Nogo-B Receptor Expression Correlates Negatively with Malignancy Grade and Ki-67 Antigen Expression in Invasive Ductal Breast Carcinoma

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Abstract. *Background: Nogo-B receptor (NgBR) has been shown to be involved in endothelial cell chemotaxis and morphogenesis. However, few studies analyzing its expression in cancer cells have been performed. Materials and Methods: We examined NgBR expression in 233 patients with invasive ductal breast carcinoma (IDC) and corresponding non-malignant breast tissues (NMBT) on mRNA (real-time polymerase chain reaction) and protein levels (immunohistochemistry; IHC and western-blot analysis). NgBR expression was found also analyzed in breast cancer cell lines of varying invasiveness. Results: NgBR expression was increased in IDC compared to NMBT on the mRNA (p=0.0007) and protein level (p=0.018). NgBR expression decreased significantly with IDC malignancy grade and correlated negatively with the Ki-67 antigen expression (r=–0.18; p=0.0005). High NgBR mRNA expression was associated with estrogen receptor negativity (p=0.0023) and the triple-negative phenotype of the tumors (p=0.0129). Conclusion: NgBR may be involved in IDC development, however, its role in its progression requires further research.*

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Key Words: Breast cancer, Nogo-B receptor, NgBR, immunohistochemistry, Ki-67 antigen.

Breast cancer poses one of the most frequent causes of cancer-related morbidity, with its highest peak being observed in women aged 20 to 59 years. In 2013, approximately 230,000 new cases of breast cancer were diagnosed with the mortality rate reaching 40,000 cases (1). Although this malignancy is now well-recognized as a heterogeneous disease with several molecular subtypes being distinguishing by utilizing expression microarrays, there is still a need for defining new accurate prognostic and predictive factors (2, 3).

Nogo isoforms are a part of a superfamily of proteins called reticulons (RTN), which have been shown to exert biological functions strongly related to their localization (4, 5). The Nogo family consists of three main isoforms originating from the *Nogo* (*RTN4*) gene: Nogo-A (RTN4A), Nogo-B (RTN4B) and Nogo-C (RIN4C). Nogo-A (200kDa) was primarily found to be expressed in the central nervous system (CNS), where it acts as a potent neurite outgrowth inhibitor. Nogo-C (25 kDa) was shown to be expressed in CNS and differentiated muscle fibers, however, its exact function remains unknown (6-9). Ubiquitously-expressed Nogo-B (55kDa), a splice variant of Nogo-A was shown to be involved in the facilitation of cellular stress responses in divergent tissues and organs, *e.g.* blood vessels, liver, lungs

and kidney (10-15). It was shown, that Nogo-B mediates the chemotaxis and morphogenesis of endothelial cells endoplasmatic reticulum *via* binding to its specific Nogo-B receptor (NgBR) (16, 17). It has been shown to facilitate dolichol monophosphate (Dol-P) synthesis by increasing the activity of *cis*-isoprenyltransferase (*cis*-IPTase) (18). Loss of NgBR expression resulted in lower *cis*-IPTase activity with diminished levels of Dol-P synthesis, resulting in decreased protein *N*-glycosylation (18). Lines of evidence suggest that *N*-glycosylation of various proteins may be implemented in the regulation of cancer cells motility, invasiveness, proliferation and apoptosis processes (19, 20). Elevated NgBR expression was recently found in invasive ductal breast carcinomas (IDC) and was associated with estrogen receptor (ER) positivity, advanced disease stages and correlated with survivin expression in tumor cells (21).

Taking into account the diverse biological roles of NgBR, we aimed to examine its expression in a subset of IDCs with emphasis on patients' clinicopathological data and breast cancer cell lines characterized by differentiated invasive potential. Immunohistochemical as well as mRNA expression levels of NgBR were correlated with patients' outcome.

Materials and Methods

Patients and tumors. This study was approved by the Bioethical Committee at Regional Specialist Hospital in Wroclaw. The study was performed on 233 tissue specimens of IDCs sampled before treatment initiation from patients treated at the Maria Sklodowska-Curie Memorial Institute of Oncology in Krakow in 2000-2006. These tissue specimens were used for immunohistochemical (IHC) analysis. The clinical and pathological data obtained from the archives of the hospital are summarized in Table I. The mean patient age at diagnosis was 56.4±11.20 (range: 27-84) years. In the follow-up period, patients were observed for 69.0±38.2 (1-145) months. During this time, 50 (21.5%) patients died and 82 (35.2%) sustained local or systemic recurrence. All the patients were treated with mastectomy or conservative quadrantectomy followed by axillary lymph node resection. Adjuvant chemotherapy was administered to 204 (87.5%) women, whereas the postoperative tamoxifen therapy was given to 145 (62.2%). Postsurgical radiotherapy took advantage in 118 (50.6%) patients. Moreover, 22 cases of benign fibrocystic breast changes (FBC) were also included in the study and assessed using IHC. The tissue samples obtained from these patients were fixed in 10% buffered formalin, dehydrated and embedded in paraffin.

Additionally, breast cancer tissues were obtained from 115 IDC patients treated in 2005-2006 and were fresh frozen in liquid nitrogen and stored at –80˚C. For the molecular studies, only cancer specimens with high tumor cell content (>75%) were used. In this study cohort the patients' age was 58.2±11.7 (32-87) and 90 (78.3%) had menopause. Primary tumor characteristics were as follows: pT1 – 31 (27.0%), pT2 – 79 (68.7%), pT3 – 3 (2.6%), pT4 – 2 (1.7%). Lymph node involvement was diagnosed in 74 patients (64.4%). The group comprised 15 (13.0%) stage I, 63 (54.8%) stage II and 37 (32.2%) stage III patients. The ER, progesterone receptors (PR) and human epidermal growth factor receptor-2 (HER2) were found positive in 66 (57.4%), 54 (46.7%) and 29 (25.2%) of the analyzed tumors, respectively. Twenty seven (23.5%) cases were characterized as triple negative (devoid of ER, PR and HER2 expression). During the follow-up period (mean 57.5±26.5, range 1-112) 19 (16.5%) patients died, whereas 41 (35.7) had local or systemic recurrence. Following radical surgical treatment (mastectomy or quadrantectomy with lymph node resection), postsurgical chemotherapy, tamoxifen and radiotherapy was applied to 88 (76.5%), 65 (56.5%) and 69 (60.0%) patients, respectively. Furthermore, 24 cases of corresponding morphologically non-malignant breast tissue (NMBT) were sampled for molecular analyses.

Immunohistochemistry (IHC). Haematoxylin and eosin-stained (H&E) preparations were utilized to verify the diagnosis and to assess the grade of malignancy according to Elston and Ellis by two independent pathologists of the cases selected for immunohistochemical studies (22). IHC was performed on 4-μm-thick paraffin sections. Target Retrieval Solution, pH 9 (97˚C, 20 min) and a PT Link Rinse Station were used to deparaffinize the sections and retrieve the antigens. The sections were then washed in TBS/0.05% Tween buffer. Endogenous peroxidase activity was blocked by 5 min incubation at room temperature (RT) with EnVision FLEX Peroxidase-Blocking Reagent. Sections were then washed in TBS/0.05% Tween and primary antibodies directed against NgBR (rabbit anti-human, 1:100; Imgenex, San Diego, CA, USA), Ki-67 (MIB-1; ready to use, RTU), ER (clone 1D5; RTU) and PR (clone 636; RTU) were applied and incubated at RT for 20 min in an automated staining platform (Link48 Autostainer; DakoCytomation, Glostrup, Denmark) in order to ensure high reproducibility of the reaction conditions. Sections were subsequently washed in TBS/0.05% Tween, and EnVision FLEX reagent was applied in accordance with the manufacturer's instructions to visualize the studied antigens. After washing the sections in TBS/0.05% Tween, EnVision FLEX/horseradish peroxidase (HRP) secondary antibodies were applied (20 min at RT). In the next step, the substrate for peroxidase, diaminobenzidine (DAB), was applied and the sections were incubated for 10 min at RT. Finally, the sections were counterstained with Mayer's haematoxylin, dehydrated in alcohol (70%, 96%, 99.8%) and xylene and then mounted using SUB-X Mounting Medium.

HER2 expression was examined using a HercepTest™ kit, following the procedure recommended by the manufacturer. In cases of equivocal IHC results (+2) a HER2 FISH pharmDx™ Kit was utilized to determine the *HER2* amplification status. All the antibodies (except for the anti-NgBR antibody), reagents and equipment were obtained from DakoCytomation. Negative controls were performed by omitting the incubation with the primary antibody, whereas tumor sections known to have high expression of the analyzed marker were used as a positive controls. NgBR expression in endothelial cells was regarded as an internal positive control.

Analysis of IHC reactions. The IHC sections were independently evaluated under a BX-41 light microscope (Olympus, Tokyo, Japan) by two pathologists who were blinded to the patients' clinical data. In doubtful cases, a re-evaluation was performed using a double-headed microscope and the staining was discussed until a consensus was achieved. For NgBR assessment, the semi-quantitative immunoreactive score (IRS) of Remmele and Stegner, which is frequently used for evaluation of antigens expressed in neoplastic cells cytoplasm, was utilized (23, 24). The scale takes into account the percentage of cancer cells presenting a positive reaction in

Table I. *Patient and tumor characteristics of the study cohort analyzed using immunohistochemistry.*

Parameter	Number	%
Age		
≤55 years	112	48.1
>55 years	121	61.9
Menopausal status		
Pre	83	35.6
Post	150	64.4
Tumor size		
pT1	114	48.9
pT2	104	44.6
pT3	12	5.2
pT4	3	1.3
Lymph nodes		
Negative	87	37.3
Positive	146	72.7
Stage		
I	47	20.2
II	127	54.5
III	57	24.5
IV	2	0.8
Grade		
G1	15	6.4
G2	132	56.6
G3	86	36.9
ER		
Positive	164	70.4
Negative	69	29.6
PR		
Positive	144	61.8
Negative	89	48.2
HER2		
Positive	40	17.2
Negative	193	82.8
Triple negative		
Yes	43	18.5
No	190	71.5
Ki-67		
≤25%	154	66.1
>25%	79	33.9

ER, Estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

relation to all cancer cells (0 points: absence of cells with positive reaction, 1 point: 1-10% cells, 2 points: 11-50%, 3 points: 51-80%, 4 points: over 80% cells with positive reaction) as well as the intensity of the reaction colour (0: no reaction, 1: low intensity, 2: moderate intensity, 3: intense colour).

The Ki-67 antigen was evaluated semi-quantitatively in whole tissue sections as in our previous study according to tumor cell positivity and encoded as follows: 0, 0% cells stained; 1, 1-10% cells stained; 2, 11-25% cells stained; 3, 26-50% cells stained; and 4, 51-100% cells stained (24). Similarly, a semi-quantitative four-grade scoring system based on tumor cell positivity was used for ER and PR expression assessment: 0, 0% cells stained; 1, 1-10% cells stained; 2, 11-50% cells stained; 3, 51-100% cells stained (24, 25). ER and PR sections scoring 1 and higher were regarded as positive according to the criteria of the 11th St. Gallen conference (26).

Cell lines. Immortalized normal breast cells (hTERT- HME1; from ATCC, Washington, USA) were cultured in Mammary Epithelial Cell Growth Medium Bullet kit (Lonza, Basel, Switzerland). Human breast cancer cell lines MCF-7, MDA-MB-231 (both ATCC), SK-BR-3, BT-474 (from the Cell Lines Collection of the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Science, Wroclaw, Poland) and BO2 cell line (courtesy of Dr Philippe Clezardin, INSERM U664, Lyon, France) were cultured in α-Minimal Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS; Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine and 0,1 mg/ml penicillin and 100 U/ml streptomycin. According to Lacroix and Leclercq, these cells can be classified into three groups on the basis of their phenotype: MCF-7 represents the luminal-epithelial phenotype showing expression of ER, PR and HER2 (ER+, PR+, HER2+) and are weakly invasive. SK-BR-3 and BT-474 cells (ER–, PR+, HER2+), defined as weakly luminal epithelial-like, are similar in their invasiveness to the MCF-7 line. MDA-MB-231 and its derivative BO2 show no ER, PR and HER2 expressions (ER–, PR–, HER2–; triple negative), express mesenchymal markers (*e.g.* α-smooth muscle actin; αSMA) and possess the highest metastatic potential in comparison to the other two groups (27).

RNA extraction, cDNA synthesis and real-time PCR. Total RNA from IDC, NMBT and cell lines were extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), in accordance with the procedures of the manufacturer. The quality of the isolated RNA was evaluated with the use of agarose gels and ethidium bromide staining by visualizing 18S and 28S bands under UV light. Concentration and quality of the isolated RNA were measured in the NanoDrop1000 (Nanodrop Technologies, Wilmington, DE, USA). Eight hundred ng of total RNA were used for cDNA synthesis utilizing the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). The reaction mixtures were incubated for 10 min at 25˚C, then for 120 min at 37˚C and terminated for 5 min at 85˚C. PCR amplifications of total cDNA were carried out for 30 cycles.

For the determination of *NgBR* mRNA expression, specific primers for *NgBR* (F: 5'-TGCCAGTTAGTAGCCCAGAAGCAA-3', R: 5'-TGATGTGCCAGGGAAGAAAGCCTA-3') and reference gene *18S rRNA* (F: 5'-CGGCGACGACCCATTCGAAC-3', R: 5'-GAATCGAACCCTGATTCCCCGTC-3') were obtained from Biomers (Jacksonville, FL, USA). cDNA was amplified in 1×Power SYBR Green Master Mix with gene-specific primers and probes utilizing the 7500 Real-Time PCR System (both Applied Biosystems). Thermal cycling conditions were as follows: polymerase activation at 50˚C for 2 min, initial denaturation at 95˚C for 10 min, followed by 45 cycles of denaturation at 95˚C for 15 s and annealing step and synthesis at 58.2˚C for 1 min. The reactions were performed in triplicates and the data were analyzed using the 7500 Real-Time PCR System. The expression of each gene was normalized against the mRNA expression of the housekeeping gene *18S rRNA*. An IDC sample that represented the average expression level of *NgBR* mRNA was used as a calibrator. The relative expression (RQ) of *NgBR* mRNA was calculated by using the ΔΔCt method (28).

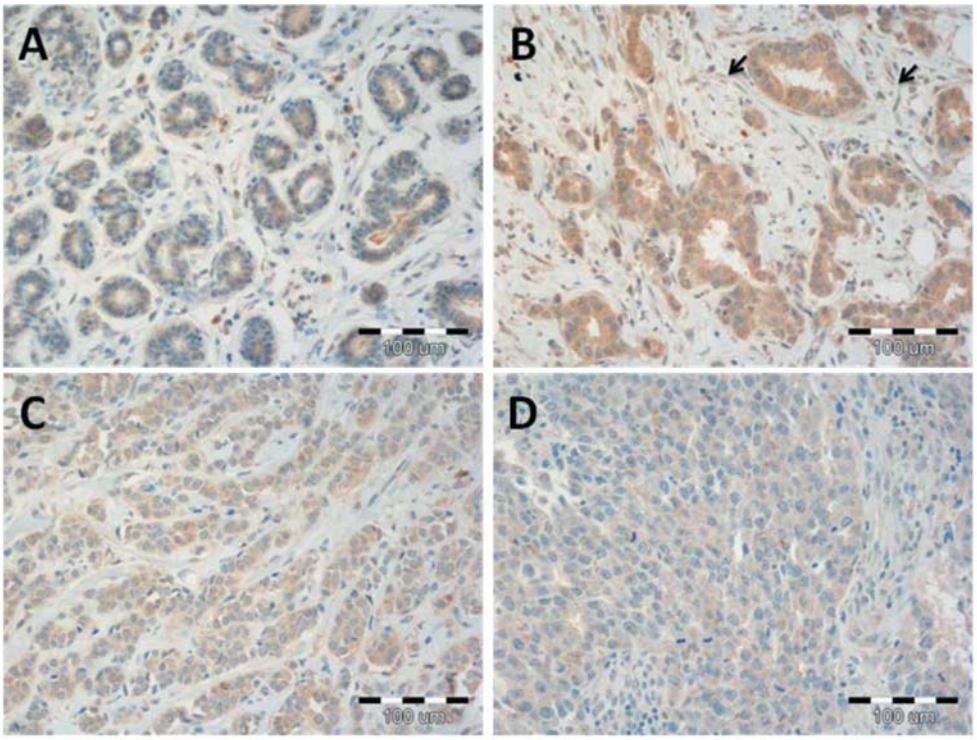

Figure 1. *Expression of Nogo-B receptor (NgBR) in non-malignant duct cells of fibroblastic breast disease (A) and invasive ductal breast carcinoma of G1 (B), G2 (C) and G3 (D) malignancy grade. Arrows indicate NgBR immuoreactivity in stromal cells.*

SDS-PAGE and western-blot. Frozen samples of IDC, NMBT and cell lines were thawed in Tissue Protein Extraction Solution T-PER (Thermo Scientific, Waltham, MA, USA) with the addition of protease inhibitors and 0.2 mM phenylmethanesulfonylfluoride (PMSF; Invitrogen). Whole protein cells lysates containing equal amounts of total protein (30 μg) were determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Protein samples were mixed with sample buffer and dithiothreitol (DTT) and resolved by SDS-PAGE electrophoresis. After the completion of electrophoresis, samples were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon; Millipore, Bedford, MA, USA) and incubated in a 4% BSA solution in TBS with the addition of 0.1% Tween-20. The membranes were incubated with the anti-human NgBR antibody (Imgenex) and anti-human β-actin (Cell Signaling, Danvers, MA, USA) overnight at 4˚C. The membranes were then incubated with the secondary antibody specific for the primary antibody (Jacksons Immunoresearch, Suffolk, UK) for 1 h, rinsed and incubated with the Immun-Star-HRP Chemiluminescent Substrate (Biorad, Hercules, CA, USA). β-actin expression was used as an internal control to normalize NgBR expression.

Statistical analysis. The statistical analysis was performed using Prism 5.0 (GraphPad, La Jolla, CA, USA). The Mann-Whitney *U*-test and the Kruskal-Wallis test with *post hoc* Dunn's Multiple Comparison analysis were applied to compare the groups of data that did not met the assumptions of the parametric test. Correlations between the scores of the examined IHC markers were tested using the Spearman's correlation test. The significance of the differences of the overall survival (OS) times was determined by the Mantel-Cox log-rank test. For each variable, the hazard ratio and 95% confidence interval (95% CI) were estimated. Results were considered statistically significant when $p<0.05$ in all the analyses.

Results

Immunohistochemical NgBR expression in fibroblastic breast changes (FBC) and IDC tissues. In immunostained sections, NgBR expression was noted in the cytoplasm of normal duct cells in FBC cases, as well as in cancer cells of IDC (Figure 1). Weak NgBR expression was also noted in the stroma of IDC (Figure 1). Statistical analysis revealed no differences in NgBR expression between the analyzed FBC (IRS 4.82±2.06) and IDC (IRS 5.34±2.8 cases) cases ($p=0.36$; Figure 2A). However, NgBR expression decreased significantly with malignancy grade of the tumors (G1 *vs*. G3, $p<0.005$ and G2 *vs*. G3, $p<0.05$; Dunn's Multiple Comparison test) (Figure 2B). Moreover, a negative correlation was noted between the expression intensity of NgBR and the Ki-67 antigen expression (r=–0.18, $p=0.0005$, Spearman correlation test) (Figure 2C) in IDC. Interestingly, NgBR expression was significantly higher in postmenopausal patients ($p=0.0023$), although no correlations were observed

with patients' age at diagnosis. No statistical differences were noted between IHC NgBR expression and primary tumor size, presence of lymph node metastases, expression status of ER, PR and HER2, as well as the triple-negative phenotype (Table II).

NgBR gene expression in IDC and NMBT tissues. Expression of *NgBR* mRNA was significantly higher in paired IDC (RQ 14.52±21.38) samples to compared to the corresponding NMBT tissue (RQ 2.15±1.98, p=0.0007; Wilcoxon matched-pairs signed rank test; Figure 3A). In all analyzed IDC cases (N=115), RQ of *NgBR* mRNA expression was 6.72±15.55 and did not differ significantly from the above-mentioned NMBT tissues. Similar observations were noted on a protein level utilizing the western-blot analysis. Measurement of the optical density of 33kDa NgBR bands (IDCs 5.0±5.68 *vs*. NMBT 0.96±0.83) revealed their significant higher levels in cancer tissues (p=0.018; Wilcoxon matched-pairs signed rank test) (Figure 3B,C). Statistical analysis revealed that ER-negative and triple-negative IDC cases were characterized by a significantly higher *NgBR* mRNA expression (p=0.0023, p=0.0129, respectively; Mann-Whitney test). *NgBR* mRNA expression did not differ significantly in relation to primary tumor size, presence of lymph node metastases, expression of PR and HER2, menopausal status and patient's age (Table II).

NgBR expression in breast cancer cell lines. NgBR expression was measured in the hTERT-HME1 breast cell line and in five breast cancer cell lines representing differentiated invasive potential. In all analyzed cell lines, NgBR expression was noted in two splice variants (bands of 33kDa and 55kDa splice) (Figure 4). Densitometric analysis showed that the expression of the NgBR 33kDA splice variant was lower in MCF-7, BT-474 and MDA-MB-231, whereas higher in SK-BR-3 and BO2 breast cancer cell lines compared to hTERT- HME1. However, in the case of the NgBR 55-kDA splice variant, almost no expression could be noted in HME1-hTERT and BO2 cell lines (Figure 4).

Univariate survival analysis. Statistical analysis revealed that immunohistochemical as well as RQ *NgBR* expression levels had no prognostic significance concerning patient' OS (Table III). Moreover, no impact of its expression (immunoreactivity as well as mRNA level) on patients' OS in particular IDC subtypes (ER-positive, HER2-positive and triple negative phenotype) was noted. In the immunohistochemical study group, only advanced clinical stage (II-IV) and G3 malignancy grade were associated with patients' poor OS (p=0.0063 and p=0.0116, respectively). None of the analyzed clinico-pathological parameters was significantly associated with the patients' outcome in the real-time PCR cohort (Table III).

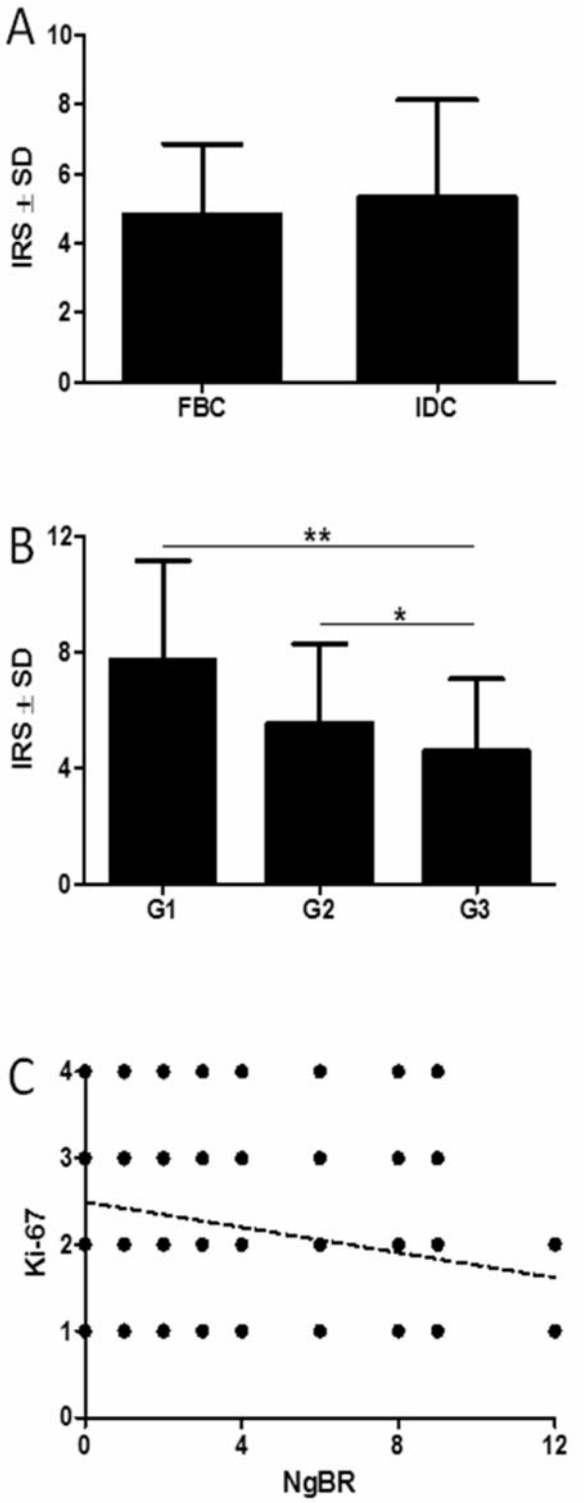

Figure 2. *Nogo-B receptor (NgBR) expression in fibrocystic breast changes (FBC) and analyzed invasive ductal breast carcinoma cases (IDC) (A) NgBR immunoreactivity with regard to the malignancy grade of the analyzed tumors (B). Correlation of NgBR and Ki-67 antigen expression (r=–0.18, p=0.0005) (C). *p<0.05, **p<0.01; Dunn's Multiple Comparison test.*

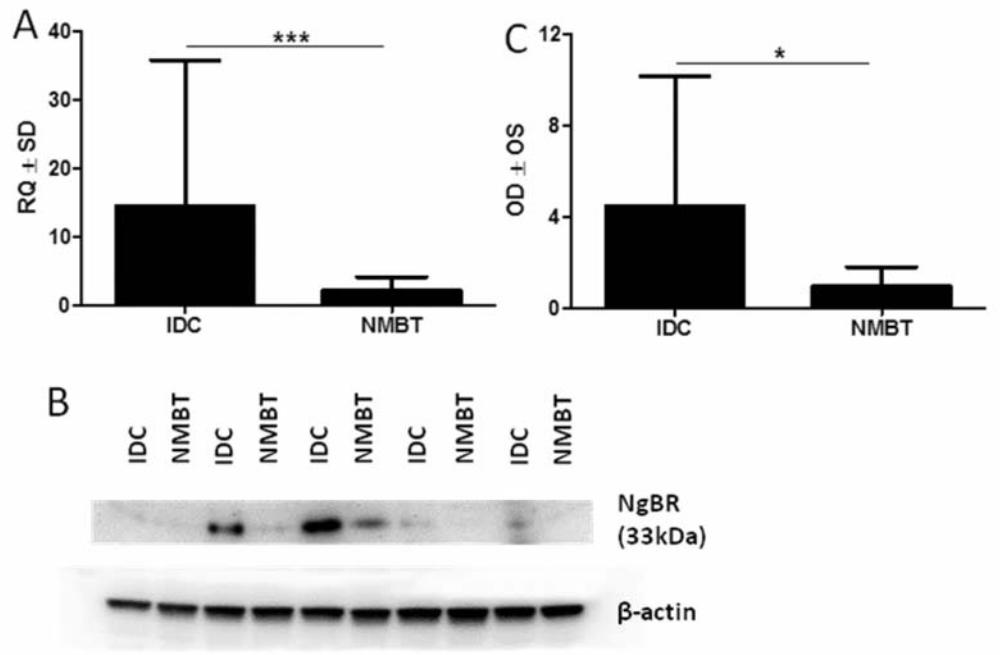

Figure 3. *Expression of Nogo-B receptor, as determined by real-time PCR in 24 (A) and by western-blot in 10 (B,C) paired samples of invasive ductal breast carcinoma (IDC) and non-malignant breast tissue (NMBT). RQ, Relative expression; OD, optical density, *p<0.05, ***p<0.001 by the Wilcoxon matched-pairs signed rank test.*

Table II. *Expression level of Nogo-B receptor (NgBR) in the immunohistochemical (233 cases) and the real-time polymerase chain reaction (PCR) group (115 cases). Data were analyzed using the Mann-Whitney test. Significant p-values are given in bold.*

	Immunohistochemistry (233 cases)				Real-time PCR (115 cases)			
Parameter	Number	%	IRS±SD	*p*-Value	Number	%	RQ±SD	*p*-Value
Menopausal status								
Pre	83	35.6	5.02±2.78	0.0233	25	21.7	6.25±12.63	0.4217
Post	150	64.4	5.99±3.16		90	88.3	6.85±16.33	
Tumor size								
pT1	114	48.9	5.54±3.00	0.3656	31	27.0	6.64±16.87	0.7839
pT2-pT4	119	41.1	5.14±2.59		84	83.0	6.75±15.14	
Lymph nodes								
Negative	87	37.3	5.12±2.86	0.2856	41	35.6	6.65±14.52	0.2749
Positive	146	72.7	5.47±2.77		74	64.4	6.75±16.19	
ER								
Positive	164	70.4	5.35±2.85	0.8727	66	57.4	3.09±6.21	**0.0023**
Negative	69	29.6	5.29±2.67		49	42.6	11.61±21.90	
PR								
Positive	144	61.8	5.47±2.84	0.3777	54	46.7	3.55±6.77	0.1368
Negative	89	48.2	5.11±2.74		61	53.3	9.52±20.05	
HER2								
Positive	40	17.2	6.00±2.69	0.0603	29	25.2	5.90±17.27	0.8217
Negative	193	82.8	5.20±2.81		86	74.8	6.99±15.03	
Triple negative								
Yes	43	18.5	4.86±2.57	0.2094	27	23.5	14.77±23.44	**0.0129**
No	190	71.5	5.44±2.84		88	76.5	4.25±11.24	

IRS, Immunoreactive score; SD, standard deviation; RQ, relative expression; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2.

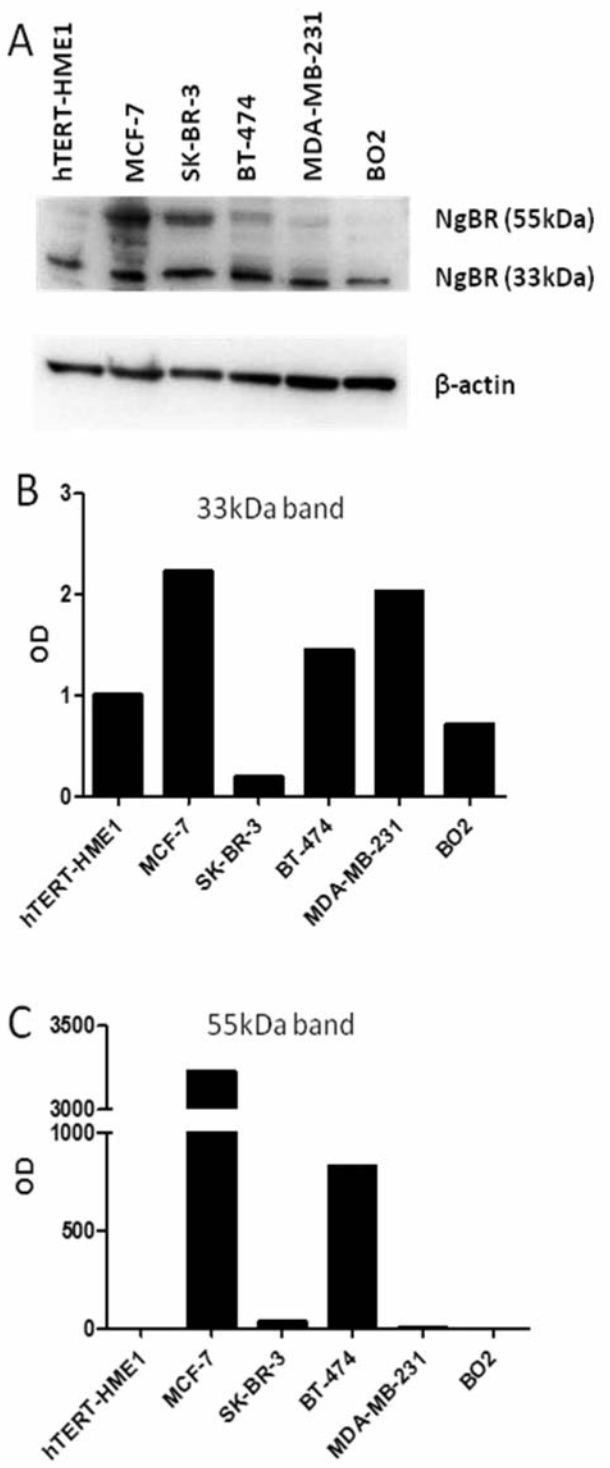

Figure 4. *Expression of Nogo-B receptor as determined by western-blot in the analyzed normal immortalized breast epithelial cells (hTERT-HME1) and breast cancer cell lines (MCF-7, SK-BR-3, BT-474, MDA-MB-231, BO2) (A). Densitometric analysis of NgBR isoform bands revealed their divergent expression in analyzed cell lines. β-actin was used to normalize the NgBR isoform expression and the bands visible in the HME1-hTERT line were used as a calibrator for the analysis (B - 33-kDa bands; C - 55-kDa bands). OD, optical density.*

Discussion

Recent studies concerning the role of NgBR expression have mainly focused on its involvement in vascular remodeling and regenerative processes, in which protein kinase B (AKT) was identified as a downstream factor of NgBR signaling (16, 29). Moreover, single studies have shown that NgBR participates in intracellular cholesterol trafficking, dolichol biosynthesis and protein *N*-glycosylation, out of which the latter two processes were shown to play a role in the carcinogenesis process of some tumors (18, 30, 31). Recently, Wang *et al*. observed that NgBR expression is increased in breast cancer cells in comparison to epithelial cells of non-cancerous breast tissues (21). Furthermore, the highest NgBR expression was observed in ER-positive and clinically advanced cases (21). However, no further associations with patients' clinico-pathological data were analyzed, including their outcome. In order to validate the results obtained by Wang *et al*., we analyzed NgBR immunoreactivity in the whole tissue, but not the tissue microarray (TMA), sections of 233 IDC cases. Similarly, real-time PCR was performed in order to analyze the *NgBR* mRNA levels with regard to patients' outcome.

In this study we have shown that NgBR is significantly up-regulated on mRNA and protein level in IDC in comparison to NMBT, what is in accordance with the results obtained by Wang *et al*. (21). Interestingly, no significant differences were noted in its immunohistochemical expression in IDC cancer cells and FBC. However, a higher NgBR immunoreactivity could be noted in IDC cancer cells, what may partially support the results of our molecular studies. Current knowledge regarding the expression of NgBR in various tissues does not allow for a clear explanation of this observation. Recently, NgBR expression in cancer cells correlated with survivin expression in ER-positive breast cancer cases (21). This observation may point to the role of NgBR in cancer cells survival in this particular breast cancer molecular subtype. Based on the results of studies concerning its role in endothelial cells, additional actions of this protein in breast cancer cells migration and invasiveness may be only hypothesized (16, 29). Up-regulated expression of *NgBR* in cancer cells may result in their increased migration. This protein was found to stimulate chemotaxis and morphogenesis of endothelial cells upon its binding to Nogo-B (16, 29). Moreover, it was shown that the knockdown of NgBR in these cells resulted in diminished activation of AKT with subsequent reduction of VEGF-A-induced migration of these cells (29). Taking this into account, a possible up-regulation in activity of the growth factor-mediated phosphatidyloinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling pathway, by increased NgBR in breast cancer cells is hypothetically possible. The increased activity of this pathway was noted in human breast cancers

Table III. *Univariate overall survival analysis (analyzed by Mantel-Cox test) in the immunohistochemical (233 cases) and the real-time polymerase chain reaction (PCR) group (115 cases). Significant p-values are given in bold.*

Clinicopathological parameter	Immunohistochemistry (233 cases)			Real-time PCR (115 cases)		
	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
NgBR IRS (≤4 *vs.*>4)	0.6533	0.3767-1.133	0.1298	NA	NA	NA
NgBR RQ (≤median *vs.* >median)	NA	NA	NA	0.8435	0.3353-2.122	0.7177
Age (≤55 yrs. *vs.* >55 yrs.)	1.111	0.6402-1.930	0.7871	0.5155	0.1971-1.348	0.1767
Menopausal status (Pre *vs.* Post)	1.140	0.6478-2.007	0.6491	1.056	0.3561-3.134	0.9212
Tumor size (pT1 *vs.* pT2-pT4)	1.395	0.8044-2.420	0.2359	0.9793	0.3678-2.608	0.9666
Lymph nodes (Pos. *vs.* Neg.)	1.730	0.9665-3.098	0.0650	0.9069	0.3344-2.467	0.8481
Stage (I *vs.* II-IV)	2.482	1.292-4.766	**0.0063**	1.267	0.4877-3.395	0.6105
Grade (G1,G2 *vs.* G3)	2.107	1.181-3.760	**0.0116**	NA	NA	NA
Ki-67 (≤25% *vs.* >25%)	1.726	0.9542-3.122	0.0711	NA	NA	NA

HR, Hazard ratio; CI, confidence interval; IRS, immunoreactive score; RQ, relative expression; Neg., negative; Pos., positive.

resistant to endocrine therapies (32, 33). Interestingly, *NgBR* mRNA expression levels were higher in the ER-negative and triple-negative IDC tumors, which may be connected to the observed high levels of phosphorylated AKT in triple-negative IDCs, corresponding to its increased activity (34). Based on these observations, NgBR could also hypothetically regulate the sensitivity of normal breast and cancer cells to VEGF-A stimulation, which was shown in numerous studies to modify cancer cells adhesion, migration, invasiveness and survival (35). Moreover, NgBR may potentially regulate the activity of mTOR *via* intercellular levels of free cholesterol. In human fibroblasts, *NgBR*-knockdown resulted in increased levels of free cholesterol, which could reduce the mTOR activity similarly as in endothelial cells (30, 36). The interaction of NgBR and mTOR pathways may results in modifications of cancer cells' metabolism, as recent studies identified mTOR as a key factor integrating cells' responses to growth factors and nutrient bioavailability (37, 38).

Abundant lines of evidence point to alterations in the pattern of protein *O*- and *N*-glycosylation in cancer cells in relation to normal, untransformed cells (19, 20, 39). The NgBR ectodomain was identified as a member of intrinsically unstructured proteins and possesses a high level of homology with the *cis*-isoprenyl synthases responsible for lipid modifications and therefore possible regulation of *N*-glycosylation of various proteins (17, 18). Indeed, it was recently shown that NgBR stabilizes the *cis*-IPTase and is crucial for Dol-P synthesis necessary for protein *N*-glycosylation. It has been already shown, that decreased NgBR expression levels in cervical cancer HeLa cells resulted in decline of its proteins *N*-glycosylation processes (18). Such mechanism may also occur in breast cancer cells, that would indirectly explain the higher NgBR expression noted in IDC as compared to NMBT specimens (18).

Taking into account the results of earlier studies (21), we aimed at evaluating the role of NgBR in IDC progression. Statistical analysis revealed that the NgBR expression decreased with the increasing malignancy grade of the tumors. In accordance with this finding was the observed negative correlation of its expression with the expression of the Ki-67 antigen, possibly pointing to a potential role of NgBR in modifying cellular proliferation. Our observation is in opposition to the earlier reported correlation of NgBR and survivin expression in breast cancer (21). Currently, it is not possible to clearly explain the differences between these studies. However additional *in vitro* experiments may help to elucidate the matter. Besides ours and Wang *et al.*'s studies, only a single work addressed the role of NgBR expression in the proliferation of non-cancerous cells (21, 29). In the study of Zhao *et al.*, NgBR knockdown had no effect on the proliferation of zebrafish angioblasts (29).

Earlier reports of Miao *et al.* revealed that NgBR is crucial for Nogo-B-stimulated chemotaxis and morphogenesis (16). This result could suggest that NgBR expression could also affect this processes in breast cancer cells with implications in regard to primary tumor size, malignancy grade or metastatic potential. In our study, we observed negative correlations of NgBR immunoreactivity with the malignancy grade and the Ki-67 antigen expression, but no relationship with primary tumor size, presence of lymph node metastasis and expression status of ER, PR and HER2 in IHC sections could be noted. Based on this observation, it is possible that the assessment of NgBR in different histological subtypes and its expression impact on patients' prognosis would require more detailed methods. These facts could in turn distinguish the different protein bands noted during hTERT-HME1 and breast cancer cells' examination by western blot. Moreover, assays analyzing NgBR activity in addition to the mRNA and protein

expression levels would be of importance to fully-determine the role of NgBR in IDC, since contrary results were achieved in our study and the study of Wang *et al*. (21).

The immunohistochemical results obtained in both the aforementioned studies may, however, be attributed to the differences in the antibodies used, the tissue size material (whole *vs*. TMA section) and the statistical analysis' cut-off points (21). Despite using the comparable real-time PCR method condition, we did not confirm the results of Wang *et al*. with regard to the observed *NgBR* mRNA levels and ER expression status of the analyzed tumors (21). Nevertheless, we confirmed the up-regulation of NgBR in IDC in comparison to NMBT, in accordance with the recently published report (21).

In summary, we have noted an up-regulation of the NgBR expression in IDC tissues, compared to NMBT. The lack of significant relationships between NgBR expression, key tumor characteristics, and patients' survival may limit its usefulness as a prognostic factor of IDC. However, taking into account the various functions of this protein, further studies are required in order to be able to fully-examine its role in IDC development and progression.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

Acknowledgements

The Authors thank Mrs. Teresa Klepuszewszka, Mrs. Aleksandra Piotrowska and Ms. Aleksandra Jethon for their technical support. This work was funded by the scientific grant No. N N401 424539 of the Polish Ministry of Science and Higher Education.

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Received May 14, 2014
Revised June 27, 2014
Accepted June 30, 2014