Evaluation of the Prognostic Value of CD44 in Glioblastoma Multiforme

KUO-CHEN WEI[1], CHIUNG-YIN HUANG[1], PIN-YUAN CHEN[1], LI-YING FENG[1], TAI-WEI ERICH WU[1], SHU-MEI CHEN[1], HONG-CHIEH TSAI[1], YU-JEN LU[1], NGAN-MING TSANG[2], CHEN-KAN TSENG[2], PING-CHING PAI[2] and JYH-WEI SHIN[3]

Departments of [1]Neurosurgery, and [2]Department of Radiation Oncology, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Taoyuan, Taiwan; [3]Department of Parasitology, National Chung Kung University, Tainan, Taiwan, R.O.C.

Abstract. *Background/Aim: Glioblastoma and astrocytoma are the most common brain tumors affecting adults 45-60 years of age. The poor prognosis for glioblastoma patients results from recurrence after treatment. There is therefore an urgent need to develop diagnostic and prognostic markers as well as new therapies. Patients and Methods: Microarray analyses of clinical specimens from glioblastoma patients were used to identify potential tumor markers. Expression of candidate genes was analyzed by real-time reverse transcription-polymerase chain reaction and by immunoblotting and immunohistochemistry. Results: Five potential markers (CD44 antigen (CD44), growth arrest and DNA-damage-inducible, alpha (GADD45A), fibronectin 1 (FN1), CD63 antigen (CD63) and secreted phosphoprotein 1 (SPP1)) showed expression patterns that correlated significantly with malignant glioma. In particular, expression of the CD44 antigen was elevated in more severe tumor types, and higher in tumor cores than in peripheral regions. However, lower levels of CD44 expression surprisingly correlated with lower survival. Conclusion: The CD44 antigen is a promising candidate for further development as a prognostic and therapeutic tool.*

Gliomas are the most common of the primary brain tumors; in the United States, they account for approximately 78% of the new cases of primary malignant brain and central nervous system (CNS) tumors diagnosed annually (1, 2). Glioblastoma multiforme (GBM) is the most common and aggressive glioma, with the poorest survival. It is a highly malignant brain tumor, typically affecting adults between 45 and 60 years of age. Although surgical techniques, radiotherapy and chemotherapy have improved, the prognoses of patients with glioblastomas are still poor (~1 year), largely because of the spread of tumor cells to other regions of the brain.

Gliomas are classified by the World Health Organization (WHO) into four major grades according to their histopathological features: pilocytic astrocytomas (grade I), low-grade astrocytomas (grade II), anaplastic malignant astrocytomas (grade III) and GBM (grade IV). These classifications are based on phenotypic changes and biological behaviors in cells and tissues, each of which reflect genes operative in neoplastic development. Establishing the molecular basis for these phenotypic changes is crucial to refining current therapies and developing new ones. It has been suggested that gene expression profiles from glioma patient specimens might predict patient outcome more accurately than pathological criteria (3, 4). This study used bioinformatics to identify potential prognostic markers in clinical specimens from patients with malignant glioma.

Correspondence to: Kuo-Chen Wei, Department of Neurosurgery, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, No. 5, Fu Shin Street, Kweishan, Taoyuan, Taiwan, R.O.C. Tel: +88633281200, ext. 2412, Fax: +88633285818, e-mail: kuochenwei@adm.cgmh.org.tw and Jyh-Wei Shin, Department of Parasitology, National Chung Kung University, No.1, University Road, Tainan, Taiwan, R.O.C. Tel: +88662353535, ext 5611, Fax: +88662387614, e-mail: hippo@mail.ncku.edu.tw

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Materials and Methods

Patient population. This study was approved and performed according to the guidelines of the Institutional Review Board of Chang Gung Memorial Hospital (approval #94-182). Written, informed consent was obtained from all patients. The patient population consisted of 73 adults whose gliomas or supratentorial GBM were verified histologically between May 2003 and April 2006 (Table I). Histopathological examinations were performed by a neuropathologist according to WHO criteria. Tumors showing prominent microvascular proliferation and/or necrosis, in addition to high cellularity, marked nuclear atypia, and remarkable mitotic activity were diagnosed as GBM.

Region-specific specimen collection. Deep-seated tumors were removed using an intraoperative navigation system (BrainLAB, Feldkirchen, Germany) that minimized invasiveness and maximized

Table I. *Patients' clinical data.*

	Glioblastoma multiforme	Glioma		
		Grade 2	Grade 3	Other grade 4[a]
Gender				
Subtotal	52	6	10	5
Male	33	4	7	2
Female	19	2	3	3
Age at diagnosis (years)				
<40	16	2	3	4
40-50	9	4	2	0
50-60	8	0	3	0
>60	19	0	2	1
Age at sampling (years)				
<40	14	1	3	3
40-50	10	4	1	1
50-60	9	1	4	0
>60	19	0	2	1
Disease status				
Primary	20	1	5	4
Recurrent	23	5	5	1

[a]Including anaplastic oligodendroglioma, giant cell glioma and malignant oligoastrocytoma.

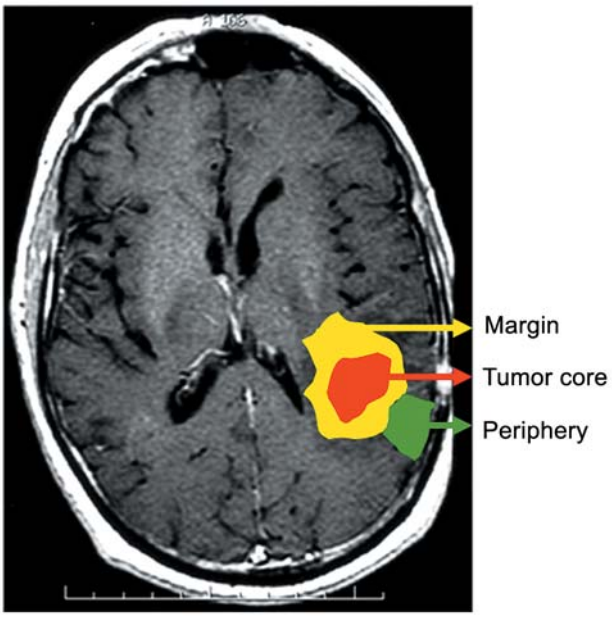

Figure 1. *MRI of representative tumor, indicating sampling areas (periphery, margin and core).*

patient safety and accurate tumor resection. Brain tissue samples were collected from the resection zone, categorized as peripheral normal brain, tumor marginal tissue or tumor core (according to the navigators' assessment; Figure 1a), and stored in liquid nitrogen.

Preparation of fluorescently labeled cDNA probes. Twenty μg of total RNA extracted from tumor samples were mixed with 2 μg of oligo-dT primer in a total volume of 13 μl, heated to 70˚C for 10 min, and cooled on ice. To this mixture was added 1.5 μl of 20× nucleotide cocktail (10 μM each 2'-deoxyadenosine 5'-triphosphate, 2'-deoxycytidine 5'-triphosphate, 2'-deoxyguanosine 5'-triphosphate, and 6 μM 2'-deoxythymidine 5'-triphosphate), 3 μl of either Cy3-2'-deoxyuridine 5'-triphosphate (dUTP) or Cy5-dUTP (GE Healthcare Bio-Sciences, Piscataway, NJ, USA), 6 μl of 5× first-strand buffer, 3 μl of 0.1 M DL-Dithiothreitol, 0.1 μl of RNAguard (GE Healthcare), and 2 μl of 200 units/ml Superscript II reverse transcriptase (Invitrogen, Taipei City, Taiwan, ROC). After 2 h at 42˚C, RNA strands were degraded by adding 5 μl of 0.5 N NaOH and incubation for 10 min at 70˚C. Samples were neutralized by adding 7.5 μl of 1 M Tris-HCl (pH 7.5). The separately synthesized Cy3- and Cy5-labeled targets were combined and mixed with 20 μg of human Cot-1 DNA (Invitrogen) in a final volume of 500 μl. Target probes were purified by centrifugation using a Microcon YM-30 filtration unit (Millipore, Billerica, MA, USA), and concentrated to a final volume of 5 μl.

DNA microarray hybridization. Whole human genome (GE Healthcare) and a customized oncogene and kinase cDNA microarray consisting of 15,488 probes (National Cheng Kung University Microarray Center, Tainan, Taiwan, ROC) were used in this study. Four sets of brain tissue were analyzed: three from glioblastoma tissues and one from trauma tissue (*i.e.* control). A total of 2.5 μl of 10 μg/ml poly(A) RNA (Roche Applied Science, Indianapolis, IN, USA), 0.5 μl of 10% SDS, 3 μl of 20× PM (0.1% BSA, 1% SDS), 15 μl of formamide, and 3 μl of 20× SSC (1.5 M NaCl, 150 mM sodium citrate, pH 7.0) were added to each concentrated target, and the volume adjusted to 30 μl with distilled water. Target mixtures were denatured for 2 min at 100˚C, incubated in the dark for 20–30 min at room temperature, and then placed on the array under a 24 × 40-mm cover glass. Hybridizations were performed for 16 h at 42˚C in a humidified chamber. After hybridization, arrays were washed at room temperature in 2xSSC with 0.l% SDS for 10 min, 0.1× SSC and 0.l% SDS for 10 min, and 0.1× SSC for 5 min. All data were calibrated against commercial human RNA (Clontech, Mountain View, CA, USA).

Scanning and data processing. Washed arrays were scanned using a GSI Lumonics ScanArray 4000 (Packard BioChip Technologies, Billerica, MA, USA). The data (16-bit TIFF images) were analyzed using QuantArray software (PerkinElmer, Waltham, MA, USA), converting the signal intensity of each spot into a text format. The background (*i.e.* the average intensity of blank spots) was subtracted. Cut-off values for each experiment were set at 5-9-fold of the lowest signal intensity, leaving ~3,000 data points with relatively high signal intensities. For each sample, data points affected by experimental artifacts were eliminated by log transforming the Cy3/Cy5 ratios and subtracting the median of all log (Cy3/Cy5) values from each log (Cy3/Cy5) value. For each gene, variances among the samples were calculated. Genes with more than three missing data values were excluded from further analyses. Potential protein–protein interactions between candidate gene products were analyzed using MetaCore™ software (GeneGo, St. Joseph, MI, USA).

Table II. *Genes significantly regulated in glioblastoma multiforme.*

Gene	Symbol	Accession #	Position	Locus	OMIM	Unigene
CD44 antigen (homing function and Indian blood group system)	*CD44*	NM_000610	11p13	960	107269	Hs.306278
CD63 antigen (melanoma 1 antigen)	*CD63*	NM_001780	12q12-q13	967	155740	Hs.445570
Fibronectin 1	*FN1*	NM_002026	2q34	2335	135600	Hs.418138
Growth arrest and DNA-damage-inducible, alpha	*GADD45A*	NM_001924	1p31.2-p31.1	1647	126335	Hs.80409
Secreted phosphoprotein 1 (osteopontin; bone sialoprotein I; early T-lymphocyte activation 1)	*SPP1*	NM_000582	4q21-q25	6696	166490	Hs.313

Real-time polymerase chain reaction. The following primers and probe for CD44 were used (Applied Biosystems, Foster City, CA, USA): forward: 5'-CAACTCCATCTGTGCAGCAAA-3'; reverse: 5'-GTAACCTCCTGAAGTGCTCGTC-3'; probe: 5'-CATATTGC TTCAATGCTTCAG CTCCACCTG-3'. Other probes included CD63 antigen (CD63; Hs00156390_m1), fibronectin 1 (FN1; Hs01549940_m1), growth arrest and DNA-damage-inducible, alpha (GADD45A; Hs00355052_g1) and secreted phosphoprotein 1 (SPP1; Hs00959010_m1). Eukaryotic 18S rRNA (Hs99999901_s1) was used as an endogenous control. Real-time PCR cycles were performed as follows: 50˚C for 2 min; 95˚C for 15 min; forty cycles at 95˚C for 15 s and 60˚C for 1 min. Experiments were performed in triplicate. Gene expression levels were calculated by the ΔΔ Ct method and normalized against the 18S control.

Immunoblotting analysis. Brain tumor samples were washed twice in ice-cold PBS and lysed on ice in ice-cold T-PER tissue protein extraction reagent (Pierce, Rockford, IL, USA) containing protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Lysates were cleared by centrifugation, and total protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA, USA). Protein samples (10 μg/lane) were separated on 12% polyacrylamide gels by SDS–PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Blots were blocked overnight in TBS (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, 0.5 μM EDTA, pH 7.4) containing 5% nonfat dry milk, incubated for 2 h with anti-human CD44 antibodies (1:50,000; R&D Systems, Abingdon, UK), and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:20,000; PerkinElmer) for 1 h. Human CD44 was detected using the Western Lightning kit (PerkinElmer) according to the manufacturer's instructions.

Immunocytochemistry. Tissue sections from peripheral, marginal and tumor core regions were deparaffinized, treated with 3% H_2O_2 for 10 min at room temperature, and then microwaved in 0.01 M citrate buffer (pH 6.0) to retrieve antigenicity. The sections were blocked with 1% BSA in PBS for 20 min at room temperature and incubated overnight with a goat anti-human CD44 monoclonal antibody (1:100 dilution in PBS containing 1% BSA). Samples were washed four times with PBS and incubated with goat anti-mouse IgG (PerkinElmer). Immunocomplexes were visualized by the LSAB 2 HRP kit (Dako, Carpinteria, CA, USA) using 3,3'-diaminobenzidine tetrachloride as a substrate. Sections were counterstained lightly with hematoxylin, dehydrated with a graded alcohol series, cleared with xylene, and mounted with coverslips.

Statistical analyses. All data were analyzed using SPSS statistical software, v. 13.0. Gene expression in different tumor regions was analyzed using nonparametric methods for paired samples. Differences between low and high gene expression groups were evaluated using the log rank test; the Spearman correlation coefficient was used to estimate the significance of the correlation. Kaplan–Meier survival curves were used for univariate survival analyses. Cox regression was also used in univariate and multivariate models with adjustments for age.

Results

This study analyzed four sets of microarray data: three from glioblastoma tissues and one from trauma tissue using a 15,488-probe array. Genes not present in both replicates or that showed discrepancies in both intra- and interarrays replicates were filtered out. After data trimming, 4,191 genes were harvested for further analysis. Among the genes with altered expression levels, 1,914 genes were up- or down-regulated more than onefold, and 154 genes more than twofold. The significantly altered genes were processed for the protein-protein interaction analysis. Our result revealed five significantly altered genes were involved with related networks and were selected for further analysis (Table II).

To validate the prognostic utility of the selected genes with respect to GBM, eighteen sets of tissue samples were collected from different brain regions (Figure 1) using an intraoperative navigation system. Expression of each of the candidate genes was up-regulated significantly in tumor core samples, although expression decreased progressively the more distally the samples were taken (Figure 2). Further analysis indicated possible protein–protein interactions between CD44 and the other candidate genes. Immunoblots confirmed intense CD44 protein expression in malignant gliomas (*e.g.* astrocytoma, GBM), consistent with the elevated transcript levels. Furthermore, immunohistochemistry verified the graded distribution of the protein in the tumors (Figure 3).

The lower levels of CD44 protein expressed in grade 3 gliomas and in normal peripheral regions suggested a correlation between CD44 and glioma malignancy. CD44 expression was up-regulated significantly in grade IV GBM relative to lower grade gliomas and nontumor controls ($p<0.05$). Although CD44 expression did not differ significantly between glioma grades II and III, both had levels significantly higher than those of nontumor controls (Figure 4a).

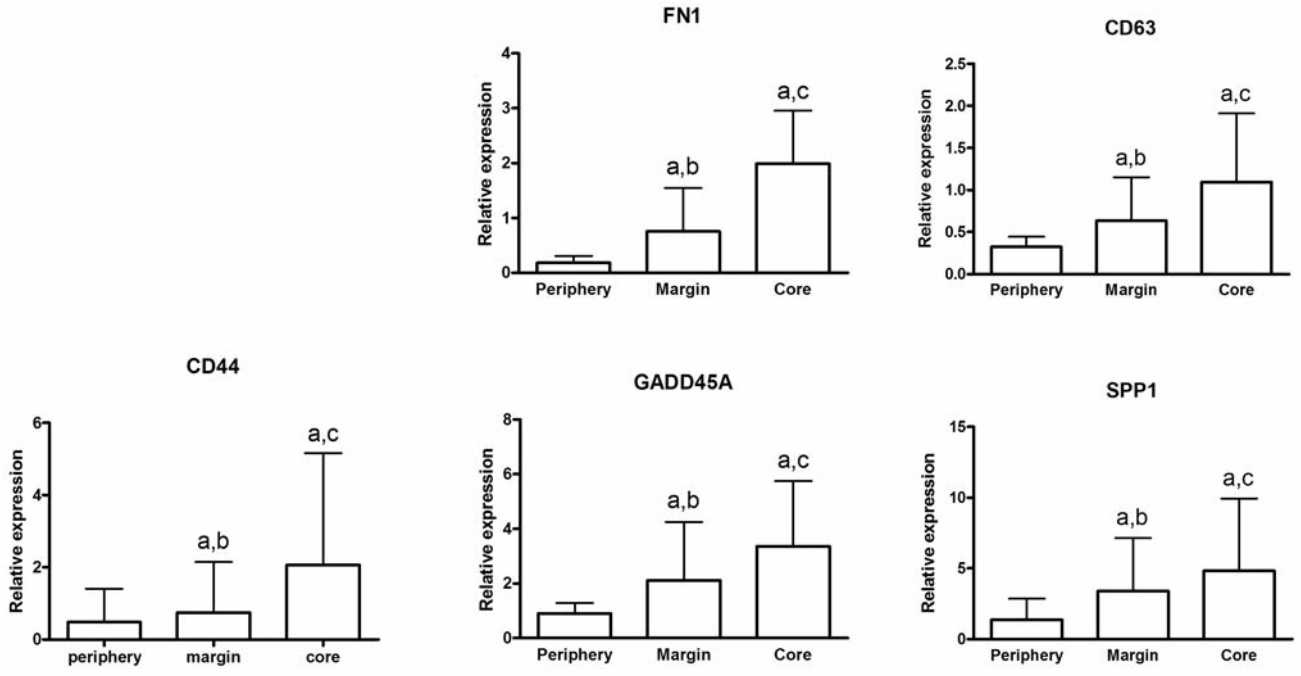

Figure 2. *Transcription levels of candidate genes in samples from GBM patients' brains were validated by real-time PCR. Data were analyzed by the two-related-sample nonparametric test. a: p<0.05 relative to periphery; b: p<0.05 relative to core; c: p<0.05 relative to margin, data were presented as mean with standard derivation.*

Based on the putative correlation between CD44 and glioma malignancy, CD44 transcription was analyzed with respect to its value as a prognosticator of survival. Kaplan–Meier survival plots indeed showed a relationship between CD44 expression and survival rates in GBM patients. Surprisingly however, patients with higher levels of CD44 transcripts actually had a better prognosis (p=0.027) (Figure 4b). Intriguingly, higher CD44 transcript levels also correlated with significantly improved survival in 23 patients with recurrent GBM (p=0.0196) (Figure 4c).

Discussion

Establishing the factors responsible for the development, metastasis and recurrence of brain tumors can help identify potential diagnostic markers and targets for new therapeutic regimens. This study identifies five potential glioblastoma tumor biomarkers: CD44, CD63, FN1, GADD45A, and SPP1. The expressions of all these genes are elevated in brain tumors, and all are involved in important cell functions that might contribute to tumorigenesis (5-9). Further studies are needed to evaluate the potential of these genes as proper tumor biomarkers. CD44 in particular is a widely distributed transmembrane cell-surface adhesion protein involved in many physiological and pathological processes, including matrix adhesion, lymphocyte homing and activation, wound healing, growth promotion, cell survival and migration, and tumor growth and metastasis (10-12). CD44 also mediates interactions between the extracellular matrix and the intracellular cytoskeleton and interacts with growth factors and matrix metalloproteases, triggering signal pathways that can induce tumor cell invasion and suppression of apoptosis (13).

Alterations of CD44 expression have been reported in several tumor types, including breast cancer, soft tissue sarcoma (14) and neuroblastoma (15). Nevertheless, the results regarding the role of CD44 in neoplasia are contradictory. CD44 expression is up-regulated in colorectal cancer (16), but its down-regulation correlates with metastatic progression in prostate cancer (17). Similarly, overexpression of CD44 suppressed metastasis and invasiveness in some studies (18, 19), whereas in others, migration and invasion were decreased by its suppression (20). Clearly, although CD44 might play important roles in malignant neoplasia and metastasis, the precise nature of the mechanisms involved can vary with the tissue and/or pathology. The data obtained in the present study indicate that CD44 is expressed, both transcriptionally and translationally, at higher levels in higher-grade brain tumors. Furthermore, CD44 is expressed more abundantly in the tumor core than in marginal and peripheral regions. Thus, CD44 expression correlates not only with the degree of malignancy, but also with the density of malignant cells in tumor-affected regions.

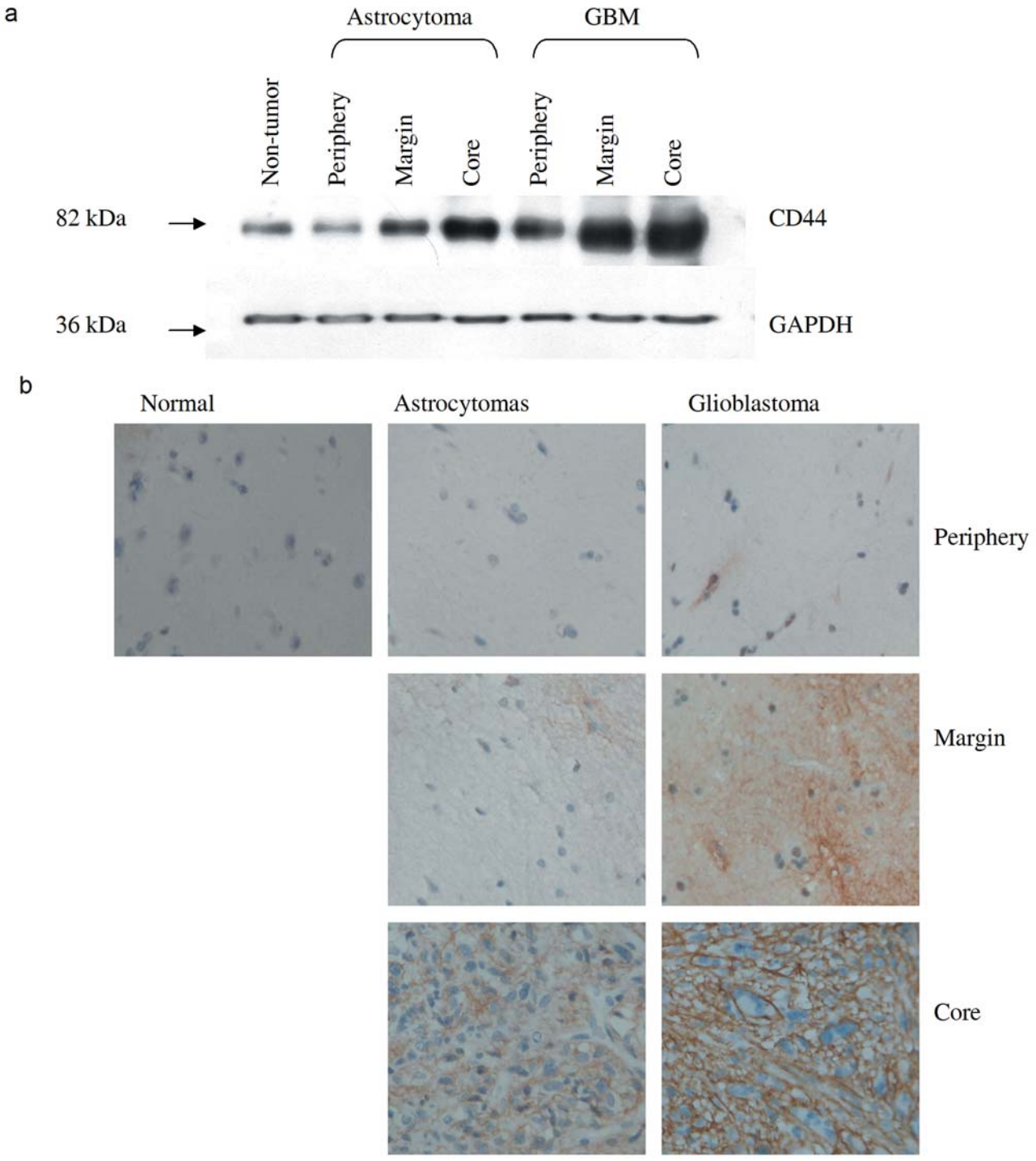

Figure 3. *CD44 protein expression in surgically defined regions was determined using antiCD44 monoclonal antibodies. a, Immunoblots of tissue samples from defined regions. b, Immunohistochemical staining of glioma tissues from defined regions.*

There are a number of potential mechanisms by which CD44 might promote malignancy. Malignant cells are thought to acquire motility by interacting with extracellular matrix components through specific receptors and involvement of the cytoskeleton *via* signal transduction (21). CD44 modulates adhesiveness, motility, matrix degradation, proliferation and cell survival, all factors that could allow a tumor to initiate a metastatic cascade (14). Specifically,

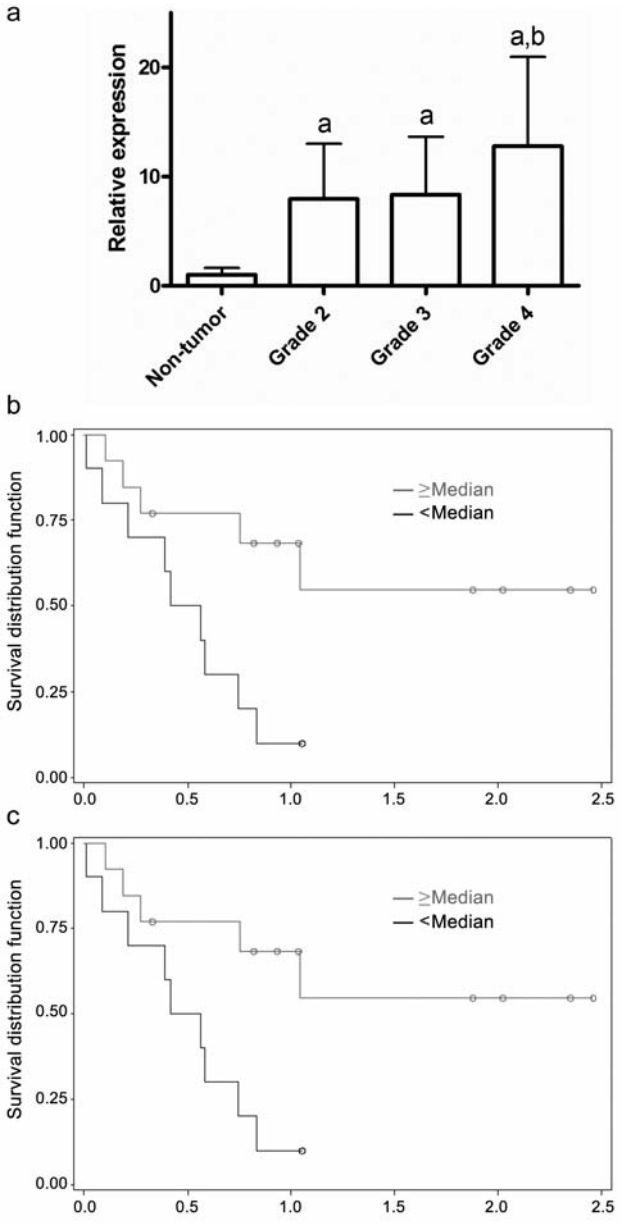

Figure 4. *a, Real-time PCR analysis of CD44 expression in samples from different grades of gliomas. a: p<0.05 relative to non-tumor; b: p<0.05 relative to grade 2 and grade 3 gliomas. b, Overall survival of 43 grade 4 glioma patients. The median CD44 expression was used as a cut-off value, p=0.0116, (Hazard ratio, HR=0.367; 95% confidence interval, CI=0.164-0.823). c, Overall survival of 21 patients with recurrent grade 4 glioma. Median CD44 expression was used as a cut-off value, p=0.0445, (HR=0.333; 95% CI=0.109-0.333).*

CD44 binding to several extracellular matrix components (including hyaluronic acid, collagen, fibronectin and osteopontin) could facilitate the arrest and subsequent growth of CD44-expressing tumor cells at secondary sites (22). Indeed, in the present study, transcription of both FN1 and SPP1 was up-regulated in the microarrays; expression of these genes might work in conjunction with CD44 to promote metastasis. Thus, the up-regulation of CD44 in the margins of malignant gliomas might be indicative of tumor cell growth and migration.

CD44 may be involved in other mechanisms as well. For example, the existence of cancer stem cells in various neoplasms is widely accepted (23), and brain tumor stem cells are involved in tumor progression (24, 25). Furthermore, CD44 is required for mesenchymal stem cell migration (26) and leukemic stem cell homing (27). Thus, the gradual increase in CD44 expression from the periphery to the tumor core might be indicative of homing signals guiding stem cells to the tumor region. Both the metastatic and homing mechanisms facilitate the progress of malignant brain tumors and therefore could contribute to the poor prognosis of GBM patients.

Despite the apparent involvement of CD44 in malignant gliomas, the utility of CD44 expression as a diagnostic tool or prognostic indicator remains debatable. In colorectal cancer, expression of standard and variant forms of CD44 were poor prognosticators (28). However, reduced CD44 expression correlated with poor survival in patients with several cancer types (14, 29-31), and in the present study, in GBM patients. These results are somewhat surprising: Given the possible roles for CD44 discussed above, one might expect that patients expressing higher levels of CD44 would be at higher risk. One mechanism that might account for this apparent contradiction is the resistance of brain cancer cells to chemotherapeutic alkylating agents (*e.g.* 1,3-*bis*(2-chloroethyl)-1-nitrosourea, temozolomide). GBM patients receiving such drugs often have an initial positive response, but tumors eventually recur. Intriguingly, the expression of CD44 is significantly lower in GBM cells resistant to such alkylating agents (32). Thus, although higher levels of CD44 expression are seen in more severe tumors, lower CD44 levels could indicate that the malignant cells are more resistant to chemotherapeutic agents. Hence, lower levels of expression might correlate with a greater likelihood of recurrence and consequently a worse outcome overall. This is supported to some degree by the observation that patients with recurrent GBM have better survival outcomes if their CD44 expression levels are higher. Thus, although the present study establishes the utility of CD44 expression as a useful prognostic indicator with respect to GBM, the mechanisms by which this gene influences tumor progression remain the subject of future studies.

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