The laminin-derived peptide C16 regulates GPNMB expression and function in breast cancer

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ABSTRACT
Breast cancer is an important public health problem, and its progression may be related to the extracellular matrix (ECM), which acts as a structural scaffold and instruction source for neoplastic cells. Laminins are ECM proteins regulating tumor biology. The laminin-derived peptide C16 regulates different properties of tumor cells. Here we analyzed C16-induced differential gene expression in MDA-MB-231 breast cancer cells. MCF-10A normal-like breast cells served as control. Among different cancer-related genes, C16 induced overexpression of GPNMB. This gene encodes a transmembrane protein GPNMB (glycoprotein non-metastatic B), involved with malignant phenotype of breast cancer cells. Immunoblot validated microarray results. To correlate gene and protein expression with cellular function, we investigated whether C16 would regulate invasion in breast cancer cells. siRNA experiments strongly suggested that C16 and GPNMB cooperate to regulate invasion of highly aggressive MDA-MB-231 cancer cells. We addressed regulatory mechanisms involved in C16-mediated increase of GPNMB protein levels in MDA-MB-231 cells, and observed that C16 stimulates β1 integrin and Src phosphorylation. Furthermore, Src inhibition decreases peptide-induced GPNMB expression levels. To contextualize in vivo our results in vitro, we addressed GPNMB immunostaining in breast cancer human tissue microarrays. Quantitative immunohistochemistry showed that GPNMB is significantly more expressed in breast cancer compared to normal tissue. We concluded that laminin-derived peptide C16 regulates gene and protein expression of GPNMB in breast cancer cells. C16 and GPNMB may cooperate to regulate invasion of highly aggressive MDA-MB-231 cells, probably through Src signaling. GPNMB presented increased expression in breast cancer in vivo compared to normal breast tissue.

1. Introduction
Breast tumors are the second most common type of cancer worldwide, affecting mostly women [1–3]. In Brazil, the National Cancer Institute (INCA) estimates that, in 2016, 57,960 new cases of breast tumors would be diagnosed; representing the second most frequently diagnosed non-skin cancer type [4]. Certain reproductive factors, age, weight and hormone levels (especially in menopause) have significant influence on breast cancer occurrence [1–3,5–8].

Like other types of tumors, breast cancer originates from a single normal cell that acquires genetic or epigenetic changes, undergoes monoclonal expansion, accumulates molecular alterations, and finally progresses to an invasive stage [9,10]. The complex pattern of alterations acquired by the tumor cells can influence a variety of cellular functions, including proliferation, migration, invasion, survival, and capacity to induce angiogenesis [9,10]. Besides molecular alterations acquired by tumor cells, during tumor progression cells are engaged in a complex interplay with the surrounding microenvironment, which can also influence tumor behavior [5,11]. The extracellular matrix (ECM) plays an important role in this context. Composed of collagens, proteoglycans, elastin and structural glycoproteins, ECM is a three-dimensional network that may act both as structural scaffold and instruction source for cells [12–17]. The basement membrane is a specialized ECM sheet-like structure predominantly composed by laminin, type IV collagen, nidogen and perlecan [18–21].

Laminins are basement membrane glycoproteins, formed by three
different polypeptide chains, termed α, β, and γ [18,22–24]. It has been shown that laminin modulates different cellular processes such as cell adhesion, migration, growth, and differentiation [18]. A crucial step in tumor development is the proteolytic cleavage of basement membrane components, including laminin, through the action of specific proteases secreted by both tumor and stromal cells [25]. This process not only removes physical barriers to cell invasion but also unmasks cryptic bioactive peptides [26]. An increasing number of evidences have shown that peptides derived from laminin cleavage may control tumor behavior [27–38]. Among them, C16 (KAFDITYVRLKF), derived from laminin-111 γ–1 chain, is a cell-adhesive peptide related to migration, metastatic activity, and angiogenesis [37,39–41]. Previous works from our group also demonstrated that C16 is a major regulator of invadopodia formation and activity [34,42].

Although these evidences suggest that C16 plays a relevant part in tumor progression, its influence in genetic programs of breast cells has not yet been investigated. This prompted us to analyze C16-induced differential gene expression in MDA-MB-231 breast cancer cells (highly aggressive, triple negative for estrogen, progesterone and HER-2 receptors). Normal-like MCF-10A cell line served as control. Our results showed that C16 induced overexpression of GPNMB gene (glycoprotein non-metastatic B) in neoplastic cells. GPNMB is a potential biomarker associated with increased cell migration, invasion, angiogenesis and bone metastasis of breast cancer cells [43–47]. We also found that MDA-MB-231 cells present high levels of GPNMB protein.

To correlate gene and protein expression with cellular function, we investigated whether C16 and GPNMB would cooperate to regulate the invasive phenotype of breast cancer cells. Regulatory mechanisms involved in C16-mediated increase of GPNMB protein levels were analyzed. Furthermore, to contextualize in vivo our results in vitro, we have addressed GPNMB immunostaining in breast cancer human tissue microarrays (TMAs).

2. Material and methods

2.1. Peptides

EZ Biolab (Westfield, IN, USA) synthesized peptide C16 (KAFDITYVRLKF) and scrambled control peptide C16SX (FKLRVYTIDFAK). Peptides purity was 98% (RP-HPLC), and molecular mass was confirmed by mass spectrometry. Peptides C16 and C16SX were diluted in culture medium without serum or supplements for all experiments.

2.2. Cell culture

MDA-MB-231 cells were purchased from the Cell Bank of Rio de Janeiro, Brazil. MCF-10A cells were kindly provided by Prof. Nathalie Cella (Department of Cell and Developmental Biology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil). This work did not involve direct manipulation of humans or animals, and was approved by ICB Ethics Committee (Protocol CEP-ICB 524/12).

Cells derived from invasive ductal breast carcinoma (MDA-MB-231, "basal-like", triple-negative for estrogen, progesterone and HER-2 receptors) were cultured in Dulbecco’s modified Eagle medium (DMEM, Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Carlsbad, CA, USA). Normal-like breast epithelial cells (MCF-10A) were grown in DMEM plus Ham’s F12 nutrient (DMEM: F12, Sigma) supplemented with 10 mg/ml insulin, 0.1 mg/ml cholaer toxin, 0.5 mg/ml hydrocortisone, 5% FBS and 10 ng/ml EGF (all from Sigma). Cells were maintained in 75 cm² flasks in an atmosphere of 5% CO₂ at 37 °C.

MDA-MB-231 and MCF-10A were cultured in 6-well plates and treated with peptide C16 (100 µg/ml) diluted in serum free medium. Control groups were treated with scrambled peptide C16SX diluted in serum-free medium. Non-peptide controls were used. Positive control was media with 10% FBS. Negative control was serum-free media.

2.3. Total RNA extraction

After treatment of cells with C16 or C16SX (100 µg/ml) for 24 h, total RNA was extracted using PureLink RNA Mini kit (Life Technologies), following manufacturer’s instructions. Total RNA concentration was determined by spectrophotometry at 260/280 nm (NanoDrop Technologies, Wilmington, DE, USA), and its structural integrity was confirmed by visual inspection of ribosomal RNA bands in 1% agarose gel stained with ethidium bromide.

2.4. cDNA microarray

We used the Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA) to analyze the expression of 28,869 human genes in MDA-MB-231 and MCF-10A cells treated with C16 in comparison to treatment with scrambled control peptide (C16SX). Ambion WT Expression Kit (Life Technologies) was used to generate sense-strand complementary DNA (cDNA) from total RNA. Briefly, 100 ng of total RNA underwent cDNA synthesis using random primers containing a T7 promoter sequence. Afterwards, complementary RNA (cRNA) was generated through an in vitro transcription, and then used to synthesize sense-strand cDNA. Sense-strand cDNA was then fragmented and end-labeled with Biotin Allomide Triphosphate. Labeled cDNA (2.5 µg) was hybridized to the Human Gene 1.0 ST Array for 17 h at 45 °C and 60 rpm. GeneChips were then washed, stained with streptavidin-phycocerythrin, and scanned. Conversion of the acquired fluorescence intensities to numerical values was performed using the Expression Console software (Affymetrix). Data was summarized using the iterPlier algorithm with background adjustment (PM - GCBG method), and quantile normalization. Normalized gene expression values were imported into the TIGR Multi-Experiment Viewer 4.6 software (http://www.tm4.org/mev/) for statistical analysis. Student’s t-test was performed to assess gene expression differences between treatments with C16 and C16SX. Genes whose expression levels were increased or decreased by more than 1.2-fold and showed statistical significance (p ≤ 0.05) were considered differentially expressed. Heatmap of differentially expressed genes (DEG) was generated using the freely available software Heatmap Builder 1.1 (Stanford University, Stanford, CA, USA) [25]. Gene Ontology term annotation was provided by web-based software Molecule Annotation System (MAS) 3.0 (CapitalBio Corporation, Changping District, Beijing, China).

2.5. Immunoblot

MDA-MB-231 and MCF-10A cells were grown on 6-well plates and incubated with either C16 or C16SX scrambled control peptide for different time intervals. All samples were subjected to immunoblot, as described elsewhere [48]. Samples were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) containing protease and phosphatase inhibitors when necessary (NaF, Orthovanadate Sodium, pepstatin A, PMSF, and E-64, Sigma). Samples were centrifuged at 10,000g for 10 min at 4 °C and the supernatant collected. Quantitation of proteins was carried out using the BCA method (Thermo, Rockford, IL USA). Samples were resuspended in Laemmli buffer containing 62.5 mM Tris–HCl pH 6.8, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 5% mercaptoethanol, 0.001% bromophenol blue. Equal amounts (30 µg) of cell lysates were electro-phoresed in 10% polyacrylamide gels. Proteins were transferred to a Hybond ECL nitrocellulose membrane (Amersham), blocked in TBS with 5% non-fat milk or BSA 3% for one hour. The following primary antibodies were used: GPNMB (1:250, sc-271415, Santa Cruz Biotechnology), and β-actin (1:10,000, A5316, Sigma). Membranes were revealed by ECL protocol.
2.6. Invasion assay

Invasion assays were carried out in Boyden chambers coated with 13 mg/ml Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Membrane pore size was 8 μm-diameter. After a starvation period of 1 h, MDA-MB-231 and MCF-10A cells (15 × 10^4) were plated in the upper chamber wells in serum-free media. Lower chamber wells were filled with media containing either C16 or C16SX (100 μg/ml), or with non-peptide positive or negative controls. Cells were cultured in these conditions for 48 h, and then fixed with 4% paraformaldehyde. Cells on the upper side of the polycarbonate membrane were removed, while invading cells, located on the lower side of the membrane, were stained with crystal violet, photographed at a final magnification of 200x, and counted. On each well, 7 random fields were evaluated. Invasion assays were conducted in triplicates.

2.7. Invasion assay in cells with depleted GPNMB by siRNA

In order to assess the involvement of GPNMB in the invasion process possibly triggered by C16, MDA-MB-231 cells were transfected with commercially available siRNA targeting GPNMB (80 nM, Santa Cruz Biotechnology, sc-60721) following the manufacturer’s instructions. One day before transfection, sub confluent MDA-MB-231 cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum. Cells were incubated with a complex formed by the siRNA, transfection reagent (Lipofectamine 2000; Invitrogen), and transfection medium (Opti-MEM; Invitrogen) for 48 h at 37 °C. Control siRNA (80 nM, Santa Cruz Biotechnology, sc-37007), a non-targeting siRNA, served as negative control. Cells were submitted to invasion assays in presence of C16 as described above. Additionally, cells were subjected to invasion assays in the presence of scrambled peptide C16SX.

At least three siRNA oligos were used in these experiments yielding similar results.

2.8. Effect of C16 in β1 integrin expression

GPNMB is described as pro-growth and pro-metastatic molecule due to its ability to bind α5β1 integrin and increase downstream signaling in breast cancer cells [49]. This prompted us to evaluate whether C16 would increase β1 integrin levels in breast cancer cells. MDA-MB-231 and MCF-10A cells were treated by either C16 or C16SX and subjected to immunoblot to detect β1 integrin (1:1000, MAB 2079Z Millipore).

We also addressed C16-induced colocalization of GPNMB and β1 integrin in MDA-MB-231 cells. Treated and control samples were fixed in 4% paraformaldehyde in PBS for 15 min, followed by blocking with 3% bovine serum albumine (BSA, Sigma). Cells were stained with antibodies against β1 integrin-conjugated with Alexa Fluor 555 (MAB2079-AF555, Millipore) and GPNMB (sc-271415, Santa Cruz). GPNMB was labeled by a secondary antibody. Samples were mounted with Pro Long Gold with DAPI (Life Technologies). Autoradiography was performed using Graph Pad Prism 5.0 software (Graph Pad, San Diego, CA, USA). Non-immune serum served as negative control. Additional statistical analyses were performed using Graph Pad Prism 5.0 software (Graph Pad, San Diego, CA, USA). Differences between groups were assessed by either ANOVA (parametric) or Kruskal-Wallis (non-parametric) followed by appropriated multiple comparison tests. Differences were considered significant when p ≤ 0.05.

3. Results

3.1. C16 regulates gene expression in MDA-MB-231 and MCF-10A cells

To correlate C16 peptide, Src phosphorylation status, and GPNMB protein levels, MDA-MB-231 cells were treated by the Src inhibitor PP2, followed by C16 treatment. Cells cultured overnight in medium with 10% FBS were serum-starved for 24 h and then incubated with Src inhibitor PP2 (5 μM, Sigma). As a control, cells were incubated with vehicle (methanol). Both PP2 inhibited and control cells were treated by C16, followed by detection of GPNMB, Src and phospho-Src by immunoblot.

To contextualize in vitro our results in vitro, we studied the presence of laminin γ1 chain and GPNMB in breast cancer and normal breast tissue, using Tissue Microarray Slides (TMAs, IMA Xenex Co, San Diego, CA, USA). TMA contained 35 infiltrating duct carcinomas, 9 normal breasts, 1 ductal carcinoma in situ, 1 atypical medullary carcinoma, 1 intraductal papillary carcinoma, 1 metaplastic carcinoma, 1 sarcomatoid carcinoma, and 10 lymph node metastatic carcinoma tissue samples. Sections were deparaffinized in xylene and hydrated in decreasing concentrations of ethanol. Antigen retrieval was carried out with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6) in water bath (95–100 °C) for 30 min. Sections were blocked for 1 h with 1% bovine serum albumin (BSA, Sigma) in phosphate-buffered saline (PBS). Laminin γ1 chain was stained by a rabbit polyclonal antibody (Santa Cruz Biotechnology, sc-17763, 1:600). GPNMB was labeled by a mouse monoclonal antibody (Santa Cruz Biotechnology, sc-60721, 1:100). Antibodies were incubated overnight at 4 °C. Endogenous peroxidase blocking was carried out for 20 min, followed by incubation with NovolinkTM Polymer Detection Systems (Leica Biosystems Newcastle Ltd, Upon Tyne, UK). Diaminobenzidine (Sigma) was used as chromogen and sections were counterstained with Mayer’s hematoxylin (Sigma) and mounted with Permount (Fisher Scientific, Fair Lawn, NJ, USA). Non-immune serum served as negative control. Immunostaining was assessed by measuring GPNMB-labeled area fraction (%). Bright field images of at least five arbitrarily selected areas in each sample were acquired in a Primostar microscope equipped with an AxioCam HRc color CCD camera (Carl Zeiss, Oberkochen, Germany), using a 40X objective. The areas stained with dianaminobenzidine were separated and segmented using the color deconvolution plug-in (ImageJ software).

3.1.1. Statistical analysis

Gene Microarrays statistical analysis was carried out in TIGR Multi-Experiment Viewer 4.6 software. Additional statistical analyses were performed using Graph Pad Prism 5.0 software (Graph Pad, San Diego, CA, USA). Differences between groups were assessed by either ANOVA (parametric) or Kruskal-Wallis (non-parametric) followed by appropriated multiple comparison tests. Differences were considered significant when p ≤ 0.05.
Thirty-three genes were upregulated and forty-seven genes were downregulated by C16 in MDA-MB-231 cells (Fig. 1B). Among these genes, GPNMB was upregulated in both cell lines studied here (Fig. 1B). Since GPNMB is involved in cell adhesion, migration, invasion, metastasis and angiogenesis we decided to investigate C16-induced GPNMB increase and its consequences in MDA-MB-231 cells. C16 clearly increased GPNMB expression in tumor cell line MDA-MB-231. However, the highest effect of C16 on GPNMB expression occurred in MCF-10A cells.

Supplementary Tables I and II illustrated differentially expressed genes in MDA-MB-231 and in MCF-10A cells treated by C16 peptide.

### 3.2. C16 increases GPNMB protein levels in breast cells

C16 significantly regulated GPNMB gene expression in normal and breast cancer cells. This prompted us to investigate whether C16 would modulate GPNMB protein levels in the highly aggressive MDA-MB-231 breast cancer cell line compared to normal-like MCF-10A cells.

MDA-MB-231 and MCF-10A were treated by either C16 or scrambled peptide control C16SX (100 µg/ml). Immunoblot demonstrated that the peptide stimulated GPNMB expression in MDA-MB-231 cells.
231 cells (Fig. 2A), compared to C16SX. Discrete GPNMB increase was
detected in MCF-10A cells treated by C16 peptide (Fig. 2A). GPNMB
detection was moderate in cells treated by non-peptide controls.

Immunoblot (Fig. 2A) also showed different forms of the GPNMB. Fast
migrating band were more intense in MDA-MB-231 cells compared to MCF-10A. GPNMB levels are discrete in MDA-MB-231 and in normal-like MCF-10A cells treated by non-peptide controls. In MDA-MB-231 cells, time course analysis demonstrates that C16 significantly increases GPNMB levels 24 h after C16 treatment (B). Results in B represent mean ± standard error of three experiments. Asterisk indicates statistically significant differences (p < 0.001).

3.3. GPNMB regulates C16-induced invasion in triple negative,
highly aggressive MDA-MB-231 breast cancer cell line

GPNMB has already been associated with invasive phenotype in
other tumor cell lines [47]. Therefore, we decided to assess whether
C16 would stimulate invasion in breast cancer cells lines. Invasion
assays in Matrigel-coated Boyden chambers showed that C16 stimu-
lated invasion activity in the highly aggressive MDA-MB-231 cell line.
Cells treated by C16 showed a two-fold increase in invasive activity
compared to C16SX peptide control (Fig. 3A, left graph). Furthermore,
C16-induced invasion increased 5-fold compared to non-peptide
negative control. C16 failed to modulate invasion rate of MCF-10A
control cells (Fig. 3A, right graph). Interestingly, the control cell line MCF-10A showed an increased invasive phenotype in presence of FBS (Fig. 3A, right panel). This behavior has been previously reported [50]. To correlate protein expression and cell function, we investigated whether GPNMB would be related to C16-induced invasion in MDA-MB-231 cells. For this purpose, we carried out invasion assays using MDA-MB-231 cells with siRNA-depleted GPNMB, followed by treatment with C16 peptide (B). Cells with reduced GPNMB expression exhibit significant decrease in C16-mediated invasion compared to controls (B). Immunoblot analysis confirmed transfection efficiency (B). The siRNA control is the group transfected with the scrambled non-depleting siRNA. Results represent mean ± standard error of three experiments. Asterisk indicates statistically significant differences (p < 0.05).

3.4. Peptide C16 increases β1 integrin expression and induces GPNMB and β1 integrin colocalization

GPNMB binds α5β1 integrin and increase downstream signaling in breast cancer cells [49]. MDA-MB-231 and MCF-10A treated by peptide C16 increased β1 integrin levels after 24 h (Fig. 4A). Immunofluorescence addressed C16-induced colocalization of GPNMB and β1 integrin. The peptide induced colocalization of GPNMB and β1 integrin at lamellipodia of tumor cells (Fig. 4B, upper panel) compared to cells treated by the scrambled peptide control C16SX (Figure 4B, lower panel).

3.5. Src signaling pathway is involved in C16-induced GPNMB expression

GPNMB cooperate with integrins to activate Src signaling pathway [49]. Furthermore, we previously demonstrated that C16 stimulates β1 integrin and Src phosphorylation in different tumor cell lines [42]. C16-induced Src phosphorylation was investigated in breast cancer cells. Here we correlated C16, Src phosphorylation, and GNPMB protein levels in MDA-MB-231 cells.

C16 enhanced Src phosphorylation in MDA-MB-231 cells (Fig. 5A). Src phosphorylation increase was significant 6 h after peptide addition compared to C16SX scrambled control peptide. Moreover, a decrease in C16-mediated GPNMB protein levels was observed in MDA-MB-231 cells treated with Src inhibitor PP2, compared to cells treated with the inhibitor vehicle (methanol) and C16 (Fig. 5B). No differences in GPNMB levels were detected in Src-inhibited cells incubated with scrambled peptide C16SX.

Taken together our results strongly suggest that Src signaling pathway modulates C16-induced GPNMB protein levels in breast cancer cells.

3.6. Immunostaining of laminin γ1 chain in breast cancer compared to normal breast

To contextualize in vivo our results in vitro, we assessed laminin γ1 chain and GPNMB immunostaining in breast cancer samples compared to normal breast tissue. Strong γ1 laminin reactivity was detected in normal mammary glands (Fig. 6A). On the other hand, GPNMB labeling was discrete or absent in similar regions (Fig. 6B). Tumor breast tissue showed diffuse and discontinuous γ1 laminin staining (Fig. 6C, arrows). Interestingly, increased GPNMB detection was seen in corresponding areas (Fig. 6D). Negative controls exhibited no staining (not illustrated).

These results suggest that γ1 laminin disruption may correlate with increased GPNMB detection.
3.7. GPNMB staining is prominent in human breast cancer

Area fraction analysis showed that GPNMB staining was significantly increased in lymph node metastatic carcinoma compared to normal breast tissue (Fig. 7A and B). Following breast cancer molecular classification [51,52], increased GPNMB immunostaining was observed in more aggressive subtypes basal-like and HER2 neoplasms compared to normal breast (Fig. 7C). Considering tumor stage, augmented GPNMB labeling was observed in IIA, IIIA and IIIC staging compared to normal breast (Fig. 7D).

4. Discussion

We have demonstrated that peptide C16, derived from laminin γ1 chain, increases the expression of GPNMB gene and protein levels in MDA-MB-231 and MCF-10A cells. This peptide also stimulated invasion of MDA-MB-231 cells. We also found that GPNMB knockdown decreases C16-induced invasive phenotype cell of MDA-MB-231 cells. β1 integrin and Src signaling pathway are related to C16-induced GPNMB expression in breast cancer cells. GPNMB has a clear relevance in human breast tumors, as shown by TMA analysis. To our knowledge, this study demonstrates for the first time that a
laminin-derived peptide and a putative breast cancer biomarker cooperate to regulate the invasive phenotype of a highly aggressive breast cancer cell line.

Tumor development involves a direct relationship between cancer cells and extracellular matrix. In this context, controlled ECM proteolysis may have an important impact regulating tumor biology [26,53–56]. Laminin is a major ECM protein and presents cryptic sites in its structure, which may be exposed by protease activity [54,58]. C16 (KAFDITYVRLKF), located at the γ1 chain, could be one of these exposed sites. The γ1 chain of laminin is found in breast epithelium and blood vessels basement membranes [18], thus being prone to proteolysis. Additionally, mass spectrometry studies showed that MCF-10A cells treated by proteases generated an amino acid sequence similar to C16 (except for the absence of the two last amino acids in the carboxy-terminal end), strongly suggesting that the peptide is a product of cell-induced proteolysis [57]. C16 has been associated with cell adhesion [41], angiogenesis [37] and secretion of metalloprotei-

Microarray analysis revealed that C16 peptide regulates expression of genes associated with breast cancer cells. This peptide increased the expression of genes involved with different aspects of cancer biology, among them adhesion, migration, invasion and metastasis. The GPNMB (glycoprotein non-metastatic melanoma protein B) gene was a strong hit in both normal and breast cancer microarrays. Microarray results were confirmed by immunoblot, showing increased GPNMB protein levels in breast cancer cells compared to normal-like cells. No correlation was found between gene expression and protein levels in normal-like MCF-10A breast cells. Biological reasons for this discrepancy include post-transcriptional and post-translational modifications [59–61]. It has also been suggested that protein concentrations correlate with the corresponding mRNA levels by only 20–40% [61].

GPNMB is a type I single-pass transmembrane protein with a large extracellular domain composed by an integrin-binding (RGD) motif and a polycystic kidney disease (PKD) domain [62]. Cytoplasmic tail is short with 53 amino acids [63]. This molecule is expressed in
approximately 40–75% of breast cancers and promotes migration, invasion and metastasis [45,46,49,62,63].

Our immunoblot results showed different forms of GPNMB. Fast migrating band were more intense in MDA-MB-231 cells compared to MCF-10A. This result would raise the possibility that GPNMB is post-translationally processed in the cancer cells but not in the control cells. It is known that proteases such as ADAM10 release a soluble ectodomain of this protein [63]. We addressed whether GPNMB fast bands would be the results of MMP-mediated proteolysis (Supplementary Fig. 1). MMP-inhibition by GM6001 clearly decreased fast migratory bands in treated and control cells. However, no difference was found in either C16 or C16SX control samples. On the other hand, GPNMB was not detected in the conditioned media. We may infer that C16 induced no GPNMB shedding, since no soluble fraction of this molecule was detected in the conditioned media.

We have already demonstrated that C16 increases invasion and invadopodia activity in cancer cell [34,42]. Here, we showed that C16 increased invasion rate of MDA-MB-231 cells. Our results showed that C16 increased GPNMB gene and protein expression in breast cancer cells. Furthermore, C16 stimulated invasion of a highly aggressive triple negative MDA-MB-231 cells. We then decided to investigate whether GPNMB would interact with C16 regulating invasion of MDA-MB-231 cells. GPNMB depletion diminished C16-induced invasion, showing that this protein and the peptide C16 may cooperate to regulate breast cancer invasiveness. Integrin-binding properties may explain GPNMB and C16 interaction. GPNMB exhibits a RGD motif [62,63]. It is well known that this RGD motif binds integrins, mostly α5β1 and αvβ3 heterodimers [64]. Moreover, integrins α5β1 and αvβ3 integrins have been reported as C16 partners [65]. We further addressed C16-induced colocalization of GPNMB and β1 integrin. MDA-MB-231 cells treated by C16 exhibited colocalization of GPNMB and β1 integrin at lamellipodia. Thus, we may conclude that integrins are involved in the cooperation between C16 and GPNMB.

C16 interactions with integrins receptors may activate different signaling pathways [34,42]. We may infer that β1 integrin would bind GPNMB, generating signals transduced by Src signaling pathway in breast cancer [44]. We observed an increase of β1 integrin by immunoblot, a significant Src phosphorylation at 6 h and an increase of GPNMB at 24 h in time-course experiments; all triggered by C16 peptide in MDA-MB-231 cells. Furthermore, cells treated with Src inhibitor decreased C16-induced GPNMB protein levels. Taken together these results strongly suggest that C16 would interact with β1 integrin, generating signals to be transduced by the Src pathway, thus regulating GPNMB expression and the invasive phenotype of a highly aggressive breast cancer cell line. Diagram portrayed in Fig. 8 summarizes our current model for the effect of C16 on GPNMB expression and how it modulates cancer cell invasion.

GPNMB is associated with bone metastasis of breast carcinoma and is overexpressed in aggressive breast cancer (basal-like or triple-negative) [43]. Our results indicate that GPNMB expression is related to laminin breakdown. We showed laminin γ1 chain in breast cancer and normal breast. Laminin γ1 chain, containing the peptide C16, is disrupted in breast cancer. Furthermore, our data suggests that γ1 laminin disruption appears to correlate with GPNMB increased immunostaining. We may assume that laminin disruption in breast cancer would release bioactive peptides, among them C16, thus stimulating GPNMB levels. TMA analysis confirmed

![Normal breast](image1)

**Fig. 6.** Laminin γ1 chain disruption may correlate with increased GPNMB detection. Strong γ1 laminin reactivity is detected in normal mammary glands (A). GPNMB labeling is discrete or absent in similar regions (B). Tumor tissue shows diffuse and discontinuous γ1 laminin staining (C, arrows). Increased GPNMB detection is observed in corresponding areas (D). Scale bars: 50 µm.
that GPNMB immunostaining was significantly increased in breast cancer compared to normal tissue. These differences were consistently observed taking into account different parameters such as tumor type, molecular classification and staging, and in all parameters analyzed here GPNMB was prominent in more aggressive subtypes.

We have attempted to elucidate mechanisms involved in C16 effects, to better understand the role played by this peptide in breast cancer. Based on our experimental findings we propose that C16 enhances GPNMB gene expression and protein levels in cells derived from breast cancer. Furthermore C16 and GPNMB may cooperate to regulate cellular invasiveness of breast cancer cells, probably through integrins and Src signaling pathway. The putative interaction between GPNMB and the laminin-derived peptide C16 regulating tumor biology would be the target of our future investigations.

Fig. 7. GPNMB staining is prominent in human breast cancer. Area fraction analysis shows that GPNMB staining is significantly increased in lymph node metastatic carcinoma compared to normal breast tissue (A and B). GPNMB immunostaining is significantly increased in basal-like and HER2 neoplasms compared to normal breast (C). Augmented GPNMB is also observed in IIA, IIB and IIC staging, compared to normal breast (D). Asterisks indicate significant results (p < 0.05). IDC = infiltrating duct carcinoma. LMC = Lymph node metastatic carcinoma. Scale bars: 20 µm.
Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.yexcr.2017.07.005].

References


Fig. 8. Diagram showing cellular events related to C16 regulating GPNMB in breast cancer cells. C16 may interact with integrin dimers containing the β1 subunit, prompting to Src phosphorylation. Src activation would stimulate GPNMB expression and the invasive property of MDA-MB-231 cells.


