture in case NCH441. Despite the overall high similarity of genomic profiles, some culture-specific alterations emerged, such as gains of the *MYC* locus. *B*, genomic alterations in adherent serum-grown cultures often do not correspond well to the genotype of the respective original tumors, with many primary cultures never showing the genetic imbalances seen in the tumors they were derived from. *C*, the majority of established glioma cell lines, exemplified by A172, do not carry genomic aberrations reported as common events in primary tumors. *D*, bar chart showing similarity of genomic profiles of adherent culture with original tumor (grey) and spheroid culture with original tumor (black) for each of the seven cases for which primary material was available. Correlations are expressed as Pearson's correlation coefficients calculated using the R package.

records on radiographic images, by telephone, and by review of death certificate. All patients were treated by surgery at diagnosis; radiotherapy and/or chemotherapy was administered for high-grade tumors. Tumor samples for the first tissue microarray were collected at the Department of Neurosurgery, Heidelberg University Hospital, Germany, from 2001 to 2003. Tumor samples for the second tissue microarray were collected at the Department of Neuropathology, Heinrich-Heine-University, Düsseldorf, Germany, from 1988 to 2004. H&E-stained sections were prepared to define representative tumor regions as previously described (14). Patient characteristics of all samples are summarized in Supplementary Table S1. All tumors were histologically classified according to the WHO Classification of Tumors of the Nervous System (15).

Immunohistochemistry. See Supplementary Methods.

Quantitative real-time PCR. To measure relative mRNA abundance of candidate genes in tumors, a reference of total RNA obtained from nonneoplastic human brain tissue samples of five individuals (BioChain) was used. Total RNA was treated with DNaseI (Invitrogen) and reverse-transcribed with SuperscriptII (Invitrogen). Each cDNA sample was analyzed in duplicate with the ABI PRISM 7900 (Applied Biosystems) using Absolute SYBR Green ROX Mix (ABgene). Endogenous housekeeping genes (*ARF1*, *DCTN2*) were used as internal standards. The quantification of the RNA of interest in comparison with the housekeeping genes was calculated according to a previously published algorithm (16). Oligonucleotide sequences are available in Supplementary Table S2.

Pathway analysis. The canonical pathway analyses were generated through the Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com). Genes that were >2-fold differentially expressed between glioblastoma spheroid cultures and normal brain and associated with a canonical pathway in the Ingenuity Pathways Knowledge Base were considered for the analysis.

PDPN silencing. Lentiviral vectors were produced by cotransfection of HEK293T cells with the psPAX2, pMD2.G, and pLKO.1 constructs (MISSION TRC-Hs 1.0; ref. 17). Transfections were carried out using TransIT (Mirus Bio). Virus was harvested 72 h after transfection. Infections of NCH421k cells were carried out in the presence of 8 µg/mL

of polybrene. Virus-containing supernatant was removed after 24 h. Two independent shRNA constructs were used: MISSION shRNA NM_006474.4-482s1c1 (shRNA B) and NM_006474.4-453s1c1 (shRNA A). Nontargeting shRNA was used as a control.

Proliferation assay. Proliferation assays were done using the MTS-based CellTiter 96 AQ Non-Radioactive Cell Proliferation Assay (Promega) following the manufacturer's recommendations. All samples were assayed in eight replicas.

Invasion assay. Drops containing 2×10^4 cells/25 µL medium were plated on the lid of a 10-cm Petri dish that was inverted. Within 48 h the cells formed spheroids that were harvested and placed into collagen gels. Cold vitrogen (Nutacon) was mixed with cold 10-fold concentrated MEM and cold 0.1 mol/L NaOH reaching a final concentration of 2.4 mg/mL collagen. Collagen solution was distributed; spheroids were placed into each well and kept at 37°C for 60 min. After solidification the gels were overlaid with medium. Cell migration out of the spheroids was monitored over a period of 10 d.

RhoA activation assay. The level of RhoA activation was measured with the G-LISA RhoA activation assay (Cytoskeleton) following the manufacturer's recommendations.

Results

Spheroid cultures are representative of the genotype of the original tumors. In order to assess the degree to which genetic imbalances seen in glioma-derived adherent and spheroid cultures are representative of those observed in the original tumors, we compared the array-CGH profiles of both types of cultures with the profiles of the respective primary tumors (Fig. 1A and B). Pairwise correlation comparisons between the array-CGH data sets of seven spheroid cultures and their matched primary tumors showed that they were highly similar in most cases, with Pearson's correlation coefficient exceeding 0.69 in 6 of 7 cases (Fig. 1D). Furthermore, spheroid cultures were homogenous with regard to their genomic aberrations, as could be attested

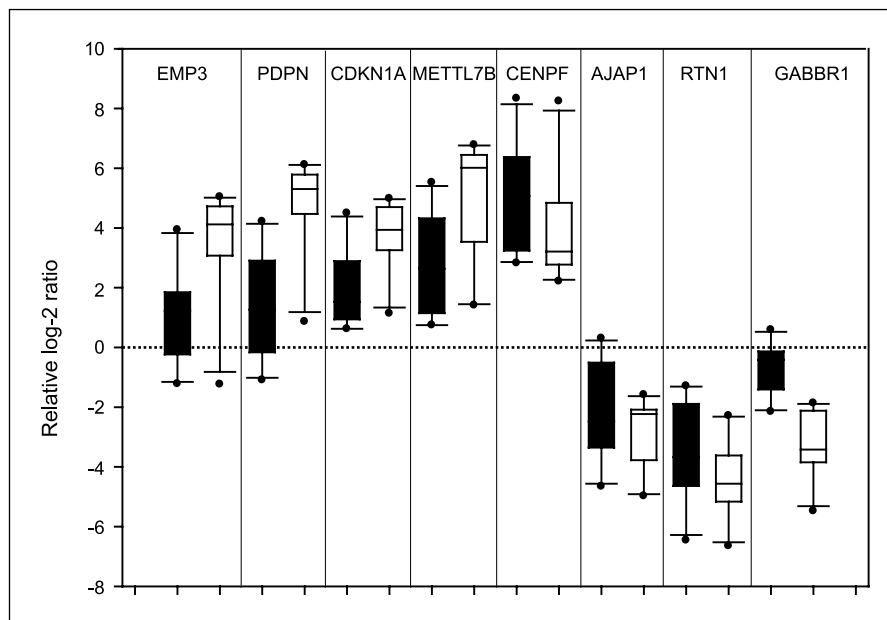


Fig. 2. Validation of candidate genes emerging from spheroid cultures analysis in glioma patients. A total of eight genes from the set of candidate genes showing most pronounced differential expression between spheroid cultures and normal brain were analyzed by quantitative real-time-PCR in primary and secondary glioblastoma tissue samples from 20 patients. Box plots show log 2-transformed gene expression ratios of differentially regulated genes in secondary glioblastoma ($n = 10$, black boxes) and primary glioblastoma ($n = 10$, white boxes) relative to normal brain ($n = 5$) defining the zero-level.

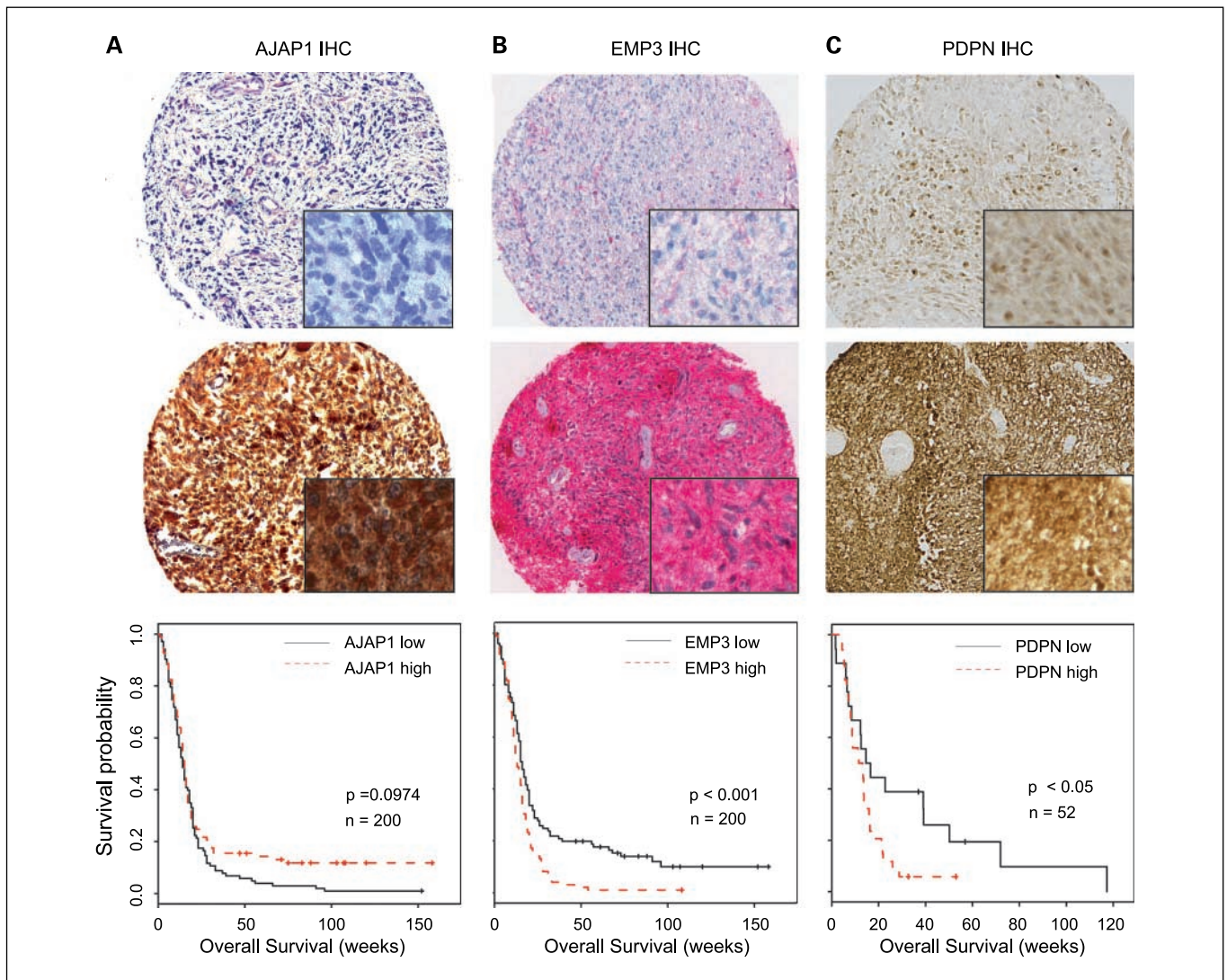


Fig. 3. Expression of AJAP1, EMP3, and PDPN is associated with clinical outcome of glioma patients. Top, immunohistochemical analysis of glioblastomas showing weak (*top*) or strong (*bottom*) expression for each of the gene products. The diameter of the tissue specimens is 0.6 mm. *A*, immunohistochemical detection of AJAP1 was done with standard streptavidin-biotin peroxidase technique. *B*, immunostain visualization of EMP3 was achieved with ultraView Red. *C*, immunohistochemical detection of PDPN was done with standard streptavidin-biotin peroxidase technique. Bottom, Kaplan-Meier analysis showing the association of expression with overall survival in patients with astrocytic gliomas (*P* values were calculated using log-rank tests).

by doing array-CGH analysis of several single neurospheres picked from the same culture. The resulting genomic profiles were almost identical and highly similar to the profiles of the whole culture and of the original tumor (Fig. 1A).

Unlike tumor-derived spheroid cultures, adherent serum-grown cultures derived from the same tumors often did not represent the genotype of the original tumors. Among adherent cultures established from 13 different primary tumors, only 4 (31%) showed at early passages (between 1 and 8) the same genomic imbalances as detected in the respective original tumors; one adherent culture did not show any genetic imbalance before reaching passage 39, two displayed a tumor profile in only one of two independent culturing attempts, and six (46%) never exhibited any genetic imbalances (Fig. 1B). Similarly, array-CGH analysis of commonly used glioblastoma cell lines (A172, CCF-SSTG1, T98G, U373MG, U178MG, TP365MG, U118MG, and U251MG) revealed that their pro-

files were quite distinct from those typically found in glioblastoma primary tumors (Fig. 1C and Supplementary Fig. S1).

Despite the overall high similarity of genomic profiles of the spheroid cultures to the original tumors, high-level amplifications of the *MYC* locus appeared in 4 of 20 cultures although these amplifications were not detected by array-CGH (Fig. 1A) or by fluorescence *in situ* hybridization (data not shown) in the original tumors.

Because the spheroid cultures apparently were more representative of the genotype of the original tumors than were the adherent serum-grown cultures, we focused our further analysis on spheroid cultures. Analysis of the DNA copy number alterations in 20 spheroid cultures via array-CGH revealed novel as well as previously described chromosomal aberrations, such as deletion of chromosome 10, gain of chromosome 7, as well as amplification of the *EGFR* and *PDGFRA* loci. The most frequent gains involved genetic material from chromosomes 7,

19p, and 20q whereas losses were most commonly found on chromosomes 10, 14q, 6q, and 22q (data not shown). We also identified loci exhibiting high-level amplifications as well as putative homozygous deletions (Supplementary Table S3). By defining minimal overlapping regions of all sample data, we were able to identify potential target genes for some of the amplified or homozygously deleted regions. Recurrent amplifications were found at 1q32.1 (*MDM4*), 4q12 (*PDGFRA*), 7p11.2 (*EGFR*), 8q24.21 (*MYC*), 12q14.1 (*CDK4*), and 12q15 (*MDM2*). Recurrent homozygous deletions were detected at 9p21.3 (*CDKN2A*) and 11q24.3. In addition, we observed several aberrations previously not reported to occur in glioblastoma, such as amplifications of the 2p23.3 and 6p22.3 loci, harboring the potential oncogenes *RAB10* and *E2F3*, respectively, and one homozygous deletion of the 1p36 locus harboring the potential tumor suppressor gene adherens junction-associated protein 1 (*AJAP1*).

Spheroid cultures can be used to identify novel tumor-relevant genes. To further pinpoint candidate genes, we analyzed gene expression in a subset of 10 spheroid cultures. Of the 16,476 analyzed transcripts, 1,009 were >2-fold overexpressed in the cultures and 1,152 were expressed at >2-fold lower levels as compared with the normal brain reference RNA pool. The top-ranked highly expressed genes are shown in Supplementary Table S4. Among these genes, we found a number of genes of well-known importance in glioblastoma pathogenesis, such as *CDK4*, *CDK6*, or *PDGFRA*, but also several genes not previously shown to play a major role in glioblastoma. We included as further control nonneoplastic neurospheres. The resulting gene signature differed from the signature obtained by the comparison with normal brain reference RNA; however, most of the top-ranked genes were also differentially expressed compared with nonneoplastic neurospheres. From the latter candidate genes, we validated eight top-ranked genes (*EMP3*, *PDPN*, *CDKN1A*, *METTL7B*, *CENPF*, *AJAP1*, *RTN1*, and *GABBR1*) by evaluating their mRNA expression level by quantitative real-time PCR in 10 primary and 10 secondary glioblastomas (Fig. 2). These analyses revealed (a) highest or exclusive overexpression of *EMP3*, *PDPN*, *CDKN1A*, and *METTL7B* in primary glioblastoma; (b) overexpression of *CENPF* in all primary and secondary glioblastomas; and (c) lower expression of *AJAP1*, *RTN1*, and *GABBR1* in all primary and secondary glioblastomas relative to the normal brain reference RNA pool.

To identify signaling pathways that are active in glioblastoma spheroids, we used the Ingenuity Pathways Analysis software. We ranked canonical pathways according to the significance of the enrichment for the most highly deregulated genes in glioblastoma spheroids among canonical pathways from the Ingenuity Pathway Analysis (IPA) library. Among canonical pathways, G₁-S checkpoint regulation and G₂-M checkpoint regulation strongly fit genes highly deregulated in glioblastoma spheroids ($P = 2.52E-07$ and $3.31E-03$, respectively). This finding is consistent with the fact that many of the known genetic alterations in glioblastoma target cell cycle regulatory genes. Most of these aberrations affect critical cell cycle regulatory components like *p16*, *CDK4*, *CDK6*, *cyclin D1*, and *RB*. Individual components in this pathway are altered in nearly all glioblastoma patients, resulting in the inactivation of cell cycle control pathways (18). The functions associated with the five most significant

networks were cell cycle, cancer, cellular development, nervous system development, and cellular movement. Three candidate genes connected to cellular movement were selected for further investigation: *AJAP1*, *EMP3*, and *PDPN*.

AJAP1 figured among the most highly downregulated genes in 10 spheroid cultures and was also a putative candidate gene for the 1p36 homozygous deletion detected in one culture. *AJAP1* protein expression was analyzed by immunohistochemistry in 356 astrocytic glioma samples from 200 patients (Supplementary Table S1, Fig. 3A). Differences in *AJAP1* expression were observed between glioblastomas as compared with WHO grade II and III astrocytomas, with *AJAP1* expression levels being low in 55% of glioblastomas but only in 28% of WHO grade II and III astrocytomas (Supplementary Table S5).

Among the top-ranked highly expressed genes in spheroid cultures compared with normal brain were the epithelial membrane protein 3 (*EMP3*) and podoplanin (*PDPN*). *EMP3* protein expression was analyzed by immunohistochemistry in tumor samples from the same cohort of 200 astrocytic glioma patients as studied for *AJAP1* (Fig. 3B). In the majority of primary glioblastomas, we detected a strong *EMP3* expression whereas the majority of the WHO grade II and III gliomas as well as most secondary glioblastomas showed low or no expression (Supplementary Table S5). *PDPN* protein expression was analyzed in 162 astrocytic glioma samples from 63 patients (Supplementary Table S1, Fig. 3C). Similar to *EMP3*, we detected a strong *PDPN* expression in 85% of the primary glioblastomas, whereas the majority of the WHO grade II and III gliomas as well as the secondary glioblastomas showed weak or no expression (Supplementary Table S5).

To investigate whether and how the expression levels of *AJAP1*, *EMP3*, and *PDPN* are related to patient outcome, we correlated the respective expression scores with PFS and OS data of the investigated patients. Univariate analysis showed significant association of high *AJAP1* expression with shorter OS in the group of glioblastoma patients ($P < 0.05$, Table 1) and significant association of high *EMP3* expression ($P < 0.001$, Fig. 3B and Table 1) and high *PDPN* expression ($P < 0.05$, Fig. 3C and Table 1) with shorter OS in the entire group of astrocytic glioma patients. However, Fisher's exact tests revealed that

Table 1. Univariate analysis identifies expression of *AJAP1*, *EMP3* and *PDPN* as prognostic factors for clinical outcome

	Overall survival	
	HR (95% CI)	P
AJAP1 expression		
WHO grade II-IV (n = 200)	0.786 (0.588-1.05)	0.097
WHO grade II-III (n = 28)	0.381 (0.138-1.06)	0.064
WHO grade IV (n = 172)	1.39 (1.02-1.89)	0.038
EMP3 expression		
WHO grade II-IV (n = 200)	1.67 (1.24-2.25)	<0.001
WHO grade II-III (n = 31)	2.76 (0.889-8.57)	0.079
WHO grade IV (n = 169)	0.934 (0.688-1.27)	0.660
PDPN expression		
WHO grade II-IV (n = 52)	1.94 (1.00-3.74)	0.049
WHO grade IV (n = 42)	0.989 (0.445-2.20)	0.980

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.

Table 2. Multivariate analysis identifies expression of AJAP1 as an independent prognostic factor for clinical outcome

Variable*	PFS		OS	
	HR (95% CI)	P	HR (95% CI)	P
WHO grade IV (n = 163)				
AJAP1 expression	1.469 (1.068-2.022)	0.018	1.541 (1.110-2.14)	0.0098
Patient age	1.002 (0.990-1.014)	0.79	1.025 (1.012-1.04)	0.00018
Extent of resection	0.703 (0.502-0.984)	0.04	0.490 (0.346-0.69)	0.000058
Karnofsky index	0.914 (0.816-1.025)	0.13	0.852 (0.760-0.95)	0.0056
WHO grade IV (n = 160)				
EMP3 expression	0.734 (0.533-1.01)	0.058	0.796 (0.579-1.09)	0.160
Patient age	1.002 (0.989-1.01)	0.80	1.024 (1.011-1.04)	0.0003
Extent of resection	0.706 (0.503-0.99)	0.043	0.469 (0.331-0.66)	0.000021
Karnofsky index	0.939 (0.837-1.05)	0.28	0.844 (0.752-0.95)	0.004
WHO grade IV (n = 41)				
PDPN expression	N.D.	N.D.	0.381 (0.137-1.06)	0.065
Patient age	N.D.	N.D.	1.039 (1.001-1.08)	0.044
Chemotherapy	N.D.	N.D.	0.489 (0.229-1.05)	0.065

NOTE: Cox proportional hazard regression analysis.

Abbreviations: N.D., not determined; PFS, progression-free survival; OS, overall survival; HR, hazard ratio; 95% CI, 95% confidence interval.

*The variables were compared in the following ways: expression, high versus low; patient age, x versus x+1; extent of resection, total versus subtotal; Karnofsky index, x+1 versus x; chemotherapy, yes versus no.

the expression of all three proteins was associated with WHO grade ($P = 0.017$ for AJAP1, $P < 0.0001$ for EMP3, and $P < 0.00001$ for PDPN).

To test whether expression of any of the three candidate genes was an independent prognostic marker, we carried out multivariate survival analysis for glioblastoma patients. Multivariate analysis identified high AJAP1 expression as a significant prognostic factor associated with shorter OS ($P < 0.01$) and shorter PFS ($P < 0.05$) in glioblastoma patients, independent of age, extent of resection, and Karnofsky index (Table 2).

Silencing of PDPN leads to decreased invasiveness. PDPN silencing was done in glioblastoma spheroids by lentiviral transduction of shRNAs. Nontargeting shRNA and MOCK-transduced cells were used as controls. Using two independent shRNAs targeting different regions of the PDPN mRNA transcript, we achieved knockdown efficiencies of 86% and 92%, respectively (Fig. 4A). Transduction with each of these two shRNAs resulted in a complete inhibition of invasiveness as shown by invasion assays in three-dimensional collagen matrices (Fig. 4B). To assess whether the decreased invasiveness was due to a slower proliferation in PDPN-silenced cells, we used the MTS assay. Proliferation of PDPN-silenced cells was not significantly decreased compared with MOCK-transduced cells and with nontargeting shRNA-transduced cells (Fig. 4C). To ascertain whether the decreased invasiveness observed upon silencing involved regulation of Rho GTPases activity, we used the G-LISA RhoA activation assay to analyze the activation state of RhoA in the transduced cells. The activity of RhoA was decreased in PDPN-silenced cells compared with MOCK cells and nontargeting shRNA-transduced cells (Fig. 4D). Thus, our results indicated a role for PDPN in invasiveness of glioblastoma cells.

Discussion

In this study, glioblastoma spheroid cultures were found to retain genetic hallmarks of human glioblastoma both at the

mRNA expression and DNA copy number levels, including for example amplification of the *EGFR* locus, which is seen in 30% to 40% of primary glioblastoma but rarely conserved in established cell lines grown under standard conditions (19). Furthermore, we report that genomic and expression profiling of glioblastoma spheroid cultures identified novel glioma-associated candidate genes that may be of clinical significance as prognostic factors. Our findings also imply that data obtained in primary cell cultures or established cell lines grown adherently in standard serum-containing medium, e.g., in studies aiming at the functional characterization of specific molecular aberrations or the preclinical evaluation of novel therapeutic reagents, should be interpreted with great caution because these standard cultures often do not genetically mirror the respective primary tumors (Fig. 1B, Supplementary Fig. S1). This assumption is also supported by a recent study investigating five of the most commonly used glioma cell lines (2) as well as two other studies (3, 4). The first study investigated the genotype and gene expression patterns of both serum-free-grown and serum-grown cultures derived from two glioblastomas. The two cultures grown under serum-free conditions harbored the genetic aberrations found within the primary tumors and recapitulated the gene expression patterns of human glioblastoma; by contrast, the two cultures derived from the same glioblastoma specimens but grown as monolayers in serum-containing media were not similar to the primary tumors. The second study investigated genomic profiles of serum-grown short- and long-term primary cultures and serum-free-grown spheroid cultures derived from seven glioblastomas. In seven of eight short-term cell cultures, the genomic profiles clustered further apart from the respective primary tumors than in spheroid cultures. An additional drawback of adherent primary cultures established in serum-containing medium is the frequent growth of nonneoplastic stromal cells, which in fact may overgrow the actual tumor cells in these cultures. This feature is the most likely explanation for the absence of any genomic aberrations detectable by array-CGH in

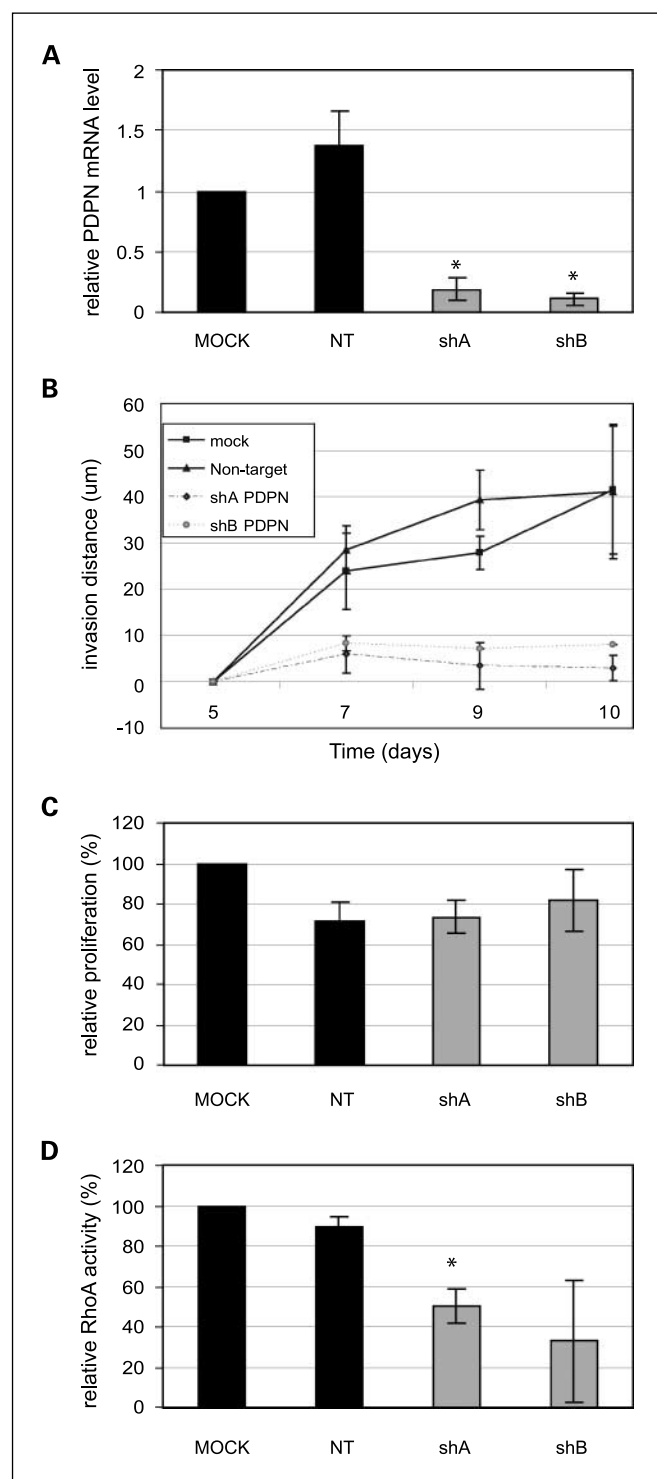


Fig. 4. A, PDPN expression was analyzed by quantitative real-time PCR in transduced glioblastoma spheroids 4 d after transduction. mRNA levels are shown as mean \pm SD of two independent experiments. B, invasive distance of transduced cells in collagen I gels was measured from day 5 to day 10 after transduction. Mean \pm SD for three to ten independent spheroids is shown. C, proliferation of glioblastoma spheroids after shRNA-mediated silencing of PDPN was assessed by the MTS assay 6 d after transduction. Error bars, SD between replicates. D, RhoA activity was assessed by the G-LISA RhoA activation assay 6 d after transduction. Mean \pm SD of two independent experiments is shown. NT, non-targeting shRNA; shA, shRNA A against PDPN; shB, shRNA B against PDPN (*, $P < 0.05$).

7 of 13 primary adherent cultures established in our study. One possible explanation for the appearance of new aberrations in adherent cultures during *in vitro* propagation might be a serum-induced higher rate of cell proliferation. In comparison, the genomic aberrations originally present in surgically resected glioma tissues are better preserved in spheroid cultures, which show a slower proliferation rate. Another potential explanation for the better preservation of genomic profiles in spheroid cultures would be the selection of a small subpopulation of cells that takes place due to serum-free conditions and thus preferentially allows for the highly malignant stem cell-like tumor cells to survive. Nevertheless, despite the high overall similarity of genomic profiles of the spheroid cultures to the original tumors, we observed higher rates of amplification of the *MYC* locus (8q24.21), as previously reported in breast, lung, and colon cancer (20). Although we cannot formally exclude that this amplification, which appeared between passage 5 and 16, might be induced by the culture conditions, it might very well be present only in a small population of cells from the original tumor. Interestingly, *MYC* is one of four factors necessary for the generation of induced pluripotent stem cells from adult human dermal fibroblasts and from adult mouse liver and stomach cells (21–23). In addition, *MYC* amplifications were frequently found within primitive/embryonal elements of central nervous system neoplasms with combined features of malignant glioma and primitive neuroectodermal tumor (24). *MYC* was also shown to be required for maintenance of glioma stem cells (25, 26). Thus, one may speculate that *MYC* amplification in the original tumor was restricted to the very small population of tumor-initiating cells, rendering it undetectable, and conferring to these cells a proliferative advantage in medium conditions favoring the growth of cancer stem-like cells.

A significant proportion of the genes that are highly expressed in spheroid cultures as compared with normal brain tissue have been previously reported as markers that distinguish between cultures showing a “full stem-like phenotype” and cultures having a more restricted differentiation capacity growing semiadherently (7). From nine genes showing high expression in the full stem-like phenotype cluster, five are among the most highly expressed genes in our data set (*NES*, *MAP2*, *BCAN*, *OLIG2*, *DLL3*). From the 13 genes associated with nervous system development overexpressed in this cluster, five (*PTPRZ1*, *DLL3*, *GPR56*, *MAP2*, *VANGL2*) were highly expressed in our glioblastoma spheroid cultures. In addition, nervous system development figured among the functions associated with the five top-ranked networks from IPA, in line with the specific expression of neurodevelopmental genes in the full stem-like phenotype cluster.

Of particular interest, we could identify a large overlap between the 38-gene signature associated with survival found by Aldape et al.¹⁰ and the most deregulated genes in our data set. Five genes of this signature (*S100A10*, *PDPN*, *EMP3*, *IGFBP2*, and *TNC*) are among the 45 most highly expressed genes in glioblastoma spheroids as compared with normal brain, and eight additional genes were either among the most highly (*TIMP1*, *COL1A2*, *IGFBP3*, *TAGLN2*, *SERPINE1*) or among the lowest expressed genes in our data set (*RTN1*, *GABBR1*,

¹⁰ K. Aldape, personal communication.

OMG). This overlap with a gene signature derived from expression profiling studies done in patients from four different data sets nicely underscores the validity of the glioblastoma spheroid culture model.

We also present evidence for a role of several novel candidate genes involved in glioma pathogenesis, whose mRNA and/or protein expression in glioblastoma patients is in line with our findings derived from the analysis of glioblastoma spheroids. *METTL7B* has not yet been reported as a potential oncogene overexpressed in glioblastoma. The role of *CDKN1A* in glioma and in stem cell function has been intensely investigated; however, our results provide first evidence suggesting *CDKN1A* expression as a putative marker to distinguish primary glioblastoma from secondary glioblastoma. Other genes overexpressed in spheroid cultures have been reported as oncogenes in various tumor entities. For example, *CENPF* may play a role in chromosome segregation during mitosis and was shown to be involved in esophageal squamous cell carcinoma, breast cancer, and Wilms' tumor (27–29). The transcript level was also previously shown to differ significantly between primary low-grade and recurrent high-grade gliomas (30).

Finally, we could show for three of the most aberrantly expressed genes in spheroid cultures that protein expression levels are associated with clinical outcome of glioma patients. *AJAP1* has been previously identified as a candidate tumor suppressor gene deleted in neuroblastomas (31), oligodendrogliomas (32), and ependymomas (33). High *AJAP1* expression is a significant independent prognostic factor for shorter OS in glioblastoma patients, as shown by multivariate analysis in our patient cohort. The *EMP3* gene has been implicated as a candidate tumor suppressor gene hypermethylated and/or aberrantly expressed in neuroblastomas as well as astrocytic and oligodendroglial gliomas (34, 35). However, primary glioblastomas mostly lacked *EMP3* hypermethylation and overexpressed *EMP3* transcripts. In line with these findings, our data confirm

at the mRNA level that *EMP3* is a marker distinguishing between primary and secondary glioblastoma. In addition, high *EMP3* expression was significantly associated with shorter OS in astrocytic glioma patients in univariate analysis, a finding related to the association of *EMP3* overexpression with primary glioblastoma. *PDPN* was proposed as a marker of malignant progression in astrocytic tumors (36). However, an association of *PDPN* expression with survival has not been established to date. Our data identified *PDPN* as marker to distinguish primary from secondary glioblastoma. Furthermore, univariate analysis of our patient cohort revealed an association between high *PDPN* expression and shorter OS of patients with astrocytic gliomas. However, similar to *EMP3*, multivariate analysis did not confirm *PDPN* as an independent prognostic factor, a finding likely again due to the association of *PDPN* overexpression with primary glioblastoma.

In conclusion, our study shows that the genomic alterations in glioblastoma spheroid cultures are similar to those detected in the respective primary tumor tissues, supporting these cultures as a valuable tool for the *in vitro* study of glioblastoma-associated pathomechanisms and the preclinical evaluation of novel therapeutic strategies. In addition, using this model system we identified a set of glioma-associated candidate genes that likely play a role in the molecular pathogenesis of glioblastoma and may represent novel prognostic biomarkers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Genomic and Expression Profiling of Glioblastoma Stem Cell –Like Spheroid Cultures Identifies Novel Tumor-Relevant Genes Associated with Survival

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