Clinical Cancer Research

Glycoprotein Nonmetastatic B Is an Independent Prognostic Indicator of Recurrence and a Novel Therapeutic Target in Breast Cancer

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Abstract

Purpose: Although the murine orthologue of glycoprotein nonmetastatic B (GPNMB), Osteoactivin, promotes breast cancer metastasis in an *in vivo* mouse model, its importance in human breast cancer is unknown. We have examined the significance of GPNMB expression as a prognostic indicator of recurrence and assessed its potential as a novel therapeutic target in breast cancer.

Experimental Design: The clinical significance of GPNMB expression in breast cancer was addressed by analyzing GPNMB levels in several published gene expression data sets and two independent tissue microarrays derived from human breast tumors. GPNMB-expressing human breast cancer cell lines were further used to validate a toxin-conjugated anti-GPNMB antibody as a novel therapeutic agent.

Results: GPNMB expression correlates with shorter recurrence times and reduced overall survival of breast cancer patients. Epithelial-specific GPNMB staining is an independent prognostic indicator for breast cancer recurrence. GPNMB is highly expressed in basal and triple-negative breast cancers and is associated with increased risk of recurrence within this subtype. GPNMB expression confers a more migratory and invasive phenotype on breast cancer cells and sensitizes them to killing by CDX-011 (*glembatumumab vedotin*), a GPNMB-targeted antibody-drug conjugate.

Conclusions: GPNMB expression is associated with the basal/triple-negative subtype and is a prognostic marker of poor outcome in patients with breast cancer. CDX-011 (*glembatumumab vedotin*) is a promising new targeted therapy for patients with metastatic triple-negative breast cancers, a patient population that currently lacks targeted-therapy options. *Clin Cancer Res; 16(7); 2147–56. ©2010 AACR.*

Breast cancer is a heterogeneous disease with respect to its histopathology and response to treatment. Gene expression analyses have classified primary human breast tumors into distinct molecular subtypes, which include normal-like, luminal, human epidermal growth factor receptor 2–positive (HER2+), and basal-like breast cancers (1, 2), which has implications for disease management (3). Recent work indicates that tumors within a particular subtype display distinct organ-specific patterns of recurrence (4, 5). Basal-like breast cancers are more aggressive in nature, preferentially metastasize to the brain and lung, and are responsible for a disproportionate number of deaths (6). Luminal breast tumors are generally responsive to hormonal therapies (7), whereas HER2+ tumors are treated primarily with HER2-targeted therapies such as trastuzumab or lapatinib. In contrast, no targeted therapeutic is currently available for patients with triple-negative breast cancers. This deficiency in targeted treatment options, coupled with the frequency and pattern of metastasis associated with this subtype, accounts for the poor outcomes of patients with basal-like breast cancer.

Glycoprotein nonmetastatic B (GPNMB), also known as osteoactivin, dendritic cell-heparin integrin ligand, or hematopoietic growth factor inducible neurokinin-1 type, is a type I transmembrane protein (8–10). The human and murine orthologues of this protein will be referred to as GPNMB and osteoactivin, respectively. GPNMB is expressed at higher levels in several malignant human tissues relative to corresponding normal tissue (9, 11, 12). Moreover, ectopic overexpression of GPNMB/osteoactivin promotes the invasion and metastasis of hepatocellular

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Translational Relevance

Triple-negative tumors constitute an aggressive subtype of breast cancer that is associated with poor disease outcome. Currently, no targeted therapies are available that effectively treat triple-negative tumors. However, there is substantial molecular heterogeneity within this subtype and some patients with triple-negative tumors do not recur. Thus, there is great interest in identifying molecular markers that can identify the most aggressive of these tumors—particularly those representing targets for therapeutic intervention. Here, we present the first evidence that glycoprotein nonmetastatic B (GPNMB) enhances the metastatic phenotype in triple-negative breast cancer cells. Moreover, its expression predicts breast cancer recurrence across subtypes and specifically among patients with triple-negative disease. Finally, GPNMB-expressing breast cancer cells are effectively killed by a novel toxin-conjugated anti-GPNMB antibody, termed CDX-011, which is currently being investigated in phase II clinical trials as a promising therapy for patients with triple-negative breast cancer.

carcinoma, glioma, and breast cancer cells (11–13). Given its role as a mediator of metastasis and its cell surface expression, GPNMB is an attractive candidate for cancer therapy. In this regard, a GPNMB-specific antibody conjugated to a cytotoxic drug, monomethylauristatin E, induces complete regression of GPNMB-expressing tumors derived from melanoma cell lines (14). This agent (formerly CR011-vcMMAE, CuraGen) has recently been assigned the generic name of *glembatumumab vedotin*, also known as CDX-011, by Celldex Therapeutics.

In this study, we investigated GPNMB as a potential therapeutic target in human breast cancer. We analyzed GPNMB expression in several published breast cancer gene expression data sets and in primary human breast tumors. Our results indicate that GPNMB may serve as an important target for therapeutic intervention in breast cancer, particularly for patients with triple-negative disease who do not benefit from currently available targeted therapies.

Materials and Methods

Analysis of published gene expression data sets. *GPNMB* expression levels were studied in published human breast cancer data sets (15–17) using the following probes: probe ID 1855, NM_002510 (15), or probe ID 201141_at (16, 17). Fold change expression values were generated by first normalizing the expression value for an individual tumor to the average expression value across all tumors. Normalized expression values were then $\log_{10}$ transformed, tumors were segregated into three equivalent groups, and subsequently defined as possessing "high," "intermediate," and "low" GPNMB expression. The Kruskal-Wallis nonparametric test, with normalized GPNMB expression used as the measurement variable, was used to measure the statistical significance of its variance according to subtype. Associated clinical data for each tumor were used to generate Kaplan-Meier survival curves. Statistical analyses were done with the MedCalc (v9) software (MedCalc Software).

Patient information and tissue microarray. Two independent tissue microarrays (TMA) were used to study GPNMB expression in breast cancer. The first study cohort (TMA1) consisted of 234 patients who underwent breast surgery between 1999 and 2003 at the McGill University Health Centre (MUHC). Paraffin blocks and corresponding slides were retrieved from the clinical pathology archive and assessed by an attending clinical pathologist for inclusion in a TMA. One hundred sixty-nine areas containing invasive carcinoma, 31 areas containing ductal carcinoma *in situ* (DCIS), 50 areas of lymph nodes with evidence of metastatic disease, and 50 areas of normal/benign breast tissue were identified under microscopic investigation. Cores (2 × 0.6 mm) were extracted from each of the corresponding areas of the paraffin blocks and used to construct TMA blocks.

The second study cohort (TMA2) consisted of 209 patients diagnosed with primary breast cancer between 2003 and 2008 at the Centre Hospitalier de l'Université de Montréal. Histologic grade was diagnosed according to the Nottingham's classification, as modified by Elston and Ellis (18). The cohort consisted of both low-grade (n = 36) and high-grade (n = 140) ductal carcinomas and typical (n = 13) and atypical (n = 20) medullary carcinomas.

Data for pathologic variables reported as per the clinical criteria in use at time of examination [pathologic stage, histologic grade, tumor size, estrogen receptor (ER), progesterone receptor (PR) and HER2 receptor status] were collected from the original pathology reports. In cases in which HER2 status was equivocal (TMA1), fluorescence *in situ* hybridization was done to derive a definitive assignment. For TMA2, only tumors that stained 3+ were considered HER2 positive. Clinical data were collected from initial interviews with patients as well as examination of medical records housed at the MUHC and the Centre Hospitalier de l'Université de Montréal. Supplementary Tables S1 and S3 contain further information on the clinicopathologic characteristics of patients whose tumors were included on TMA1 and TMA2, respectively. Clinical follow-up for patients on both TMAs was conducted through an annual review of medical records between the surgery date and November 2009. In this period, we documented death from breast cancer or from other causes unrelated to cancer (TMA1), as well as distant metastasis and/or local recurrence of disease (TMA1 and TMA2). These studies were approved by the Research Ethics Board of the MUHC (TMA1; studies SDR-99-780, SDR-00-966, and SDR-04-022) or the Research Centre Ethics Committee at the Centre Hospitalier de l'Université de Montréal (TMA2; study SL05.019).

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TMA immunohistochemical staining and analysis. Immunohistochemical staining was done according to standard procedures using a polyclonal goat anti-GPNMB antibody (1:500 dilution; R&D Systems) and a biotin-conjugated donkey anti-goat secondary antibody (1:500 dilution; Jackson ImmunoResearch Laboratories). Sections were developed with 3-3-diaminobenzidine-tetrahydrochloride and counterstained with hematoxylin.

The initial analysis of GPNMB staining on TMA1 (normal, DCIS, tumor, and lymph nodes) is described in detail in the Supplementary Methods. Subsequent analysis of both TMA1 and TMA2 was done as follows: each individual core was evaluated for GPNMB positivity using a two-tiered system. Staining intensity (0, negative; 1+, mild; 2+, moderate; 3+, strong) and percentage of positive cells belonging to either the epithelial or stromal compartments were reported by a pathologist (L.G.) and an independent observer (A.R.).

Statistical analysis of TMA data. Survival curves were calculated according to the Kaplan-Meier method with a log-rank test for probability of survival. Recurrence-free survival was computed from the date the primary tumor was surgically removed to the date of disease recurrence or last follow-up. Dichotomization for survival analysis using multivariate Cox proportional hazards model (TMA1) were done as follows: GPNMB status as epithelial or nonepithelial (stromal or negative), age as ≥45 or <45 y, HER2 status as positive or negative, estrogen receptor status as positive or negative, histologic grade as grade 3 versus grades 1 or 2, and tumor size as >20 or ≤20 mm. The median follow-up period for survival analysis was 6.11 y (range, 0.03-9.18 y). All statistical analyses were done with MedCalc (v9).

Cell culture, transfections, and fluorescence-activated cell sorting analysis. Cell lines used in this study were obtained from the American Type Culture Collection (ATCC) and

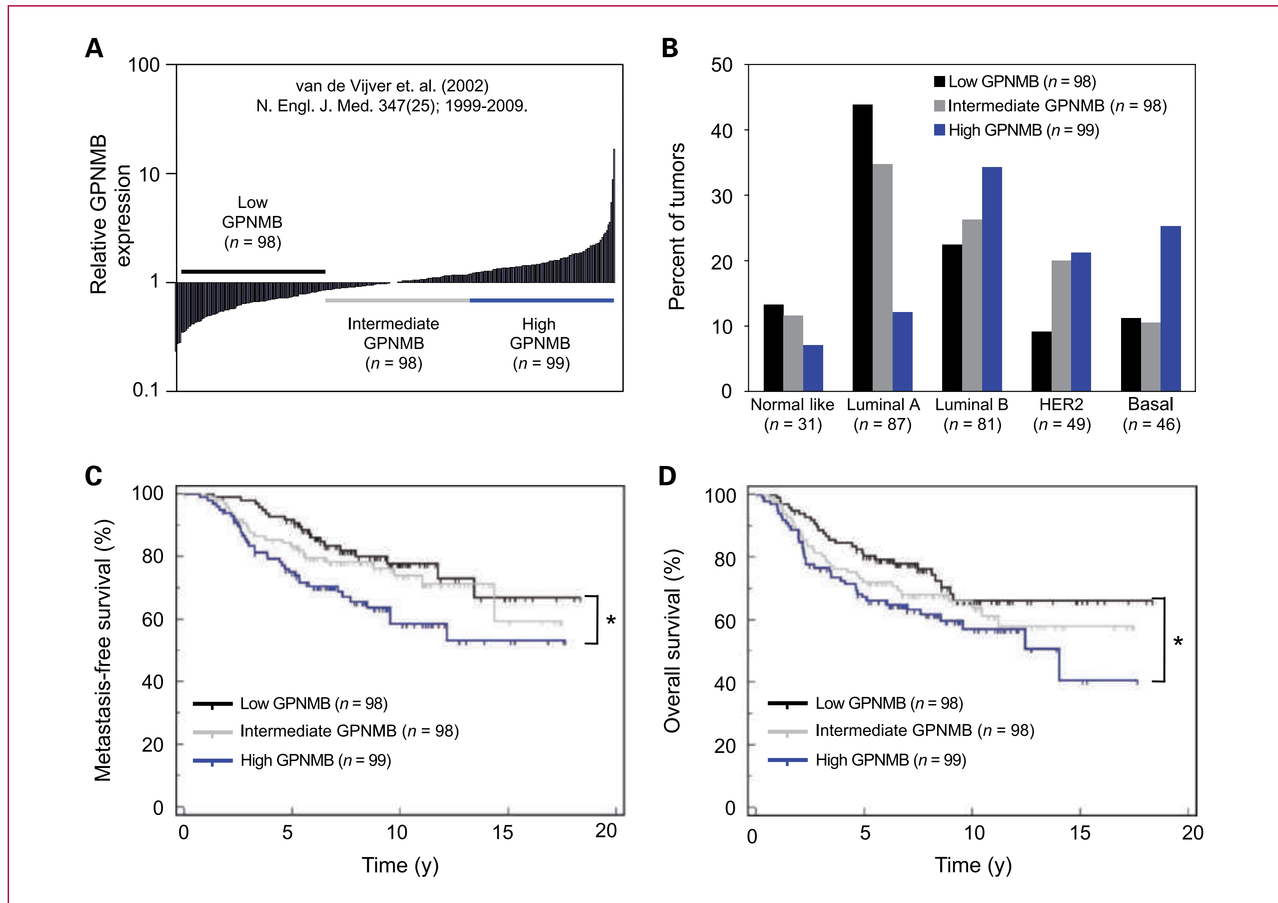

Fig. 1. High *GPNMB* mRNA levels are associated with poor prognosis in human breast cancer. A, relative *GPNMB* mRNA levels in a human breast tumor gene expression data set (15). B, distribution of high, intermediate, and low *GPNMB*-expressing breast tumors with respect to molecular subtype. Statistically significant differences between the variance in *GPNMB* expression across subtypes were determined using the Kruskal-Wallis test ($P < 0.0001$). Specifically, the distribution of low, intermediate, and high GPNMB-expressing tumors in the HER2 and basal subtypes were distinct ($P < 0.05$) from both the luminal A and normal subtypes, and luminal B was distinct ($P < 0.05$) from the luminal A subtype. Kaplan-Meier survival analysis reveals that patients with high *GPNMB*-expressing tumors had significantly shorter (C) metastasis-free survival (*, $P = 0.032$) and (D) overall survival (*, $P = 0.007$).

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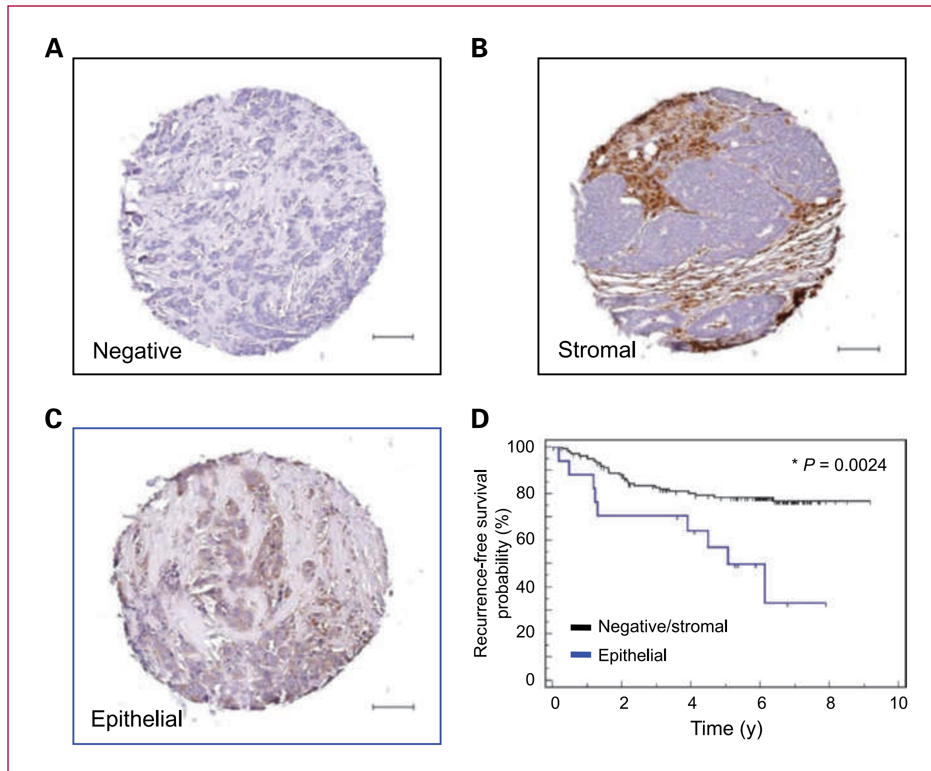

Fig. 2. GPNMB expression in breast tumor epithelium is a novel predictor of breast cancer recurrence. Representative images of breast tumor cores from TMA1 illustrating negative (A), stromal-specific (B), or epithelial-specific (C) GPNMB staining. D, Kaplan-Meier analysis of recurrence-free survival for patients with GPNMB-epithelial–positive tumors and those with negative or GPNMB-stromal staining (*, $P = 0.0024$ for patients with GPNMB-epithelial–positive tumors versus all other patients). Scale bar, 100 μm (A-C).

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maintained following ATCC guidelines. SUM1315 cells were obtained from Asterand, Inc. The pEF1-GPNMB vector was constructed by ligating the full-length human GPNMB cDNA (Open Biosystems) into a pEF1/V5-His expression vector (Invitrogen) using 5′ *Eco* RI and 3′ *Not* I restriction enzyme sites. BT549 and MDA-MB-453 cell lines were engineered to express GPNMB by Lipofectamine 2000 (Invitrogen)–mediated transfection. GPNMB-expressing cells are pools of three independent clones. Stable cell lines were maintained under 1 mg/mL G418 antibiotic selection. For flow cytometric analysis, cells were stained for cell surface GPNMB expression as previously described (11). Data analysis was done with the FlowJo software (v7.5; Tree Star, Inc.).

Immunoblotting. The antibodies used were as follows: GPNMB (1:2,500 dilution; R&D Systems) and α-tubulin (1:10,000 dilution; Sigma-Aldrich). Appropriate horseradish peroxidase–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used at a dilution of 1:10,000 and proteins were visualized by chemiluminescence (Millipore).

Motility and invasion assays. Motility and invasion assays were done as previously described with minor modifications (13). Briefly, 2×10^4 BT549 cells were used for migration assays. For invasion assays, 5×10^4 BT549 cells were used and Transwell inserts were precoated with a 6% Matrigel solution.

Transient knockdown of GPNMB in SUM1315 cells was accomplished by transfection (Lipofectamine 2000, Invitrogen) using 75 nmol/L of the ON-TARGET*plus* SMARTpool [pool of four GPNMB-targeted small interfering RNAs (siRNA), Dharmacon] at t = 0 h. An ON-TARGET*plus* pool of four nontargeting (scrambled) siRNAs was used as a control. Cells were plated in Transwell inserts for invasion assays at t = 24 h. Protein lysates were prepared at the end of the invasion assays (t = 48 h) to confirm efficient GPNMB knockdown over the duration of the experiment. For the invasion assays, 4×10^5 SUM1315 cells were plated in triplicate wells, and results are cumulative from two experiments.

In vitro *and* in vivo *growth inhibition/cytotoxicity assays.* Breast cancer cells (5×10^4 cells for MDA-MB-453 and MDA-MB-361, 2.5×10^4 cells for MDA-MB-468, and 1×10^4 cells for BT549) were seeded in 24-well tissue culture plates and allowed to adhere overnight. The following day, the medium was changed and cells were grown for 4 d in the absence or presence of the indicated concentrations of CDX-011, as previously described (9). Viable cells were counted by trypan blue exclusion using an automated cell counter (Cellometer Auto-T4, Nexcelcom Bioscience). CD1 nude mice (Charles River) were injected with 5×10^6 MDA-MB-468 breast cancer cells and monitored until tumors reached 125 mm^3. Tumor-bearing animals were divided into two groups. One cohort was injected i.v. with a single dose of CDX-011 (20 mg/kg) suspended in PBS, whereas the other was injected with PBS as a control. Tumor growth was monitored weekly by caliper measurement for 6 wk posttreatment. Results from two independent experiments are shown.

Table 1. Cox regression analysis for recurrence-free survival in 145 breast cancer patients (TMA1)

Prognostic factors	Univariate			Multivariate		
	RR	95% CI	*P*	RR	95% CI	*P*
Epithelial GPNMB expression	2.63	1.21-5.74	0.0155	2.73	1.18-6.32	0.0199
Histologic grade (III vs I and II)	7.06	2.74-18.17	<0.0001	7.08	2.54-19.77	0.0002
ER (positive vs negative)	0.34	0.15-0.55	0.0002	0.49	0.24-1.00	0.0505
HER2 (positive vs negative)	2.74	1.37-5.50	0.0047	2.03	0.95-4.29	0.0666
Age (≥45 vs <45 y)	0.22	0.26-1.35	0.2167	0.24	0.09-0.66	0.0057
Tumor size (>20 vs ≤20 mm)	3.73	1.89-7.32	0.0001	3.88	1.83-8.25	0.0004

Results

GPNMB mRNA expression in human breast tumors is associated with reduced metastasis-free and overall survival. Our previous studies showed that osteoactivin enhances breast cancer cell motility, invasion, and metastasis (13). To determine the clinical relevance of this observation, we compared *GPNMB* mRNA levels with clinical outcome in three published data sets. *GPNMB* expression varied widely among the 295 breast tumors comprising the first data set (15), with a 74-fold difference between tumors with highest and lowest *GPNMB* expression (Fig. 1A). Fewer tumors with high *GPNMB* expression belonged to the luminal A subtype (12.1%) compared with tumors with low and intermediate *GPNMB* expression (43.9% and 34.7%, respectively). Conversely, high *GPNMB*-expressing tumors were preferentially classified as basal like (25.3%) relative to low and intermediate *GPNMB*-expressing tumors (11.2% and 10.5%, respectively; Fig. 1B). Moreover, high *GPNMB* expression was associated with shorter metastasis-free (Fig. 1C) and overall survival times (Fig. 1D). We examined *GPNMB* transcript levels in two additional data sets, which contained 118 (Supplementary Fig. S1A; ref. 16) and 99 breast tumors (Supplementary Fig. S2A; ref. 17). High *GPNMB* expression was again enriched in the basal-like subtype (Supplementary Figs. S1B and S2B) and correlated with poor outcome in breast cancer patients (Supplementary Figs. S1C and D, and S2C).

GPNMB protein expression in tumor epithelium is associated with poor outcome in human breast cancer. We next performed immunohistochemical staining for GPNMB using a breast TMA (TMA1; Supplementary Table S1) that contains normal breast, DCIS, tumor, and lymph node metastasis tissue samples. All tissues were first classified as either GPNMB negative (<5% positively stained tissue) or GPNMB positive (≥5% positively stained tissue). Using these criteria, only 3.5% of the normal breast tissue samples were considered GPNMB positive (Supplementary Fig. S3A). In contrast, significantly higher percentages of DCIS lesions (26.8%) and malignant tissues (41.3% of tumors; 15% of lymph node metastases) expressed GPNMB (Supplementary Fig. S3A).

Given that GPNMB is expressed in both the tumor epithelium and stroma (Supplementary Fig. S3B), we assessed the association between tumor epithelial or stromal GPNMB positivity and poor clinical outcome. GPNMB

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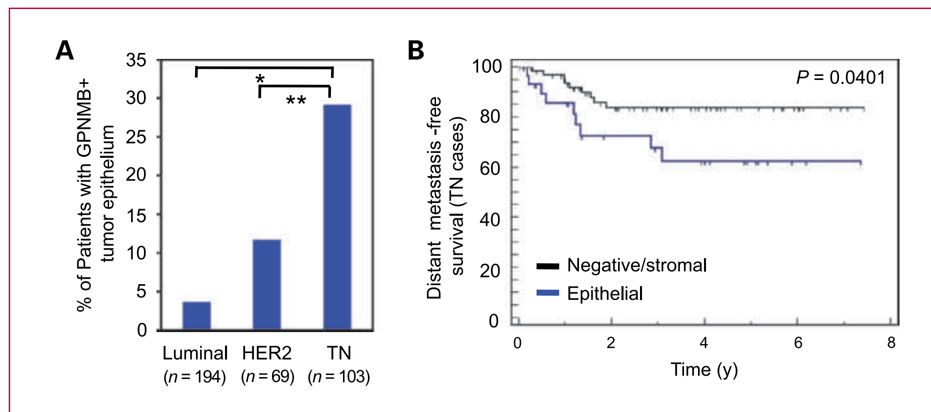

Fig. 3. GPNMB expression is associated with recurrence in triple-negative breast tumors. A, tumors on the TMAs (TMA1 and TMA2) were classified as luminal (*n* = 194), HER2+, (*n* = 63) or triple-negative (TN; *n* = 101). The percentage of tumors within each subtype that are GPNMB-epithelial positive is shown. *; *P* < 0.0001 for patients with triple-negative tumors relative to luminal tumors; **, *P* = 0.0082 for patients with triple-negative tumors versus HER2 tumors. B, Kaplan-Meier analysis of recurrence-free survival for patients with triple-negative breast cancer. *, *P* = 0.0401 for GPNMB-positive (GPNBM+) triple-negative tumors (*n* = 30) versus GPNMB-negative/GPNMB-stromal–positive triple-negative tumors (*n* = 70).

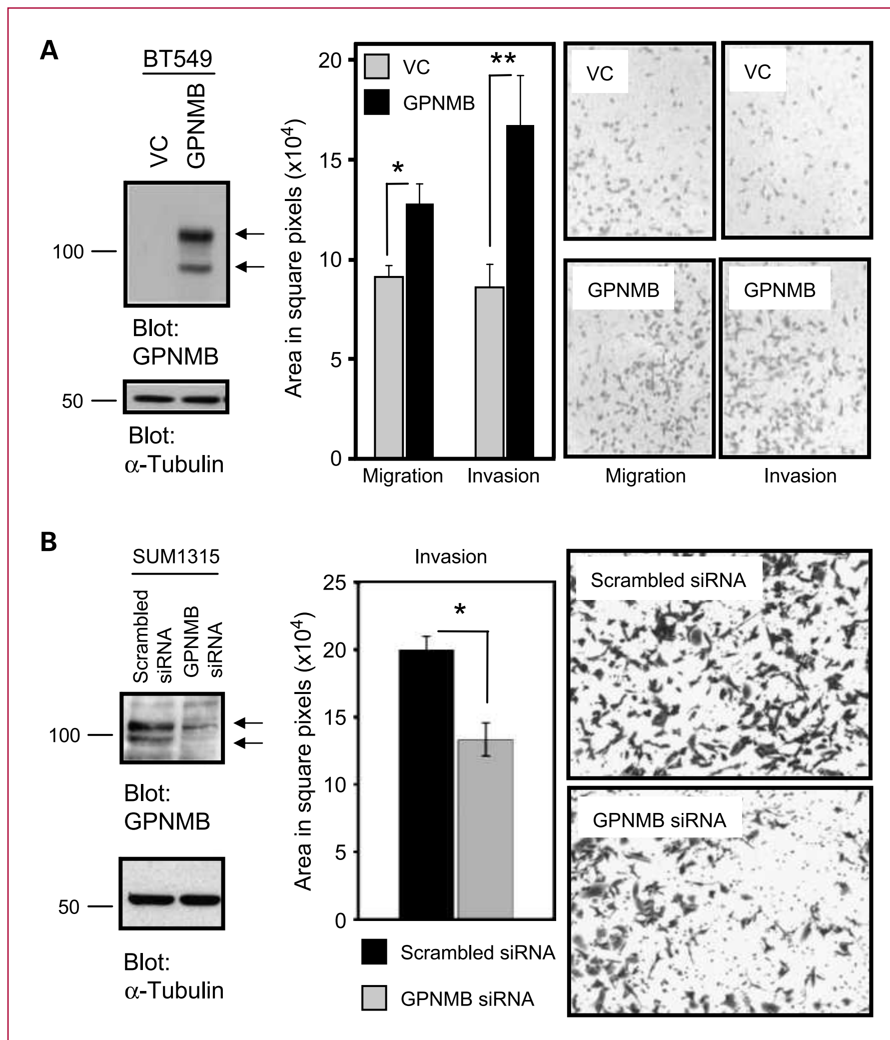

Fig. 4. GPNMB expression is necessary and sufficient to promote breast cancer cell invasion. A, left, immunoblot analyses showing ectopic expression of GPNMB in BT549 cells relative to empty vector controls (VC). Arrows, two glycosylated forms of GPNMB, which migrate at 115 and 80 kDa. α-Tubulin (55 kDa) was used as a loading control. Modified Boyden chamber assays were used to assess the migration and invasion of VC and GPNMB-expressing BT549 cells. Center, quantification of migration and invasion assays; right, representative images. *, $P < 0.001$; **, $P < 0.0001$, t test for independent samples (two tailed). B, left, immunoblot analysis showing transient siRNA-mediated reduction in GPNMB expression in SUM1315 cells versus cells treated with a scrambled siRNA control (as described in A). Center, quantification of invasion assays; right, representative images. *, $P < 0.0008$. t test for independent samples (two tailed).

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was localized in both epithelial and stromal tissue within the majority of GPNMB-positive cores (Supplementary Fig. S3B). However, the degree to which GPNMB staining segregated between these tissue compartments was highly variable. We therefore classified tumors into three categories: negative (Fig. 2A), stromal positive (Fig. 2B), or epithelial positive (Fig. 2C) based on where the predominant GPNMB staining occurred. Following these criteria, the majority of tumors were found to be GPNMB-stromal (64.1%), followed by GPNMB-negative (25.2%), and finally GPNMB-epithelial (10.7%; Supplementary Fig. S4A). We show that high GPNMB levels within the tumor epithelium is significantly associated with reduced recurrence-free survival relative to patients that either lack or display predominantly stromal patterns of GPNMB expression (Supplementary Fig. S4B; Fig. 2D). Moreover, no significant difference was observed in recurrence-free survival in patients with GPNMB-negative versus GPNMB-stromal breast cancers ($P = 0.3822$; Supplementary Fig. S4B). When analyzed concurrently with established prognostic factors in a multivariate Cox model for recurrence-free survival, epithelial GPNMB staining stood out as an independent prognostic indicator of recurrence ($P = 0.0199$; Table 1).

To validate the observation that GPNMB-epithelial staining is specifically associated with decreased overall survival, we interrogated a gene expression data set derived from laser capture–dissected epithelial and stromal tissues from breast cancer patients (19, 20). High *GPNMB* mRNA expression within the epithelial, but not stromal compartment, was associated with reduced overall survival in breast cancer patients (Supplementary Table S2). Taken together, these results show that high GPNMB expression within the tumor epithelium functions as an independent prognostic indicator of breast cancer recurrence.

GPNMB expression correlates with recurrence within the triple-negative breast cancer subtype. The correlation between epithelial GPNMB expression and disease recurrence may be explained by the observation that GPNMB is most often expressed in aggressive basal/triple-negative

breast cancers (Fig. 1B). To facilitate and extend these analyses, we interrogated a second independent cohort of breast cancers (TMA2), which are enriched in triple-negative breast cancer samples (Supplementary Table S3). Using both TMAs (TMA1 and TMA2), we defined three subtypes based on immunohistochemical staining for ER, PR, and HER2. Thus, ER and/or PR-positive tumors that were HER2 negative were classified as luminal (n = 194); HER2-expressing tumors were classified as belonging to the HER2 subtype (n = 69); and finally, those tumors lacking ER/PR and HER2 expression were defined as triple negative (n = 103). We investigated whether GPNMB expression, specifically in the tumor epithelium, correlated with histologic subtype among 366 breast tumors. We observe that 29.1% of triple-negative tumors are GPNMB-epithelial positive compared with only 3.6% of luminal and 11.6% of HER2 tumors (Fig. 3A). Given that epithelial GPNMB staining is an independent prognostic indicator of recurrence (Table 1), we determined whether epithelial-specific GPNMB expression is associated

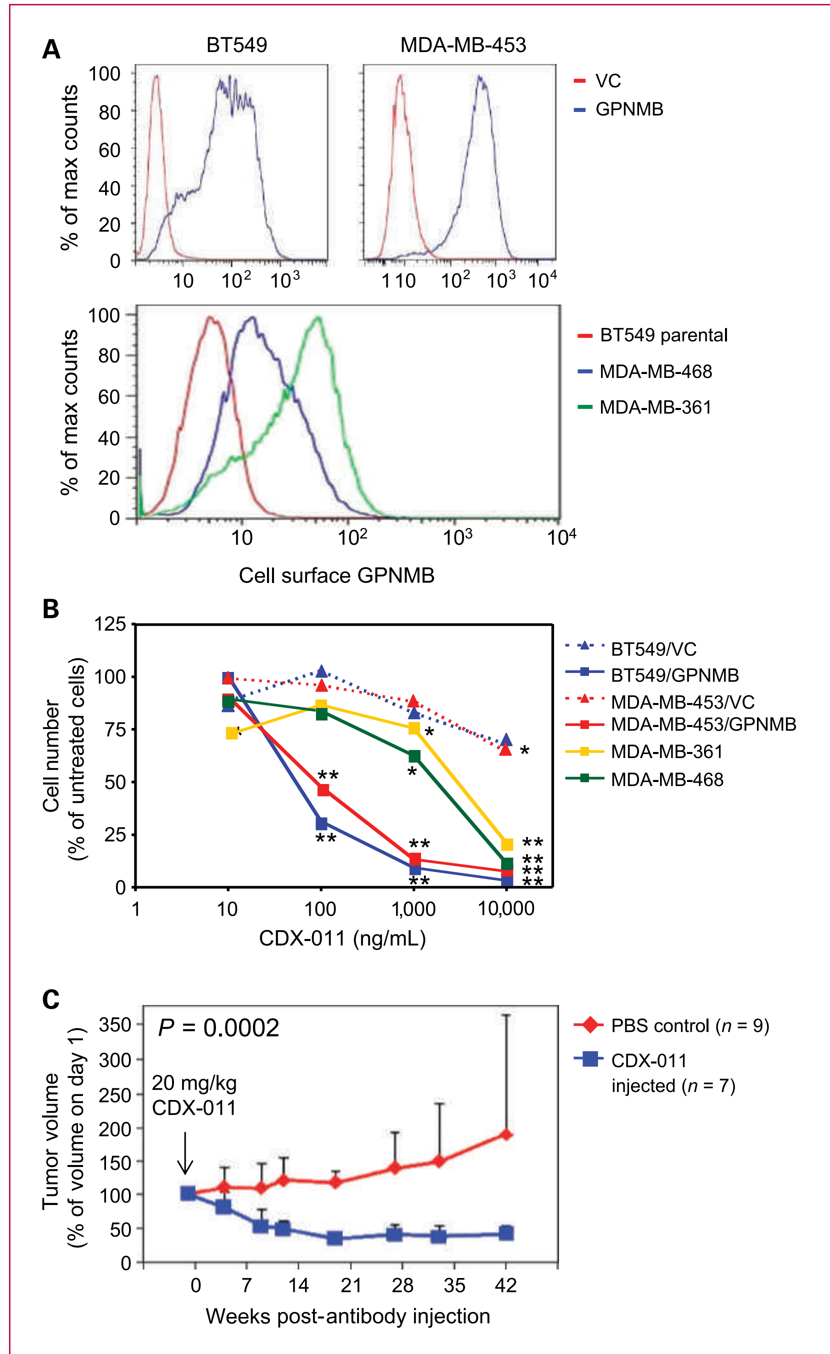

Fig. 5. GPNMB is expressed at the cell surface of breast cancer cells and is a target of the novel therapeutic, CDX-011. A, fluorescence-activated cell sorting analysis on breast cancer cells that exogenously (BT549 and MDA-MB-453) or endogenously (MDA-MB-468 and MDA-MB-361) overexpress GPNMB. B, cells were incubated with CDX-011 for the times indicated and the percentage of remaining adherent cells was quantified. The data are represented as a percentage of the adherent cells remaining in mock-treated cultures. Experiments were done in triplicate wells for a minimum of three independent experiments. *, $P < 0.05$; **, $P < 0.0001$, t test for independent samples. C, mice were injected with MDA-MB-468 cells into the mammary fat pad, allowed to reach a tumor volume of 125 mm^3, and then injected with a single dose of CDX-011 (20 mg/kg; n = 7) or PBS (n = 9) as a control. Values represent the percent tumor volume relative to the tumor volume measured 1 d before injection of the toxin-conjugated antibody or PBS control. Statistical differences in tumor growth between antibody-treated and PBS-injected controls were determined using the nonparametric Mann-Whitney test for serial measurements (P = 0.0002).

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with breast cancer recurrence specifically within the triple-negative subtype. Within the triple-negative subtype, Kaplan-Meier survival analysis shows that patients with GPNMB-epithelial–positive tumors (n = 30) display significantly shorter recurrence-free survival times relative to patients with GPNMB-negative or GPNMB-stromal–positive tumors (combined, n = 70; Fig. 3B). Moreover, multivariate Cox regression survival analysis revealed that GPNMB still functioned as an independent prognostic indicator of distant metastasis in triple-negative tumors (Supplementary Table S4). This association between high GPNMB expression and increased incidence of distant metastasis was corroborated in an independent gene expression data set (17) consisting of 30 triple-negative breast cancer patients (Supplementary Table S5). Thus, not only is GPNMB-epithelial expression more common in triple-negative breast cancers, but even within this subtype, its expression correlates with an increased risk of recurrence.

GPNMB is a therapeutic target for CDX-011 in breast cancer cells. We have reported that GPNMB levels vary widely across established human breast cancer cell lines (13). To determine whether GPNMB is sufficient to promote a motile and invasive phenotype in human breast cancer cells, we selected BT549 cells that represent a basal cell line (21) lacking endogenous GPNMB expression (Fig. 4A). Ectopic GPNMB expression significantly increased the invasiveness of BT549 breast cancer cells (Fig. 4A). Importantly, overexpression of GPNMB did not induce cell growth in BT549 cells (Supplementary Fig. S5), indicating that GPNMB-mediated effects on breast cancer cell invasion cannot be attributed to the enhancement of cell growth. To examine whether GPNMB expression is necessary for an invasive phenotype, we transiently expressed a GPNMB siRNA in SUM1315 cells, a human basal breast cancer cell line that expresses high endogenous GPNMB levels. We confirmed that GPNMB protein levels were reduced in SUM1315 cells transfected with GPNMB-specific siRNAs relative to cells treated with a scrambled control. We observed a statistically significant reduction in breast cancer cell invasion in GPNMB-siRNA–expressing cells relative to control cells (Fig. 4B). These results confirm our earlier results and support a role for GPNMB in promoting the motility and invasion of basal breast cancer cells.

Our data establishing an association between GPNMB expression and poor outcome in triple-negative breast cancer, coupled with its cell surface expression and ability to promote breast cancer motility and invasion, make it an attractive candidate for targeted therapies. Indeed, cell surface expression of GPNMB was readily detectable in cells that endogenously (MDA-MB-361, MDA-MB-468) or exogenously (BT549, MDA-MB-453) expressed GPNMB (Supplementary Fig. S6A; Fig. 5A). To determine if GPNMB represents a feasible target for breast cancer therapy, we tested the effects of CDX-011, an antibody-drug conjugate that specifically targets GPNMB, on tumor cell growth and survival. This antibody conjugate can kill GPNMB-expressing melanoma cells (9, 14). Breast cancer cells expressing low (MDA-MB-453-VC, BT549-VC), moderate (MDA-MB-361, MDA-MB-468), or high (MDA-MB-453-GPNMB, BT549 GPNMB) levels of cell surface GPNMB were incubated with increasing concentrations of CDX-011 (Fig. 5B). The growth of both moderate and high GPNMB-expressing cells was inhibited by CDX-011 in a dose-dependent manner, whereas an IC_{50} was not achieved with concentrations up to 10 μg/mL CDX-011 in low GPNMB-expressing cells. Unconjugated CDX-011 or isotype control antibodies were unable to induce this effect (Supplementary Fig. S6B). Treatment of breast cancer cells with the CDX-011 drug conjugate lead to elevated apoptosis in GPNMB-expressing breast cancer cells, as indicated by increased cleaved caspase-3 levels (data not shown).

We next examined whether the administration of CDX-011 could impair breast cancer growth *in vivo*. To accomplish this, MDA-MB-468 cells were injected into the mammary fat pads of nude mice and allowed to grow to a tumor volume of 125 mm^3. Tumor-bearing mice were then divided into two groups: one that received a single injection of 20 mg/kg of CDX-011, whereas the other cohort received control PBS injection. We observed a significant diminishment in tumor growth in mice receiving the CDX-011 conjugate compared with PBS controls (Fig. 5C). These data show that CDX-011 effectively targets and kills breast cancer cells that express GPNMB at the cell surface. Given that tumor epithelial-GPNMB expression is associated with poor outcome within the triple-negative subtype, this GPNMB-targeted conjugate represents a novel therapeutic option for treating basal-type breast cancer patients.

Discussion

The molecular classification of breast cancer underscores the heterogeneity of this disease (1–3). The poor prognosis associated with triple-negative breast cancer, coupled with the lack of therapeutic targets, has created intense clinical interest in these tumors (22–25). Recent studies have reported that triple-negative tumors with basal-like features (those expressing some or all of the following proteins: CK5/6, CK14, CK17, epidermal growth factor receptor) are associated with worse clinical outcomes than triple-negative tumors lacking these markers (23, 26). Although it is unknown whether GPNMB contributes to the basal-like phenotype, our observations identify GPNMB as a prognostic marker in triple-negative breast cancers and support the clinical development of GPNMB-targeted therapies. Interestingly, recent evidence suggests that signaling through the estrogen receptor can suppress GPNMB expression (27, 28), which is consistent with our observation that GPNMB is more commonly expressed in triple-negative breast cancers.

An unexpected finding of this study was the heterogeneous GPNMB staining observed among the various tumor compartments, with high levels of GPNMB evident in tumor stroma relative to normal tissue. This is supported by independent gene expression profiling studies that reveal

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higher GPNMB levels in tumor-associated stroma compared with that derived from normal breast (19, 29). Within the stromal compartment, an independent study identified increased GPNMB expression in tumor-derived endothelium relative to normal endothelial cells (30). GPNMB is highly expressed in dendritic cells (31) and macrophages (8), raising the possibility that some of the stromal staining within primary breast tumors may represent immune cell infiltrates. Moreover, osteoactivin expression has been linked to fibroblast activation (32) and, thus, might be expressed in cancer-associated fibroblasts. Although our studies indicate that GPNMB expressed within the cancer epithelium is associated with disease recurrence, the role of stromal GPNMB in supporting the tumor microenvironment is intriguing, and warrants further investigation.

We provide the first evidence of a relationship between GPNMB expression in primary breast tumors and metastatic occurrence. We are the first to show that GPNMB-expressing breast cancer cells can be selectively killed by a toxin-conjugated antibody directed against GPNMB (CDX-011). Cancer therapy using toxin/drug-conjugated antibodies is becoming increasingly popular (33) and includes a cytotoxin-conjugated version of Herceptin, Trastuzumab-DM1, which is currently being investigated in clinical trials for metastatic breast cancer (33, 34). In a phase I/II clinical trial for the treatment of melanoma, CDX-011 was shown to have clinical activity and was well tolerated (33). Moreover, initial results from an ongoing phase I/II trial show that tumor shrinkage was observed in CDX-011–treated patients with metastatic breast cancer. Our observations that GPNMB is highly expressed in recurrent breast cancers but rarely in normal breast tissue, coupled with our observations that CDX-011 effectively inhibits the growth of GPNMB-expressing breast cancer cells *in vitro*, suggest that GPNMB represents a promising therapeutic target in breast cancer.

We show that epithelial-specific GPNMB expression is an independent prognostic indicator of recurrence. Therefore, immunohistochemistry staining of biopsy material for epithelial GPNMB expression could be used to predict responders to CDX-011 in future clinical trials. The molecular processes that modulate cell surface expression of GPNMB, such as trafficking, internalization, and shedding of its extracellular domain (35, 36) must be characterized to optimize GPNMB-targeted therapies. Such research will provide important insights into the molecular mechanisms through which GPNMB exerts its effects on breast cancer progression.

Disclosure of Potential Conflicts of Interest

R. Simantov was an employee of CuraGen Corp.

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