Abstract

Purpose: More brain tumor markers are required for prognosis and targeted therapy. We have identified and validated promising molecular therapeutic glioblastoma multiforme (GBM) targets: human transmembrane glycoprotein nonmetastatic melanoma protein B (GPNMBwt) and a splice variant form (GPNMBsv, a 12-amino-acid in-frame insertion in the extracellular domain).

Experimental Design: We have done genetic and immunohistochemical evaluation of human GBM to determine incidence, distribution, and pattern of localization of GPNMB antigens in brain tumors as well as survival analyses.

Results: Quantitative real-time PCR on 50 newly diagnosed GBM patient tumor samples indicated that 35 of 50 GBMs (70%) were positive for GPNMBwt+sv transcripts and 15 of 50 GBMs (30%) were positive for GPNMBsv transcripts. Normal brain samples expressed little or no GPNMB mRNA. We have isolated and characterized an anti-GPNMB polyclonal rabbit antisera (2640) and two IgG2b monoclonal antibodies (mAb; G11 and U2). The binding affinity constants of the mAbs ranged from 0.27 × 10^8 to 9.6 × 10^8 M^-1 measured by surface plasmon resonance with immobilized GPNMB, or 1.7 to 2.1 × 10^8 M^-1 by Scatchard analyses with cell-expressed GPNMB. Immunohistochemical analysis detected GPNMB in a membranous and cytoplasmic pattern in 52 of 79 GBMs (66%), with focal perivascular reactivity in 27%. Quantitative flow cytometric analysis revealed GPNMB cell surface molecular density of 1.1 × 10^4 to 7.8 × 10^4 molecules per cell, levels sufficient for mAb targeting. Increased GPNMB mRNA levels correlated with elevated GPNMB protein expression in GBM biopsy samples. Univariate and multivariate analyses correlated expression of GPNMB with survival of 39 GBM patients using RNA expression and immunohistochemical data, establishing that patients with relatively high mRNA GPNMB transcript levels (wt+sv and wt), >3-fold over normal brain, as well as positive immunohistochemistry, have a significantly higher risk of death (hazard ratios, 3.0, 2.2, and 2.8, respectively).

Conclusions: Increased mRNA and protein levels in GBM patient biopsy samples correlated with higher survival risk; as a detectable surface membrane protein in glioma cells, the data indicate that GPNMB is a potentially useful tumor-associated antigen and prognostic predictor for therapeutic approaches with malignant gliomas or any malignant tumor that expresses GPNMB.
cytotoxins or radioisotopes, antibodies that are developed against cell surface or extracellular matrix antigens with tumor-restricted distribution can offer a more specific delivery system for cytotoxic reagents resulting in a high degree of selective destruction of tumors. The search for appropriate brain tumor-associated antigens has been a key challenge for immunotherapeutic approaches to central nervous system neoplasia (4–8). Development and progression of giall tumors arises as the result of accumulation of multiple genetic alterations in a single cell. As a result, malignant gliomas are composed of heterogeneous populations of cells both in genotype and phenotype (9,10). Therefore, glioma cells exhibit a wide variation in antigenic profile even within each individual tumor (11). Because there is no single gene product that was reported overexpressed in all GBM cases, success in mAb-based therapy for GBM will require the identification of a panel of glioma-associated antigens and the proper selection of target-specific mAbs for each patient.

Recent advances in the development of comprehensive molecular analysis tools for genome and gene expression provide a basis to discover novel target molecules with tumor-specific distribution (12). In an attempt to identify novel glioma-associated antigens, we have previously reported several genes that are preferentially expressed in GBMs by the serial analysis of gene expression method (13). Among these candidate GBM marker genes, glycoprotein nmb (GPNMB) showed a >10-fold induction of mRNA expression over normal brain samples in 5 of 12 GBM cases (13).

GPNMB is a type I transmembrane protein that was isolated from a subtractive cDNA library based on differential expression between human melanoma cell lines with low and high metastatic potential in nude mice. GPNMB mRNA was expressed at high levels in low-metastatic melanoma cell lines and xenografts (14). The human GPNMB gene encodes a predicted 560 amino acid protein, the deduced amino acid sequence of which shows that GPNMB is made up of three domains, a long extracellular domain (ECD) preceded by a signal peptide, a single transmembrane region, and a relatively short cytoplasmic domain (Fig. 1). The human GPNMB amino acid sequence had homology of 71.1% to DC-HIL (15), 69.8% to osteoactivin (16), 56% to the precursor of pMel 17 (17), and 51% to QNR-71 (18). The human GPNMB gene was localized to human chromosome 7q15 (National Center for Biotechnology Information Unigene Cluster Hs.82226 GPNMB), a locus involved in the human inherited disease cystoid macular dystrophy. Recently, Bachner et al. (19) suggested that human GPNMB may be a candidate gene for the dominant cystoid macular edema because they found high expression of murine GPNMB mRNA within the retinal and iris pigment epithelium. The function of GPNMB has not been fully described, and paradoxical effects have been noted in transfection studies. Transfection of our in vitro minimally transformed human fetal astrocyte line THRG (20) with GPNMB cDNA altered the phenotype of both s.c. and intracranial tumors growing in athymic mice from a minimally invasive to a highly invasive and metastatic phenotype. Conversely, transfection of a partial GPNMB cDNA into a highly metastatic melanoma cell line resulted in slower s.c. tumor growth and also in reduction of the potential for spontaneous metastasis in nude mice (14).

If the overexpression of GPNMB RNA by gliomas and lack of expression in normal brain is reflective of GPNMB protein expression, GPNMB, as an integral membrane protein, could be an important target for immunotherapy. Moreover, there is evidence that GPNMB may be involved in the invasive malignant phenotype of gliomas, making evaluation of GPNMB-expressing cells important (21). In our previous analysis, we could not detect GPNMB transcripts in four normal brain cortex samples, two whole brain samples, or one sample each of cerebellum, spinal cord, heart, kidney, lung, trachea, tonsil, or bone marrow (13). We have therefore proposed to investigate the suitability of this GPNMB marker as a glioma therapeutic target.

In this study, we have done genetic and immunohistochemical evaluation of human gliomas to determine the incidence, distribution, and pattern of localization of GPNMB antigens in brain tumors. To evaluate the therapeutic potential of GPNMB as a GBM-associated antigen, a series of 50 newly diagnosed GBM biopsy specimens were examined for GPNMB RNA transcript levels by real-time reverse transcription-PCR (RT-PCR). In addition, a larger panel of 79 newly diagnosed GBM cases, including 39 of the 50 samples in the mRNA study, was assessed for GPNMB protein expression by immunohistochemical analysis, and survival analyses were done based on these two variables. We have shown that significant numbers of GBMs overexpress GPNMB at the mRNA and protein levels, and that expression levels correlate with poorer prognosis for those patients. We conclude that therapeutic strategies designed to target GPNMB may be successful in the treatment of malignant glioma.

**Materials and Methods**

**Cell lines, cell transfection, and tumor xenografts.** Human malignant glioma–derived cell lines D54MG, D245MG, D247MG, and D392MG were established and maintained in our laboratory, whereas the glioma cell lines U87MG, T98G (MG), and U251MG, and the melanoma cell lines SK-Mel-28, A375, C32, and Malme-3M were obtained from the American Type Culture Collection (Manassas, VA). Malignant glioma and melanoma cell lines were grown in Zinc Option medium supplemented with 10% fetal calf serum. The propagation, storage, and testing of these cell lines to ensure the absence of HeLa cell
contamination, inter- or intra-cell line contamination, or Mycoplasma infection have been published previously (22). The THRG cell line (20, 21), a genetically defined human fetal astrocyte line transfected to express GPNMB, was used as the positive control cell line for GPNMB expression. Human malignant glioma xenografts D256 MGX, D270 MGX, D320 MGX, D456 MGX, and D2159 MGX were established and maintained in our laboratory; the malignant glioma xenografts 684-7 and 12A-7 were generous gifts from Dr. David James (Mayo Clinic, Rochester, MN).

For transient expression experiments, the ECD of GPNMB (nucleotides 1-1,458), adenosine of the start codon as 1) was ligated into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA). The resulting pcDNA3.1-GPNMBEC, was introduced into HEK293 cells by using the LipofectAMINE 2000 reagent (Invitrogen), and transiently transfected cells were harvested 48 hours later. To generate stable GPNMB transformants, the EcoRI-XhoI fragment of the pW77 vector containing the full-length GPNMB cDNA (a generous gift from H.P. Bloemers; ref. 14) was cloned into the retroviral vector pBabeBleo or pBabeNeo (23). The glioma cell lines D247MG, D54MG, and U251MG were infected with retrovirus as described previously (21), and stably transfected clones were selected in medium containing 200 μg/ml zeocin (Invitrogen) or 1 mg/ml genetin (Invitrogen).

cDNA cloning of GPNMB from glioma cells. For cDNA cloning of the GPNMB gene from human glioma cells, mRNA was isolated from D392MG cells by using the FastTrack 2.0 kit (Invitrogen). After cDNA synthesis using random primers and SuperScript II RNaseH− Reverse Transcriptase (Life Technologies, Rockville, MD), the DNA fragment encoding the ECD of GPNMB was amplified by one cycle of 95°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute with the following primers:

GPNMBEC FWD (sense): 5'-ATGGAACTGCTCTACTAT-3′
GPNMBEC REV (antisense): 5'-GTITTCATCCTTIAAGG-3′

The PCR products were further subcloned into pCR2.1TOPO vector (Invitrogen) and sequenced on both strands by using ABI377 automatic sequencer (Applied Biosystems, Foster City, CA).

Quantitative real-time RT-PCR assay. Total RNA was isolated from subconfluent cultured cells and sample tissues using the RNAeasy Mini kit (Qiagen, Valencia, CA), and then treated with RNase-free DNase I (Ambion, Austin, TX). Total RNA sample of normal adult whole brain was purchased from Clontech (Palo Alto, CA). Total RNA (0.2 μg) was converted to cDNA with random primers and SuperScript II RNaseH− Reverse Transcriptase (Life Technologies, Rockville, MD), the DNA fragment encoding the ECD of GPNMB was amplified by one cycle of 95°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute with the following primers:

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Relative GPNMB mRNA levels were expressed in terms of fold induction rate over control normal whole brain sample, which was determined by dividing the GPNMB/b-actin ratio of tumor sample by that of normal whole brain. All measurements were done in triplicate and the experiments were repeated twice.

Recombinant protein preparation and exoglycosidase treatment. The ECD of GPNMB protein, GPNMBEC, was produced with a hexa-histidine tag at the carboxy terminus. The extracellular segment of GPNMB (nucleotides 64-1,458), excluding the signal peptide region, was cloned into a T7-based prokaryotic expression vector (25). GPNMBEC was expressed in Escherichia coli BL21-CodonPlus (DE3) RIL (Stratagene, La Jolla, CA) as inclusion bodies. After dissolution in a buffer containing guanidine, GPNMBEC was purified with an Ni-NTA column (Qiagen) and renatured as described previously (26).

To produce GPNMBEC protein in the baculovirus system, the same DNA fragment was fused with baculoviral vector pVL1393 (BD PharMingen, San Diego, CA). After infecting High Five insect cells with high titer viral stock, GPNMB protein was purified from culture supernatant with a Ni-NTA column according to instructions of the manufacturer.

For exoglycosidase digestion, total cell lysates of 20 μg protein were denatured by boiling at 100°C for 10 minutes with 0.5% SDS, and then incubated with 10,000 units/ml of the peptide N-glycosidase F (New England Biolab, Beverly, MA) in the presence of 1% NP40 at 37°C for 2 hours. Samples were subjected to SDS-PAGE and immunoblotting to determine change in molecular mass.

Immunization. Immunization protocols used an initial DNA immunization with the plasmid vectors encoding the ECD of GPNMB, followed by boosting with the corresponding recombinant protein produced in bacteria. Rabbits were given a primary s.c. immunization with 250 μg mammalian expression vector pcDNA3.1-GPNMBEC or were immunized with 250 μg bacterial recombinant GPNMBEC protein emulsified 1:1 in Freund’s complete adjuvant; this was followed by twice boosting with 250 μg GPNMBEC protein + Freund’s incomplete adjuvant at 4-week intervals. Rabbits were bled 12 days after the last boost. Titers in serum were monitored by ELISA with GPNMBEC as the capture antigen and live-cell ELISA using GPNMBEC-expressing D54MG cells as targets.

For mice, the first immunization protocol (called “B”) was a combination protocol using plasmid DNA for initial immunizations, followed by purified, bacterially expressed GPNMB protein later in the immunization protocol, because titers insufficient for fusion were obtained after plasmid DNA immunization alone. On days 1, 49, and 77, BALB/c, C3H/He, and C57Bl/6 mice received 15 μg GPNMB encoding plasmid DNA intradermally; 50% end-point titers versus GPNMB protein determined with serum obtained on day 89 were <1/1,000. The mice then received 30 μg GPNMB protein + Freund’s
complete adjuvant on day 156 and a similar boost in Freund’s incomplete adjuvant on day 176. The 50% end-point serum titers versus source of GPNMB determined on day 188 were in excess of 1/5,000. Following a minimum interval of 30 days, BALB/c recipients were boosted i.p. with 5 μg protein, and spleens were harvested for fusion 3 to 4 days later. From the fusion using protocol B, mAb G11 was obtained. The second immunization protocol (UI) consisted solely of protein immunization: day 1, 30 μg GPNMB + Freund’s complete adjuvant; days 21, 42, and 63, 15 μg protein + Freund’s incomplete adjuvant. The 50% end-point serum titers obtained on day 74 were in excess of 1/10,000. On day 105, C57BL/6 recipients were boosted i.p. with 5 μg protein, and spleens were harvested for fusion 3 to 4 days later. From the fusion using protocol B, mAb G11 was obtained.

**Fusion, isolation, and screening of GPNMB reactive mAbs.** Fusions were done with the nonimmunoglobulin-secreting Kearney variant of P3X63Ag8.653 by using our standard procedure (27). Supernatants were screened for positivity on the bacteria derived GPNMB protein by ELISA and on THRG cells by fluorometric microvolume assay technology (FMAT) assay (see below). Hybridoma supernatant reactivity for plated protein was done as previously published (27), with the exception that the secondary reagent used was goat anti-mouse IgG-Fc specific (Sigma, St. Louis, MO), the tertiary reagent was horseradish peroxidase – Streptavidin (Zymed, South San Francisco, CA), and development was by the SigmaFast o-phenylenediamine dihydrochloride kit (Sigma) according to instructions of the manufacturer.

**FMAT analysis.** Analysis of mAb binding (supernatant samples or purified mAbs) to intact cell surfaces was done on the FMAT 8100 HTS system (Applied Biosystems). THRG cells grown in Zinc Option medium supplemented with 10% FCS were harvested and fixed with 10% formaldehyde/PBS for 6 minutes at room temperature, then plated into an FMAT 96-well plate at a concentration of 10,000 cells per well; 30 μL purified anti-GPNMB mAbs or hybridoma supernatants were added to the wells; the positive primary antibody control was the serum pool derived from immunized spleen donors, and negative background controls included Zinc Option medium supplemented with 10% FCS (hybridoma supernatant control). 1% bovine serum albumin/PBS

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![Fig. 2. Expression of GPNMB RNA in glioblastoma and melanoma cell lines and glioma xenografts. A, alternative splicing of GPNMB RNA transcripts generating a 36 bp insert (gray box) in the ECD. Arrows, positions of oligonucleotide primer sets used in RT-PCR analyses. RT-PCR reactions done with primer pairs are shown in gels for the amplification of overall GPNMB transcripts (GPNMBwt), the wild-type (GPNMBwt) mRNA, and the splice variant (GPNMBsv) mRNA. Bottom, β-actin amplification. Lane M, 100 bp DNA ladder. B, relative overall GPNMB mRNA levels were measured in seven human GBM and four melanoma cell lines as well as seven human glioma xenografts by quantitative real-time RT-PCR using primer pair A and B1. Relative GPNMB mRNA levels are expressed in terms of fold increase over normal whole brain sample. Columns, mean of triplicates; bars, SD.](https://www.aacrjournals.org/doi/fig/10.1158/1078-0432.CCR-05-2000)
buffer, or irrelevant isotype controls (IgG1 or IgG2b) at concentrations identical to those of the primary reagents. Secondary antibody goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) coupled to FAMT-blue dye according to instructions of the manufacturer (Applied Biosystems) was added to the wells at a final concentration of 0.13 μg/mL in 1% bovine serum albumin/PBS, and the plates were incubated for 2 hours in the dark at room temperature before measuring emitted fluorescence (650-685 nm) with FAMT 2.0.1 software.

**Western blotting.** Total cell pellets were lysed in buffer [50 mmol/L Tris·HCl (pH 8.0), 150 mmol/L NaCl, 1% N-940, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.045 mg/mL aprotinin]. Aliquots of sample lysates or purified GPNMB protein were subjected to electrophoresis on Bis-Tris SDS polyacrylamide gel and blotted onto polyvinylidene difluoride membranes according to the standard method (28). Nonspecific binding sites were blocked by using 3% nonfat milk in PBS-0.05% Tween 20. Incubations with primary antibodies were carried out overnight at 4°C with rabbit anti-GPNMB antiserum 2640 (5 μg/mL) or mAbs (10 μg/mL) in PBS-0.05% Tween 20 containing 1% milk; irrelevant IgG1 or IgG2b was used to control for nonspecific binding. After washing membranes, specific protein bands were detected with horseradish peroxidase–linked secondary antibodies (Amersham Biosciences, Piscataway, NJ) and developed with SuperSignal West Pico Chemiluminescence kit (Pierce, Rockford, IL) according to instructions of the manufacturer.

**Indirect analytic flow cytometry.** Our standard procedures for these assays have been published extensively (22, 27, 29, 30). Indirect analytic flow cytometry was done as previously described (30) on a Becton Dickinson FACSort equipped with Lysys software (Becton Dickinson, San Jose, CA). Assays were done at 4°C; all washes were done with ice-cold media to facilitate the detection of cell surface receptors without allowing internalization to occur. As profiles obtained with cells maintained in the culture media used were identical, the latter was used for background; this is a conservative estimate of the total positive staining population.

To examine the cell surface expression of GPNMB proteins, target cultured or biopsy-derived GBM cells were stained with anti-GPNMB antibodies, G11 or U2, or rabbit polyclonal antibody 2640 under nonpermeabilized conditions as previously described (30). Subconfluent cultures were detached from culture flasks by incubation with 0.02% EDTA/PBS, 1 × 10^6 cells were maintained in 0.5% paraformaldehyde/PBS for 10 minutes at 4°C, washed, resuspended in 150 μL PBS containing 10% fetal bovine serum, and blocked for 20 minutes at 4°C. After two washes, the samples were reacted with anti-GPNMB mAbs (10 μg/mL) or rabbit polyclonal antibody 2640 (5 μg/mL) and irrelevant mouse IgGs (10 μg/mL) or rabbit IgGs (5 μg/mL) in PBS. After two additional washes, cells were incubated with FITC-labeled secondary antibody for 30 minutes at 4°C and analyzed on a Becton Dickinson FACSort instrument (Becton Dickinson).

**Quantitative fluorescence-activated cell sorting determination of receptor density.** The number of GPNMB molecules expressed per cell by cell populations was determined by quantitative fluorescence-activated cell sorting (FACS) determination of receptor density using the Quantum Simply Cellular system (Bangs Laboratories, Fishers, IN) as described (30). The microbead solution used is a mixture of five species, each of which bound one GPNMB molecule; a biotinylated secondary antibody was used to capture GPNMB protein coupled to magnetic beads (34). Plotting the total divided by the specifically bound activity versus the reciprocal of the antigen concentration yielded a linear plot, the intercept of which represents the inverse of the mean specific activity. A modified Scatchard analysis was used to measure the binding affinity of iodinated mAbs beginning with serially diluted, labeled mAb starting at 10 μg/mL versus THRG MG cells, incubation at 4°C for 4 hours, and measurement of cell-bound activity as a proportion of input activity; nonlinear regression analysis to calculate K_d was done with GraphPad Prism software (GraphPad Prism Software, San Diego, CA).

**Tumor samples.** Samples of primary brain tumors were obtained from 90 newly diagnosed GBM patients from the Preston Robert Tisch Brain Tumor Center Tissue Bank, Duke University Medical Center (Durham, NC). The tumors were histologically diagnosed and graded as GBM according to WHO criteria (35). Among those 90 GBM patient samples, 50 cases were studied in quantitative real-time PCR analysis for GPNMB mRNA transcript detection, 79 GBM cases were analyzed by immunohistochemistry staining using anti-GPNMB antibodies, and 39 cases were done by both analyses. No patient had any history of chemotherapy or radiotherapy before surgery. The samples were immediately snap-frozen in liquid nitrogen and stored at −80°C until analysis.

**Immunohistochemistry.** Immunohistochemical analysis of acetone-fixed (~70°C, 30 minutes) 5- to 8-μm frozen tissue sections of human tumor tissue was done as described previously (22, 30). For detection of GPNMB, exposure to primary reagent (polyvalent rabbit antiserum 2640/irrelevant negative control rabbit IgG, or mAb G11/murine IgG2b isotype control at 10 and 5 μg/mL) was done for 1 hour at room temperature. Slides were washed in PBS, the appropriate dilution of biotinylated goat anti-mouse IgG or goat anti-rabbit IgG (Vector, Burlingame, CA) established by previous titration was applied, and the slides were incubated for 1 hour. Slides were washed again in PBS, exposed to horseradish peroxidase–avidin complex (Vector, standard ABC kit) for 30 minutes, and following PBS washes, developed with diaminobenzidine (Metal Enhanced DAB Substrate kit; Pierce). Slides were counterstained with hematoxylin, dehydrated, mounted, and read independently by two investigators, including a neuropathologist. Slides were scored on the basis of staining intensity (none to intense, 0-3), and staining distribution and localization (0-25%, 1+; 26-50%, 2+; 51-75%, 3+; 76-100%, 4+), with notation of parenchymal, perivascular, or nuclear staining. Positive tissue control was provided by D256G myelogenous rat xenografts.

**Statistical analysis.** The relationship between relative GPNMB mRNA expression levels (GPNMBwt, GPNMBsv, and GPNMBmut) and described above for indirect analysis. The techniques for disaggregation of biopsy and xenograft-derived cells and preparation for flow cytometry have been thoroughly described (30).
immunohistochemical score was examined by using Spearman’s correlation coefficient. For survival analyses in which patient survival was computed from the date of pathology sample to date of death or last contact, relative GPNMB mRNA expression levels and immunohistochemical scores were categorized as ≤3.0- versus >3.0-fold and zero versus positive, respectively. Survival data were current as of August 19, 2005. Cox proportional hazards model analysis was used to check the statistical significance for each individual predictor of survival (SAS Statistical Analysis Package, Cary, NC). A predictor was considered for the multivariate model if P < 0.25 in the univariate model. Age was included as a continuous predictor in the model due to a lack of dichotomy.

Results

Alternative splicing of GPNMB RNA transcripts in glioma cells. We have cloned the GPNMB gene from the human glioblastoma cell line D392MG by isolation of polyadenylate mRNA and RT-PCR (Fig. 1). Through the cDNA sequence analysis of individual clones, in addition to the published GPNMB RNA sequence (14), we found that one clone, EX1, had an in-frame insertion of a 36 bp fragment at nucleotide position 1019 in the ECD of GPNMB (Fig. 1). By Basic Local Alignment Search Tool search (National Center for Biotechnology Information, Bethesda, MD), we determined that this 36 bp fragment insert created by alternative splicing completely matches the human genomic GPNMB intron DNA clone CTA-271G13 from chromosome 7 (Genbank accession no. AC005082). This 12-amino-acid insertion variant is designated as GPNMB<sub>sv</sub> (splice variant), and the normal gene as GPNMB<sub>wt</sub> (wild type). In CTA-271G13, an additional 99 bp matching the GPNMB<sub>sv</sub> cDNA sequence was found directly downstream from this 36 bp fragment. Further sequence analysis revealed that CTA-271G13 contains the human genomic GPNMB sequence. The 5′ and 3′ ends of each intronic fragment matched the consensus sequences for 3′ acceptor site and for 5′ donor site (36, 37), confirming that GPNMB<sub>sv</sub> is generated by the alternative splicing of GPNMB RNA transcripts.

Quantification of GPNMB mRNA in GBM cell lines, xenografts, and biopsy samples. After our initial screening analyses (13), we used real-time RT-PCR to measure the expression of the GPNMB gene in seven GBM cell lines, four melanoma cell lines, seven malignant glioma xenografts, and 39 cases of newly diagnosed GBM samples (Fig. 2; Table 1). First, human GBM-derived cell lines and melanoma cell lines were analyzed for GPNMB mRNA expression (Fig. 2B). We have designed specific primer sets to identify the expression of GPNMB<sub>sv</sub>, GPNMB<sub>wt</sub> as well as GPNMB<sub>sv+wt</sub> (Fig. 2A; details in Materials and Methods). As shown in Fig. 2A, all seven glioma cell lines examined expressed both types of GPNMB RNA transcripts (i.e., the wild-type GPNMB<sub>sv+wt</sub> and the splice variant GPNMB<sub>sv</sub>). By quantitative real-time RT-PCR, D54MG, D392MG, and D247MG cells exhibited high levels of overall GPNMB mRNA, whereas U251MG, T98G, and D245MG exhibited moderate to marginal levels, and U87MG exhibited very low or no GPNMB mRNA expression (Fig. 2B). Therefore, we used D54MG, D392MG, and D247MG cells in studies of GPNMB protein expression. For the melanoma cell lines we obtained from American Type Culture Collection, C32, Malme-3M, and SK-mel-28 exhibited high levels of GPNMB RNA expression ranging from 20- to 60-fold higher relative to normal brain tissue by using β-actin as an internal control (Fig. 2B); however, the A375 melanoma line expressed a low level of GPNMB RNA. We also found that malignant glioma xenografts D256 MGX and 12A-7 exhibited a 3-fold increase over normal brain samples (Fig. 2B).

Real-time RT-PCR analysis of GPNMB<sub>sv</sub> alone in the same cell line panel revealed that a subset of GPNMB<sub>sv</sub>-positive cell lines also express GPNMB<sub>sv</sub>. Relative transcript levels of GPNMB<sub>sv</sub> RNA were 10- to 100-fold lower than those for GPNMB<sub>sv</sub> RNA, and no significant GPNMB<sub>sv</sub> or GPNMB<sub>sv+wt</sub> transcripts were detected in normal brain samples (data not shown). Similar analysis of established glioma cell lines revealed that significant levels were present in three potential glioma target cell lines, D247MG, D392MG, and D54MG; the latter two were chosen as reference controls on the basis of reproducible growth in vitro.

Goings from cultured cell lines to patient tumors, we measured GPNMB mRNA levels in 50 GBM biopsy samples. We found that 35 of 50 (70%) cases were positive (at least 3-fold increase over normal whole brain sample) for GPNMB<sub>sv+wt</sub> mRNA, 26 of 50 (52%) cases were positive for GPNMB<sub>sv</sub> mRNA, and 15 of 50 (30%) cases were positive for GPNMB<sub>sv</sub> mRNA (Table 1). High transcript expression (a >10-fold increase over normal brain of GPNMB<sub>sv+wt</sub> mRNA transcripts) was found in 19 of 50 (38%) cases; for GPNMB<sub>sv</sub> and GPNMB<sub>sv+wt</sub> mRNA, high expression was found in 11 of 50 (22%) and 3 of 50 (6%) biopsy samples, respectively (Table 1). As the GPNMB<sub>sv</sub> mRNA profile was virtually identical to the GPNMB<sub>sv+wt</sub> profile, it is conceivable that GPNMB<sub>sv</sub> is the predominant detected moiety. No significant expression for the GPNMB gene was detected in normal brain samples assayed simultaneously.

Isolation and quantitative analyses of anti-GPNMB antibodies for cell surface GPNMB. Although high levels of RNA transcripts can be predictive of protein levels, the presence of detectable protein in human tumor material must be established for targeting applications. Thus, we developed specific antibodies to detect the presence of GPNMB protein. Purified rabbit anti-GPNMB IgG 2640 was shown to detect the cell surface GPNMB protein by performing the indirect FACS analysis as shown in Fig. 3A. Antiserum 2640 reacted with GPNMB-positive cell lines D392MG and D54MG, as indicated by GPNMB RNA expression, but not with a negative cell line, such as HEK293 (Fig. 3A) or U251MG cell lines (data not shown). Antiserum 2640 also reacted with a GPNMB-transfected HEK293 cell line, which expressed GPNMB on the cell surface (Fig. 3A, right).

Table 1. Incidence of GPNMB mRNA expression from newly diagnosed GBM patients

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Incidence</th>
<th>Positive</th>
<th>&gt;10-fold</th>
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<tr>
<td>Overall GPNMB&lt;sub&gt;sv+wt&lt;/sub&gt; transcripts</td>
<td>35/50 (70%)</td>
<td>19/50 (38%)</td>
<td></td>
</tr>
<tr>
<td>GPNMB&lt;sub&gt;sv&lt;/sub&gt; transcripts</td>
<td>26/50 (52%)</td>
<td>11/50 (22%)</td>
<td></td>
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<tr>
<td>Splice variant GPNMB&lt;sub&gt;sv&lt;/sub&gt; transcripts</td>
<td>15/50 (30%)</td>
<td>3/50 (6%)</td>
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*Detected by real-time RT-PCR analysis; positive cases were defined as those with GPNMB RNA levels 3-fold higher than normal brain.

†More than 10-fold greater than normal whole brain sample.
In addition, we have immunized three different standard mouse strains (BALB/c, C57Bl/6, and C3H/He) by the intradermal route with cytomegalovirus-based plasmid DNA encoding the ECD of normal GPNNMBwt or GPNNMBsv. Those animals were subsequently boosted i.p. with bacterially expressed or insect cell–expressed GPNNMB ECD protein. Two separate fusions were done as described in Materials and Methods. As derivation of mAbs specific for the extracellularly expressed portion of GPNNMB was desired, supernatants from outgrowing hybridomas were screened for reactivity on the D54MG cell line and GPNNMB-transfected cell line THRG, previously shown to express GPNNMB (20, 21); U251MG, which does not express GPNNMB, was used as the negative control in FMAT screening. From the two protocols, two hybridomas (B: IgG2b mAb G11; U: IgG2b mAb U2) were selected for cloning and further analysis.

Detection of cell surface GPNNMB was done with anti-GPNNMB mAb G11. Representative indirect FACS histograms of purified mAb G11 are shown in Fig. 3B; this analysis establishes that mAb G11, generated following immunization with plasmid DNA of GPNNMB and with bacterially produced GPNNMB protein, is reactive with a cell surface epitope on the purposefully transfected THRG cell line (data not shown) and on D54MG (Fig. 3B, left). Indirect FACS analysis revealed reactivity of G11 with GPNNMB-expressing D247MG cells under

![Fig. 3. Detection of GPNNMB on malignant glioma cells, GBM biopsy sample, and GPNNMB-positive cells.](image-url)

A, indirect FACS analysis using rabbit polyclonal antisera 2640. Reactivity of 2640 for nonpermeabilized GPNNMB-positive glioma lines, D392MG and D54MG (dotted lines), and negative control HEK293 cells. Filled peaks, control staining with normal rabbit IgG. Right, reactivity of 2640 for nonpermeabilized HEK293 cells transfected with GPNNMB (dotted line). B, FACS analysis for mAb G11 and U2. Left, reactivity of mAb G11 for formalin-fixed, nonpermeabilized D54MG (dotted lines). Filled peaks, control staining with normal mouse IgG2b for D54MG. Middle, reactivity of G11 for formalin-fixed, nonpermeabilized D247MG cells (solid line) and D247MG cells transfected with full-length GPNNMB (dotted line). Filled peak, control staining with normal mouse IgG2b for D247MG. Note the increase in mAb binding following transfection (shift to the right), indicating a higher number of cell surface GPNNMB molecules than in the parental cells. Right, quantitative FACS analysis of biopsy GBM 2180–derived cells with anti-GPNNMB mAb U2 in noninternalizing conditions. The “B” peaks refer to the beads that bind to increasing quantities of FITC-mAb U2. Estimated median cell surface density is $7 \times 10^4$ GPNNMB molecules per cell. C, Western blot analysis of GPNNMB protein. Total cell lysates containing 20 μg protein were separated by SDS-PAGE and probed with anti-GPNNMB rabbit antisera 2640. Left, cell lysates from insect Sf9 GPNNMB ECD transfectants were incubated with or without N-glycosidase (PNGase F), and electrophoresed along with recombinant GPNNMB ECD protein produced in bacteria E. coli. Note the mobility shift after N-glycosidase treatment from $M_r$ ~ 70 to ~ 54 kDa. Middle and right, detection of GPNNMB proteins in cultured human GBM cells, including D392MG and D54MG, HEK293, and its GPNNMB transfectant, as shown on top of the gel. Note that the GPNNMB protein expression increased after transfection; the protein migrated predominantly as two bands of apparent molecular weights $M_r$ ~ 80 and ~ 100 kDa. The locations of protein molecular weight markers are shown between panels. D, Western blot analysis of GPNNMB protein by anti-GPNNMB mAb G11 and irrelevant isotype control IgG2b against GPNNMB ECD protein purified from insect cells Sf9 and whole cell lysates (20 μg) prepared from U251MG, U251MG/GPNNMB, and D247MG/GPNNMB transfectants. Short arrow, apparent molecular weights for glycosylated GPNNMB ECD purified from Sf9 insect cells (~ 70 kDa). Three arrows on the right side of gel, GPNNMB proteins detected in human glioma cell lines and transfectants representing differentially glycosylated forms.
nonpermeabilized conditions (Fig. 3B, middle). Furthermore, stable transfectant D247MG-GPNMB showed a peak shift compared with that of the parental line D247MG, indicating an increase in the number of surface GPNMB molecules after transfection. Irrelevant control IgG2b was unreactive with both lines. Similar profiles were obtained with all of the components of this mAb panel on SK-Mel-28 cell lines (data not shown); the non–cell surface GPNMB-expressing cell line U251MG was negative with these mAbs detected by FACS.

mAbs G11 and U2 were fluoresceinated for quantitative FACS analysis, and cell surface GPNMB densities were determined on a panel of cell lines, disaggregated xenografts, and biopsy samples (data not shown). mAb U2 was determined to be the optimal FITC-labeled reagent for these analyses. An example of such an analysis of patient biopsy GBM 2180 is provided in Fig. 3B (right); from the regression equation calculated for FITC-mAb binding to quantitated receptor site beads, the fluorescent channel value obtained with GBM 2180 cells predicts a median GPNMB density of $7 \times 10^4$ GPNMB molecules per cell. Fifty percent (four of eight) of long-term cultured GBM cell lines expressed GPNMB in excess of 1 molecule per cell; only three of nine established xenografts did so. Analysis of freshly disaggregated cells from GBM ($n = 27$) biopsies revealed that 11 of 27 (41%) GBM expressed cell surface GPNMB with a range of densities from $1.1 \times 10^4$ to $7.8 \times 10^5$; one GBM expressed $>10^6$ GPNMB molecules per cell. Positive GPNMB-expressing cell lines THRG and SK-Mel-28 exhibited $1.4 \times 10^3$ to $3.9 \times 10^3$ and $3 \times 10^4$ to $9 \times 10^4$ GPNMB molecules per cell, respectively. No GPNMB expression was detected by quantitative FACS in medulloblastoma cultured cells, xenografts, or biopsies (data not shown).

**Detection of GPNMB protein by Western blotting in glioblastoma cells.** The integrity of the GPNMB coding sequence was first investigated by expressing the recombinant protein in insect cells. By Western blotting, purified rabbit anti-GPNMB IgG 2640 reacted with the lysates of Sf9 insect cells transiently transfected with the ECD of GPNMB (Fig. 3C, left). GPNMBECD expressed in Sf9 cells migrated with an apparent molecular weight of $M_\text{r} \sim 70$ kDa, which was significantly larger than the molecular weight of GPNMBECD protein deduced from the amino acid composition (54 kDa). The ECD of human GPNMB contains 12 potential N-glycosylation sites. N-glycosidase treatment of Sf9 lysates significantly reduced the molecular size of GPNMBECD almost identical to that produced in *E. coli* (Fig. 3C, left), indicating that the discrepancy in the molecular mass is due to glycosylation of the GPNMB protein.

We further investigated the GPNMB protein expression in cultured human GBM cell lines. Detection of GPNMB proteins using antiserum 2640 was carried out in several cultured human GBM cells, including D392MG and D54MG as shown in Fig. 3C (middle). In GBM cell lines, GPNMB proteins were detected by antiserum 2640 in D392MG and D54MG as multiple bands between ~80 and ~100 kDa as shown by arrow lines and a faint band at 120 kDa (Fig. 3C, middle); we also found two nonspecific bands reacted with 2640 as shown by arrow heads at 70 and 40 kDa positions. The GPNMB protein expression increased significantly after transfection of U251MG, D54MG, or D247MG cells with GPNMB expression vectors (data not shown). The proteins appeared as multiple bands in Western blots, presumably due to different degrees of glycosylation; essentially, the GPNMB proteins migrated predominantly as two bands of apparent molecular weights $M_\text{r} \sim 100$ and ~75 kDa. In addition, Western blot of a parent control line HEK293 and its GPNMB transfectant with 2640 indicated that two nonspecific bands were also seen in the negative control; however, the Western blot results of HEK293/ GPNMB transfectant lysate exhibited highly specific reactivity to recombinant GPNMB protein as shown in Fig. 3C (right).

To establish the restricted specificity of these mAbs, Western blots were done by using the glycosylated purified GPNMBECD isolated from Sf9 cells as control and cell lysates prepared from the U251MG cell line as well as the GPNMB-transfected cell lines, U251MG and D247MG. As shown in Fig. 3D, mAb G11 recognized the purified GPNMBECD protein (~70 kDa mass, small arrow). mAb U2 exhibited similar pattern (data not shown). All mAbs detected bands between ~75 to 100 kDa and 120 kDa in the GPNMB-transfected D247MG line in a pattern similar to that of rabbit polyclonal antiserum 2640 (Fig. 3C and D). mAb G11 was capable of consistently identifying these bands in the untransfected glioma cell lines, U251MG and D54MG, as well as their GPNMB-transfectants in a pattern similar to that of rabbit polyclonal antiserum 2640, whereas IgG2b irrelevant isotype control was negative (Fig. 3D).

**Kinetics of mAb-to-GPNMB binding.** A kinetic analysis of the interaction of purified mAbs with immobilized GPNMBECD by surface plasmon resonance (BIAcore) was conducted to determine the association and dissociation rate constants and to calculate the affinity constants. Determination of the association and dissociation rates from the sensorgrams revealed a $k_{\text{assoc}}$ of $1.1 \times 10^8$ (mol/L)−1 and a $k_{\text{diss}}$ of $1.1 \times 10^{-3}$ second−1 for mAb G11. The $K_D$ at binding equilibrium, calculated as $K_D = k_{\text{assoc}} / k_{\text{diss}}$, was 9.6 $\times 10^8$ (mol/L)−1 for G11 and 2.7 $\times 10^7$ (mol/L)−1 for U2. Anti-GPNMB mAbs G11 and U2 were also each analyzed by conventional Scatchard analysis versus the THRG cell line. mAbs G11 and U2 exhibited $K_A$ values of $1.7 \times 10^8$ and $2.1 \times 10^8$ (mol/L)−1, respectively, measured by Scatchard analysis versus cell surface–expressed GPNMB and immunoreactive fractions in the range of 75% to 91% for G11 and 71% to 75% for U2, respectively. The estimated cell surface receptor densities obtained from the $K_{\text{max}}$ values for G11 and U2 were quite similar (ranges of $4 \times 10^8$–$8 \times 10^8$) in multiple assays.

**Immunohistochemical analysis.** The immunohistochemical analysis of GBM tissue samples from newly diagnosed, pretherapy patient cases was done using rabbit anti-GPNMB serum 2640 and/or mAb G11 to stain frozen sections of the tumor samples (39 GBMs) that were also used for GPNMB mRNA analysis. The Spearman’s analysis (one sided, assuming a direct relationship between mRNA presence and production of protein) revealed that the correlation of GPNMB mRNA and protein expression was significant ($P < 0.0001$); the higher the detected level of mRNA, the more probable a positive immunohistochemistry result. Spearman’s correlation coefficients equal to 0.58 and 0.57 for all ages and those >45, respectively.

In addition to those 39 GBM cases, an additional 40 GBMs were stained with rabbit anti-GPNMB serum 2460; of these total 79 cases, 32 GBMs were concurrently stained with mAb G11 and appropriate irrelevant IgG2b isotype control. Results of this analysis are summarized in Table 2 (79 cases of GBM) and are illustrated in Fig. 4 for some representative cases. A high
percentage of positive anti-GPNMB staining was observed in those 79 patient samples; 52 of 79 (66%) were stained as positive (Table 2). As shown in Fig. 4, GPNMB localization can present in different patterns, whereas the majority of cases (58%; Table 2) exhibit focal or multifocal tumor parenchymal staining panels (E, H, I, and K) with clear membrane staining (E, F, and K), a subset of GBMs (42%; Table 2) exhibits pronounced perivascular accumulation of GPNMB either with (27%, panels B and C) or without (15%) accompanying parenchymal staining.

Survival analysis. Analyses were conducted to examine the effect on survival of select predictors (RNA expression and immunohistochemical data) in specific patient subgroups: 39 newly diagnosed GBM patients (Table 3) and a subgroup of patients older than 45 years (data not shown, n = 34). Among all 39 patients, the relative GPNMB\textsubscript{wt+sv} RNA expression level was a strong predictor of survival across all analyses as shown in Fig. 5A. The hazard ratio of death for patients with relative GPNMB\textsubscript{wt+sv} RNA expression level >3-fold was 2.98 [95% confidence interval (95% CI), 1.26-7.06] as shown in Table 3A (as rounded numbers). The estimated 2-year survival probability was 0.42 (95% CI, 0.21-0.81) for patients with relative GPNMB\textsubscript{wt+sv} RNA expression levels <3-fold, and 0.15 (95% CI, 0.06-0.37) for patients with relative GPNMB\textsubscript{wt+sv} RNA expression level >3.0-fold. The median patient survival for low GPNMB\textsubscript{wt+sv} RNA expression level (<3-fold of normal brain sample) and high GPNMB\textsubscript{wt+sv} RNA expression level (>3-fold) are 90 and 55 weeks, respectively (Table 3B). For patients older than 45 years, the 2-year survival estimate was 0.40 and 0.13 for the low GPNMB\textsubscript{wt+sv} RNA and high GPNMB\textsubscript{wt+sv} RNA groups, respectively (Cox hazard ratio, 3.00; 95% CI, 1.19-7.57). The median patient survival for low GPNMB\textsubscript{wt+sv} RNA expression level (<3-fold of normal brain sample) and high GPNMB\textsubscript{wt+sv} RNA expression level (>3-fold) are 94 and 55 weeks, respectively (data not shown).

The relative GPNMB\textsubscript{wt} RNA expression level was also a strong predictor of survival. The hazard ratio of death for patients with relative GPNMB\textsubscript{wt} RNA expression level >3.0-fold was 2.21 (95% CI, 1.08-4.55) as shown in Table 3A. The estimated 2-year survival probability was 0.35 (95% CI, 0.19-0.64) for patients with relative GPNMB\textsubscript{wt} RNA expression levels <3.0-fold, and 0.11 (95% CI, 0.03-0.39) for patients with relative GPNMB\textsubscript{wt} RNA expression levels >3.0-fold (Table 3B). For patients older than 45 years, the survival probability at 104 weeks for low levels of GPNMB\textsubscript{wt} RNA was 0.35, whereas the probability for high levels of GPNMB\textsubscript{wt} RNA, 0.06 (Cox hazard ratio, 2.38; 95% CI, 1.09-5.20; data not shown).

In addition, immunohistochemistry score was also a strong predictor of survival as shown in Fig. 5B. The hazard of death for patients with a positive immunohistochemical score was...
2.80 (95% CI, 1.24-6.32); that is, patients with positive immunohistochemical staining have higher risk of death (2.80 times) than patients with negative staining (Table 3A). The estimated 2-year survival probability was 0.39 (95% CI, 0.19-0.77) for patients with zero immunohistochemical scores and 0.15 (95% CI, 0.06-0.38) for patients with positive immunohistochemical scores. The median patient survival for negative GPNMB immunohistochemical staining and positive GPNMB immunohistochemical staining are 90 and 50 weeks, respectively (Table 3B). For patients older than 45 years, the 2-year survival estimate was 0.36 and 0.13 for the zero and positive immunohistochemical groups, respectively (Cox hazard ratio, 2.71; 95% CI, 1.14-6.45; data not shown).

Two bivariate models containing age were generated based on multicollinearity in a Cox model with age, immunohistochemistry, and mRNA expression levels as predictors (Table 3C). The first model shows that patients with high relative $GPNMB_{wt}$ RNA expression levels have a higher risk of death (2.6 times higher) than patients with low relative $GPNMB_{wt}$ RNA expression level after adjusting for age. The second model shows similar results with positive immunohistochemistry scores that patients with positive GPNMB immunohistochemical score have a higher risk of death (2.6 times higher) than patients with zero immunohistochemical score after adjusting for age.

### Discussion

Effective mAb-based targeted therapy depends on several factors, which include characteristics of the target antigens, the

### Table 3

<table>
<thead>
<tr>
<th>A. Univariate Cox model analyses of the relationship between patient survival and levels of $GPNMB$ RNA and the presence of GPNMB protein by immunohistochemistry</th>
<th>Hazard ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative RNA$_{wt}$ expression: $&gt;3.0$ vs $\leq 3.0$</td>
<td>3.0 (1.3-7.1)</td>
<td>0.013</td>
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<tr>
<td>Relative RNA$_{wt}$ expression: $&gt;3.0$ vs $\leq 3.0$</td>
<td>2.2 (1.1-4.5)</td>
<td>0.031</td>
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<td>Relative RNA$_{wt}$ expression: $&gt;3.0$ vs $\leq 3.0$</td>
<td>1.4 (0.7-2.8)</td>
<td>0.406</td>
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<tr>
<td>Immunohistochemistry: positive vs zero</td>
<td>2.8 (1.2-6.3)</td>
<td>0.013</td>
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<table>
<thead>
<tr>
<th>B. Kaplan-Meier survival estimates</th>
<th>n</th>
<th>Dead</th>
<th>2-y survival probability %, (95% CI)</th>
<th>Median survival, wk, (95% CI)</th>
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<tbody>
<tr>
<td>RNA$_{wt} \leq 3.0$</td>
<td>12</td>
<td>8</td>
<td>42 (21-81)</td>
<td>90.0 (44.9-∞)</td>
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<tr>
<td>RNA$_{wt} &gt; 3.0$</td>
<td>27</td>
<td>27</td>
<td>15 (6-37)</td>
<td>55.1 (40.3-83.0)</td>
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<tr>
<td>RNA$_{wt} \leq 3.0$</td>
<td>20</td>
<td>16</td>
<td>35 (19-64)</td>
<td>61.6 (50.1-∞)</td>
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<tr>
<td>RNA$_{wt} &gt; 3.0$</td>
<td>19</td>
<td>19</td>
<td>11 (3-39)</td>
<td>53.4 (39.7-92.3)</td>
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<tr>
<td>RNA$_{wt} \leq 3.0$</td>
<td>28</td>
<td>24</td>
<td>25 (13-48)</td>
<td>54.9 (40.3-89.4)</td>
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<tr>
<td>RNA$_{wt} &gt; 3.0$</td>
<td>11</td>
<td>11</td>
<td>18 (5-64)</td>
<td>82.4 (49.9-∞)</td>
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<tr>
<td>Negative immunohistochemistry</td>
<td>13</td>
<td>9</td>
<td>39 (19-77)</td>
<td>90.0 (53.4-∞)</td>
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<tr>
<td>Positive immunohistochemistry</td>
<td>26</td>
<td>26</td>
<td>15 (6-38)</td>
<td>50.1 (39.1-86.6)</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>C. Multivariate Cox models of the relationship between the levels of $GPNMB$ RNA, presence of GPNMB protein by immunohistochemistry and patient survival</th>
<th>Hazard ratio (95% CI)</th>
<th>P</th>
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<tr>
<td>Age</td>
<td>1.1 (1.0-1.1)</td>
<td>0.002</td>
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<tr>
<td>Relative RNA$_{wt}$ expression: $&gt;3.0$ vs $\leq 3.0$</td>
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<tr>
<td>Age</td>
<td>2.6 (1.2-5.9)</td>
<td>0.021</td>
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<tr>
<td>Immunohistochemistry: positive vs zero</td>
<td>2.6 (1.2-5.9)</td>
<td>0.021</td>
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GPNMB mRNA were noted in normal brain RNA. 38% of GBM showed a >10-fold increase over normal brain in the correlation of GPNMB mRNA presence and production of protein (assumed a direct relationship between mRNA and protein expression) revealed that analysis (one sided, assuming a direct relationship between mRNA levels and protein expression) was significant (i.e., the higher the detected level of mRNA, the more probable a positive immunohistochemistry result). However, the sensitivity of mRNA detection is far higher than that of immunohistochemical assays, which accounts for some of the discrepancies noted. In cases where protein is detected in tissue in the absence of mRNA, the most likely explanation is that the mRNA is short-lived and chronically produced, leaving a relatively long-lived protein in situ (38). GPNMB antigens exhibit diffuse staining in frozen sections with distinct membrane staining and absence of blood vessel staining by both polyvalent rabbit antiserum and mAbs demonstrably specific for GPNMB. These observed staining patterns corroborate the cell membrane staining observed in quantitative FACS assays as described above. These data show the establishment of specific and reliable antibody probes for analysis of tissue and biopsy-derived cell populations. Concerning subcellular localization, indirect FACS analysis with anti-GPNMB mAb G11 under nonpermeabilizing conditions showed that GPNMB proteins are expressed at the surface cell membrane of human glioblastoma cell line D247MG. Furthermore, a shift of peak was observed following stable transfection with full-length GPNMB, indicating an increase in the number of surface GPNMB protein molecules following transfection. This supertransfectant cell line will likely prove useful in our preclinical mAb localization and tumor immunotherapy models.

Although some extracellular matrix antigens, such as tenascin, and some noninternalizing surface antigens are suitable for immunotherapeutic approaches, the ideal antigens for brain tumor immunotherapy would be those that fulfill the following criteria: (a) the general consensus for minimum surface antigen density is ≥1 × 10⁴ protein molecules per cell (30, 39); (b) stability at the cell surface and lack of antigen shedding (8); and (c) internalization following binding to ligand or antibody (8). Glioma cell surface–expressed GPNMB fulfills these criteria in terms of adequate cell surface diversity, dynamics of protein synthesis, half-life, internalization, and mechanisms of degradation (8). Little is known about the distribution and function of GPNMB proteins in normal human organs (13, 14, 40). However, for tumors localized within the central nervous system, the optimal route for the administration of mAb-based therapeutic agents is through surgically created resection cavities or saturation of an entire hemisphere by intracranial microdiffusion (41). The expression of GPNMB in distant normal tissues should not compromise the compartmental delivery of GPNMB-related immunologic agents within the central nervous system, because only small amounts of conjugates reach systemic organs (42).

The mechanistic biological significance of the aberrant expression of GPNMB in high-grade gliomas remains to be determined. In another study, the expression levels of GPNMB RNA transcripts showed a positive correlation with increasing grade of tumors (data not shown). The GBM group exhibited higher GPNMB mRNA levels than those in anaplastic astrocytoma; in addition, the appearance of perivascular accumulation of GPNMB protein was noted in GBM, but was absent in anaplastic astrocytoma (data not shown). The ECD of GPNMB contains 12 potential N-glycosylation sites, an RGD integrin-binding motif, and a heparin-binding motif (14). Other functional motifs found are a polycystic kidney disease motif and a proline-rich region that presumably forms a hinge and can mediate protein-protein interaction. In a genetically defined human glioma model using minimally transformed human fetal

![Graph](image_url)
astrocytes (20), transfection of GPNMB resulted in the drastic change of tumor phenotype, with invasion of brain tissues and formation of spontaneous metastasis (21). Thus, GPNMB may serve as an adhesion molecule mediating cell-cell and/or cell-matrix interaction (15) and may contribute to the acquisition of the invasive nature of malignant glioma cells. For survival analysis, newly diagnosed GBM patients over the age of 45 years had a higher risk of death (data not shown), as has been known for decades. Similarly, in this population, univariate analyses show that patients with high relative GPNMB mRNA expression levels (wt and wt+sv) had a higher risk of death. The relative GPNMB protein expression levels (positive and negative immunohistochemical results) held the same significance. Taken together, the results of survival analysis suggest that the relative GPNMB mRNA levels and immunohistochemical staining represent a strong prognostic predictor of poor GBM patient survival. There are only a few molecular markers that are prognostic for survival in malignant gliomas. High GPNMB expression in GBM patient samples, strong glutathione S-transferase/π protein expression in human gliomas (43), and a functional polymorphism in epidermal growth factor gene (44) are associated with clinically more aggressive tumors and are useful and powerful prognostic markers of poor patient survival.

In conclusion, increased GPNMB mRNA levels correlated strongly with elevated GPNMB protein expression in GBM biopsy samples and higher risk of death. Statistically significant predictors of survival among patients with GBM are age, the mRNA expression variables (wt and wt+sv), and immunohistochemical positivity. In a Cox model examining RNA for the subgroup of patients over 45 years old in these populations, mRNA expression and immunohistochemistry were shown to add significant prognostic value beyond that provided by age alone. These data indicate that GPNMB is a potentially useful tumor-associated antigen in immunotherapeutic approaches for malignant gliomas. We propose that therapeutic strategies designed to target GPNMB may be successful in malignant glioma treatment.

Acknowledgments

We thank Dr. H.P. Bloemers (University Nijmegen, Nijmegen, the Netherlands) for providing human GPNMB cDNA, Dr. Jeremy Rich (Duke University Medical Center, Durham, NC) for providing TRG cell lines, T. Shelley Davis for Western blot analysis, R. Ian Cumming for quantitative FACS assays, Charles Pegram for BIACore analysis, Shawn Connelly and Ling Wang for immunohistochemical analyses, Diane Satterfield and Melissa Ehinger for procuring tissue, and Dr. Roger McLendon for neuropathology consultation.

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Glycoprotein Nonmetastatic Melanoma Protein B, a Potential Molecular Therapeutic Target in Patients with Glioblastoma Multiforme

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