Expression of Nogo Isoforms and Nogo-B Receptor (NgBR) in Non-small Cell Lung Carcinomas

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Abstract. *Background: Nogo-B was recently shown to be involved in proliferation, apoptosis and invasiveness of cancer cells, whereas its specific receptor (NgBR) was found to be up-regulated in estrogen receptor-α positive breast cancer. No data are currently available concerning their expression in non-small cell lung carcinomas (NSCLC). Materials and Methods: Expression of Nogo isoforms and NgBR was studied in 191 NSCLC. Results: Higher Nogo-A/B immunoreactivity was noted in cancer cells of squamous cell carcinomas (SQC) compared to adenocarcinomas (p<0.001). Stage II-IV tumors had the lowest Nogo-A/B expression (p<0.0001) compared to stage I cases. Nogo-A/B expression decreased with increasing SQC malignancy grade (p=0.026). Significant NgBR mRNA down-regulation was associated with larger primary tumor size (p=0.039), lymph node involvement (p=0.039) and advancement stage (p=0.0054). Low NgBR mRNA expression predicted poor patients outcome (p=0.029). Conclusion: The current data may point to the involvement of Nogo isoforms and NgBR in the pathogenesis of NSCLC.*

Lung cancer is the most common malignancy worldwide, accounting for approximately 12% of new diagnosed cancers. Additionally an increasing tendency of its incidence may be observed (1, 2). Two main histological types of this malignancy can be distinguished, the small cell carcinomas (SCLC) and the non-small cell lung carcinomas (NSCLC). The latter may be further divided into squamous cell carcinomas (SQC), adenocarcinomas (AC) and large-cell carcinomas (LCC) (3). Regardless of their histological entity, these tumors are frequently diagnosed at an advanced disease stage (3). Therefore, development of new prognostic and predictive factors for NSCLC is immensely important.

Nogo isoforms belong to the superfamily of proteins called reticulons (RTN), which exert various biological functions strongly dependent on their localization (4, 5). The fourth group of RTN (RTN4), the Nogo family, comprises of three main isoforms of the *Nogo* gene: Nogo-A (RTN4A), Nogo-B (RTN4B) and Nogo-C (RTN4C). Nogo-A (200kDa) is expressed specifically in the central nervous system (CNS), especially in oligodendrocytes, and acts as a potent neurite outgrowth inhibitor. The least characterized Nogo-C (25 kDa) was shown to be expressed in the CNS and differentiated muscle fibers (6-10). Nogo-B (55 kDa) is a splice variant of Nogo-A and was shown to be expressed ubiquitously with two isoforms being identified (11). It is implicated in the facilitation of cellular stress responses in divergent tissues and organs *e.g.* blood vessels, liver, lungs and kidney (12-17). Nogo-B mediates chemotaxis and morphogenesis of endothelial cells *via* its binding to the specific Nogo-B receptor (NgBR) expressed on their surface (18, 19). High expression levels of the latter have been noted in mouse heart, liver, kidney and pancreas (18). Moreover, NgBR was identified to mediate the process of protein *N*-glycosylation (20). The latter was shown in numerous studies to regulate cancer cell motility, invasiveness, proliferation and apoptosis processes (21, 22).

The exact role of Nogo-B and NgBR expression in the pathogenesis and progression of various tumors is poorly-characterized and to date, the obtained results strongly vary depending on tumor type. Nogo-B was first regarded as a

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potent oncosupressor, as its overexpression led to increased apoptosis of several cancer cell lines (23). Furthermore, decreased Nogo-B expression levels were observed in aggressive forms of adult T-cell lymphoma/leukemia (ATLL) and SCLC (23, 24). However, some studies point to the limited role of its expression on cancer cells proliferation and apoptosis, as well as progression *e.g.* in breast cancer (25). Interestingly, overexpression of Nogo-B in the human cervical cancer cell line HeLa resulted in increased fibulin-5 expression and induced the process of epithelial-to-mesenchymal transition (EMT) (26). To date, NgBR expression in cancer cells was studied only in a single study. Its increased expression was observed in estrogen receptor-α (ERα)-positive invasive ductal breast cancer (IDC) and cases in advanced clinical disease stages indicating its involvement in the pathogenesis of this malignancy (25).

Taking into account the ambiguous results of Nogo-B expression and the poorly-defined role of NgBR in cancer cells, we aimed at determining their expression by using immunohistochemical (IHC) and real-time polymerase chain reaction (PCR) methods in NSCLC with regard to patients' clinico-pathological data.

Materials and Methods

Patients and tumors. This study was approved by the Bioethical Committee at the Regional Specialist Hospital in Wroclaw. All samples utilized for the study were collected before chemotherapy and radiotherapy administration. The IHC studies were performed on archival material sampled from 191 patients during surgical resection of NSCLC in 1999-2010 in the Lower Silesia Centre of Pulmonary Diseases in Wroclaw. The study group comprised 88 squamous cell carcinomas (SCC), 92 adenocarcinomas (AC) and 11 large-cell carcinomas (LCC). In the study, 26 cases of non-malignant lung tissue (NMLT) sampled from the NSCLC periphery were also included. The pTNM classification of the tumors was performed according to the recommendations of The International Association of the Study of Lung Cancer (IASLC) (27). Patients' clinical data was obtained from the archives of the hospital (Table I). The mean observation time was 25.48±35.78 (range: 1-147) months, during which 88 (46.1%) patients died of the disease.

Additionally, real-time PCR analysis of mRNA levels of *Nogo* isoforms and *NgBR* were studied in 87 NSCLC patients and corresponding 30 NMLT tissues (Table I). Samples were fresh frozen in liquid nitrogen and stored at –80˚C. For the molecular studies, only cancer specimens (45 SQC, 36 AC and 6 LCC) with high tumor cell content (>75%) were analyzed. In the follow-up period, patients were observed for 10.89±8.01 (1-30) months and 17 (19.5%) of them died.

Immunohistochemistry. The obtained tissue samples were fixed in 10% buffered formalin, dehydratated and embedded in paraffin. Haematoxylin and eosin-stained (H&E) 6-μm-thick slides were made to verify the diagnosis and define the malignancy grade of the analyzed NSCLC tumors by two independent pathologists. IHC was performed on 4-μm-thick paraffin sections. Deparaffinization and antigen retrieval were performed in Target Retrieval Solution, pH 9 (97˚C, 20 min) in a Pre-Treatment Link Rinse Station (DakoCytomation, Glostrup, Denmark). 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 (DakoCytomation) and washed once in TBS/0.05% Tween. Primary antibodies directed against Nogo-A (goat anti-human, S-19, 1:800; Santa Cruz Biotechnology, Dallas, TX, USA), Nogo-A/B (goat anti-human, N-18, 1:3200; Santa Cruz Biotechnology) and NgBR (rabbit anti-human, 1:100; Imgenex, San Diego, CA, USA) were applied and incubated at RT for 20 min in an automated staining platform (Link48 Autostainer; DakoCytomation) to ensure high reproducibility of the reaction conditions. Sections were subsequently washed in TBS/0.05% Tween, and EnVision FLEX reagent (DakoCytomation) was applied in accordance with the manufacturer's instructions in order 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). Next, the substrate for peroxidase, diaminobenzidine (DAB), was applied for 10 min at RT. Finally, sections were counterstained with Mayer's haematoxylin, dehydrated in alcohol (70%, 96%, 99.8%) and xylene and mounted using SUB-X Mounting Medium (DakoCytomation).

Negative controls were performed by omitting the incubation with primary antibody, whereas tumor sections known to have high expression of the analyzed marker were used as positive control. Nogo-A/B and NgBR expressions in endothelial cells were regarded as an internal positive control.

Analysis of IHC reactions. The IHC sections were evaluated under a BX-41 light microscope (Olympus, Tokyo, Japan) independently by two pathologists who were blinded to the patients' clinical data. Re-evaluation was performed in doubtful cases using a double-headed microscope and the staining was discussed until a consensus was achieved. All the analyzed markers were assessed utilizing the semi-quantitative immunoreactive score (IRS) of Remmele and Stegner, which is used for evaluation of cytoplasmic localized antigens (28, 29). The scale is based on the percentage of cells with a positive reaction in relation to cancer cells in the whole tissue section (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 final score is the product of both parameters and it ranges from 0 to 12.

RNA extraction, cDNA synthesis and real-time PCR. Total RNA from NSCLC and NMLT samples was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) in accordance with the procedure 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 was measured in the NanoDrop1000 (Nanodrop Technologies, Wilmington, DE, USA). 2 μg of total RNA were used for cDNA synthesis utilizing the High Capacity cDNA Reverse Trancription 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.

The determination of the analyzed genes, mRNA expression specific primers (Table II) obtained from Biomers (Jacksonville, FL,

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

	All cases (N=191)		SQC (N=88)		AC (N=92)		LCC (N=11)		*Real-time PCR (N=87)	
Mean age (range)	63.65±8.7 (39-87)		64.58±8.5 (41-87)		62.78±8.9 (39-80)		63.45±7.87 (56-77)		65.62±7.19 (51-82)	
Parameter	No	%	No	%	No	%	No	%	No	%
Gender										
Male	147	76.9	70	79.5	71	77.2	6	54.5	59	67.8
Female	44	23.1	18	20.5	21	22.8	5	45.5	28	32.2
Tumor size										
pT1	55	28.8	24	27.3	27	29.4	4	36.4	30	34.5
pT2	99	51.8	48	54.5	45	48.9	6	54.5	44	50.6
pT3	17	8.9	10	11.4	6	6.5	1	9.1	11	12.6
pT4	20	10.5	6	6.8	14	15.2	0	0.0	2	2.3
Lymph nodes										
pN0	102	53.4	52	59.1	42	45.7	8	72.7	57	65.5
pN1, pN2	89	46.6	36	40.9	50	54.3	3	27.3	30	34.5
Stage										
IA	43	22.5	20	22.7	19	20.7	4	36.4	25	28.7
IB	44	23.0	22	25.0	19	20.7	3	27.3	22	25.3
IIA	21	11.0	11	12.5	10	10.9	0	0.0	8	9.2
IIB	17	8.9	10	11.4	6	6.5	1	9.1	9	10.3
IIIA	44	23.0	19	21.6	22	23.9	3	27.3	21	24.2
IIIB	15	7.9	4	4.5	11	11.9	0	0.0	0	0.0
IV	7	3.7	2	2.3	5	5.4	0	0.0	2	2.3
Malignancy grade										
G1	10	5.6	5	5.7	5	5.4	-	-	2	2.5
G2	128	71.1	65	73.9	63	68.5	-	-	61	75.3
G3	42	23.3	18	20.4	24	26.1	-	-	18	22.2

*Group comprised of: 45 SQC, 36 AC and 6 LCC cases.

USA) were utilized. 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 sec 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 mRNA expression of the housekeeping gene *18S rRNA*. A NMLT sample that represented the average mRNA expression level of the studied genes was used as a calibrator and their relative expression (RQ) was calculated using the ΔΔCt method (30).

Statistical analysis. Statistical analysis was performed using Prism 5.0 (GraphPad, La Jolla, CA, USA). The Mann-Whitney test and the Kruskal-Wallis test with *post-hoc* Dunn's Multiple Comparison test were used to compare the groups of data that failed to satisfy the assumptions of the parametric test. Correlations between the scores of the examined IHC markers were tested using Spearman's correlation test. Significance of differences of the overall survival (OS) times were determined by the Mantel-Cox log-rank test. Hazard ratio and 95% confidence interval (95% CI) were estimated for each variable. In all the analyses, results were considered statistically significant when $p<0.05$.

Table II. *Primers utilized for the real-time polymerase chain reaction studies.*

Gene	Primers
Nogo-A	F: 5'-ATA CTT CTT TCC CCA GTA CGC-3'
	R: 5'-CAT CGG TCT TAT TTT TCT GAA GTA GG-3'
Nogo-B1	F: 5'-GCA GGG GCT CCG GCT CAG TG-3'
	R: 5'-GTT CAC ATG ACC AAG AGC AG-3'
Nogo-B2	F: 5'-GCT CTT CCT GCT GCA TCT GAG-3'
	R: 5'-GTT CAC ATG ACC AAG AGC AG-3'
NgBR	F: 5'-TGC CAG TTA GTA GCC CAG AAG CAA-3'
	R: 5'-TGA TGT GCC AGG GAA GAA AGC CTA-3'
18S rRNA	F: 5'-CGG CGA CGA CCC ATT CGA AC-3'
	R: 5'-GAA TCG AAC CCT GAT TCC CCG TC-3'

F, Forward; R, reverse.

Results

Immunohistochemical expression of tested antigens in NMLT and NSCLC. We have noted differentiated expressions of the analyzed antigens. Positive Nogo-A staining was absent in

Table III. *Expression intensities of Nogo-A, Nogo-A/B and Nogo-B receptor (NgBR) determined according to the immunoreactive score of Remmele and Stegner in pneumocytes of non-malignant lung tissues and cancer cells of non-small cell lung cancer (NSCLC). Values are given as mean±standard deviation (median).*

Structure	Nogo-A	Nogo-A/B	NgBR
Pneumocytes	0.0 (0.0)	10.13±1.87 (9.0)	3.42±1.61 (3.0)
Bronchi epithelia	0.0 (0.0)	4.50±2.32 (4.0)	5.08±2.30 (4.0)
NSCLC cancer cells	0.98±1.88 (0.0)	3.64±2.44 (4.0)	4.37±2.65 (4.0)
SQC cancer cells	0.66±1.53 (0.0)	4.43±2.24 (4.0)	4.27±2.68 (4.0)
AC cancer cells	1.37±2.18 (0.0)	2.96±2.46 (3.0)	4.39±2.65 (4.0)
LCC cancer cells	0.36±0.92 (0.0)	3.00±2.05 (3.0)	5.00±2.61 (6.0)

SQC, Squamous cell carcinoma; AC, adenocarcinoma; LCC, large-cell carcinomas

NMLT tissues, however its cytoplasmic immunoreactivity could be noted in NSCLC cancer cells (Figure 1A-C). We have noted a cytoplasmic and cytoplasmic/membrane expression of Nogo-A/B in pneumocytes, bronchial epithelial cells, endothelial and smooth muscle cells of vessels in the analyzed NMLT cases. In addition, differentiated cytoplasmic and cytoplasmic/membrane expression of Nogo-A/B could be noted in NSCLC cancer cells (Figure 1D-F). Similar, although only cytoplasmic, immunoreactivity specificity was observed for NgBR (Figure 1G-I). In addition to cancer cells, Nogo-A/B and NgBR immunoreactivity was noted in fibroblastic cells of the tumor stroma. Expression intensities of Nogo-A, Nogo-A/B and NgBR in pneumocytes, bronchial epithelial cells of NMLT and NSCLC cancer cells are summarized in Table III. Statistical analysis using the Mann-Whitney test revealed that Nogo-A/B expression intensity was significantly higher in pneumocytes compared to cancer cells of all analyzed cases, as well as particular histological types of NSCLC ($p<0.0001$). Significantly higher NgBR immunoreactivity could be noted in pneumocytes compared to its expression intensity in cancer cells of the analyzed NSCLC cases ($p=0.047$). However when the particular histological types were analyzed separately in relation to pneumocytes, the differences in NgBR expression intensity were only on the threshold of significance *e.g.* SQC ($p=0.062$), AC ($p=0.076$) and LCC ($p=0.055$). No significant differences were noted in the expression intensity of Nogo-A/B and NgBR between immunoreactivity in bronchial epithelial and cancer cells. Furthermore, no significant correlations between Nogo-A, Nogo-A/B and NgBR expression intensity in cancer cells, as well as bronchial epithelial cells and pneumocytes could be observed (data not shown).

Immunoreactivity of analyzed markers in relation to patients' clinico-pathological data. The highest Nogo-A expression was noted in the AC subtype, however the post-hoc analysis did not show significant differences between particular NSCLC subtypes (SQC *vs.* AC *vs.* LCC) (Figure 2A). Significant differences were also seen with regard to Nogo-A/B immunoreactivity in relation to the NSCLC subtype, with the highest expression noted in the SQC tumors. Nogo-A/B expression intensity was significantly higher in SQC as compared to AC cases ($p<0.001$, Dunn's Multiple Comparison test; Figure 2B). No significant differences were noted between NSCLC types with regard to NgBR expression intensity.

Expression intensities of Nogo-A, Nogo-A/B and NgBR in relation to patients clinico-pathological parameters are presented in Table IV. We have noted a significant decrease of Nogo-A/B immunoreactivity in advanced NSCLC (stage II-IV) compared to stage I ($p<0.0001$, Mann-Whitney test) cases (Figure 3A). Furthermore, we observed that the expression intensity of Nogo-A/B decreased with increasing malignancy grade of SQC tumors ($p=0.026$, Kruskal-Wallis test), with the highest differences noted between G1 (7.00±2.00) and G3 (3.83±1.98) cases (Figure 3B). No other significant associations were noted between the immunoreactivity of the studied markers and patient age, sex, primary tumor size, presence of lymph node metastases and clinical advancement stage (data not shown).

mRNA expression levels of Nogo-A, Nogo-B1, Nogo-B2 and NgBR. Statistical analysis in paired NSCLC and NMLT samples (Wilcoxon matched-pairs signed rank test) revealed that mRNA expression levels of *Nogo-B1* ($p=0.0017$) and *Nogo-B2* ($p=0.029$) were down-regulated in analyzed tumors compared to non-malignant control tissue (Table V). In regard to the whole NSCLC patients cohort, in which significant down-regulation of *Nogo-B1*, as well as Nogo-A was noted ($p=0.0027$ and $p=0.037$, respectively, Mann-Whitney test). No significant differences in *NgBR* mRNA expression levels between NMLT and NSCLC tissues were revealed. Significant correlations were, however, noted between mRNA expression levels of the analyzed genes in the studied NSCLC cases (Table VI).

Significant *NgBR* mRNA down-regulation (Mann-Whitney test) was associated with larger primary tumor size ($p=0.039$), lymph node involvement ($p=0.039$) and higher

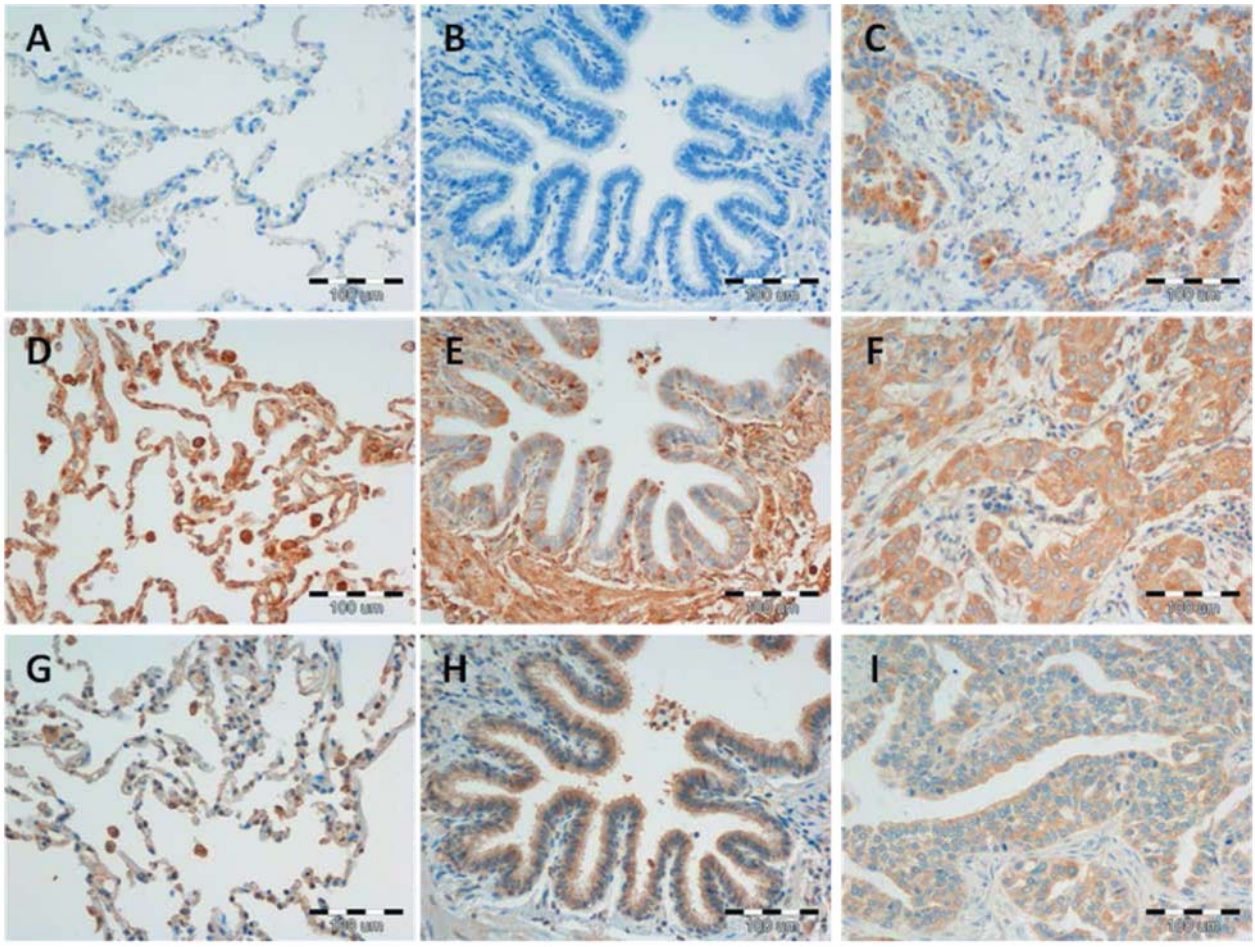

Figure 1. *Expression of Nogo-A (A-C), Nogo-A/B (D-F) and Nogo-B receptor (NgBR; G-I) in pneumocytes (A,D,G), bronchial epithelial cells (B,E,H) and cancer cells (C,F,I) of adenocarcinoma (C, I) and squamous cell carcinomas (F). Magnification ×200.*

clinical advancement stage (p=0.0054) (Figure 4). No significant associations were noted with patients' clinico-pathological data with regard to *Nogo-A, Nogo-B1* and *Nogo-B2* mRNA expression levels (data not shown).

Prognostic significance of analyzed antigens. Statistical analysis revealed that the intensity of Nogo-A, Nogo-A/B and NgBR immunoreactivity yielded no prognostic significance in the IHC patient cohort (Table VII). In this group, advanced patient age (>65 years; p=0.003), primary tumor size (pT2-pT4; p=0.0017), stage (II-IV; p<0.0001), presence of lymph node metastases (p<0.0001) and high malignancy grade (G3, p=0.016) predicted poor patient outcome. In the molecular study group in which mRNA expression of studied Nogo isoforms were analyzed (*Nogo-A, Nogo-B1, Nogo-B2*) and *NgBR*, the low expression of the latter was associated with poor patients outcome (p=0.029; Table VII). Moreover, in the studied group advanced primary tumor size (pT2-pT4; p=0.015), stage (II-IV; p=0.015) and presence of lymph node metastases (p<0.0001) were poor prognostic factors.

Discussion

Recent studies suggested that Nogo-B is a potent oncosupressor as its diminished levels were shown in aggressive forms of ATLL and SCLC (23, 24). However, in a recent study of Wang *et al*. Nogo-B as well as NgBR immunoreactivity was increased in breast cancer cells in comparison to normal breast epithelial cells (25). Furthermore, increased NgBR levels were associated with ERα positivity of the studied tumors and advanced disease stages contradicting the proposed suppressive role of Nogo-B (25). Overexpression of Nogo-B in squamous cervical cell line HeLa led to its increased migratory and invasive capabilities and resulted in the induction of EMT (26, 31). It

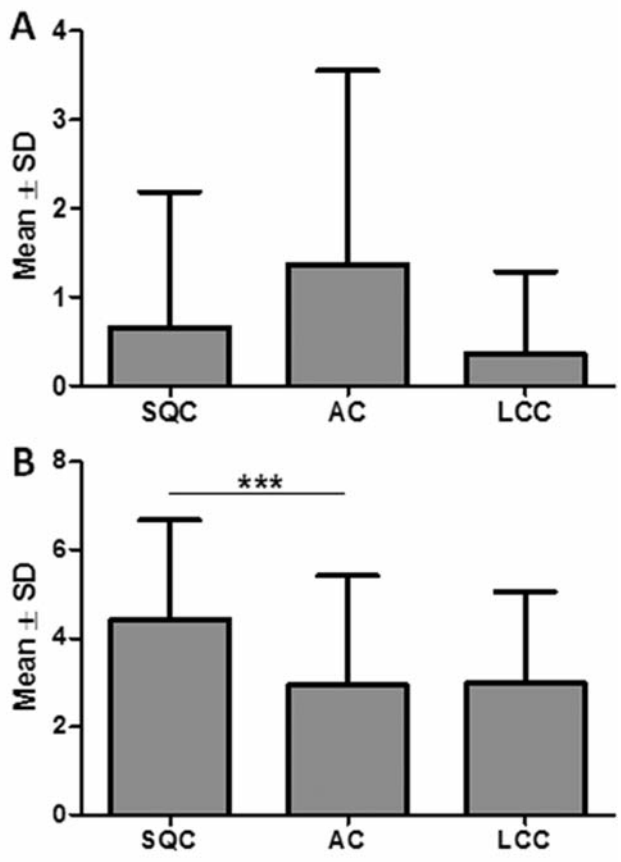

Figure 2. *Expression levels of Nogo-A (A) and Nogo-A/B (B) in particular non-small cell lung carcinoma subtypes: squamous cell carcinomas (SQC), adenocarcinomas (AC) and large-cell carcinomas (LCC). ***p<0.001 Dunn's Multiple Comparison test.*

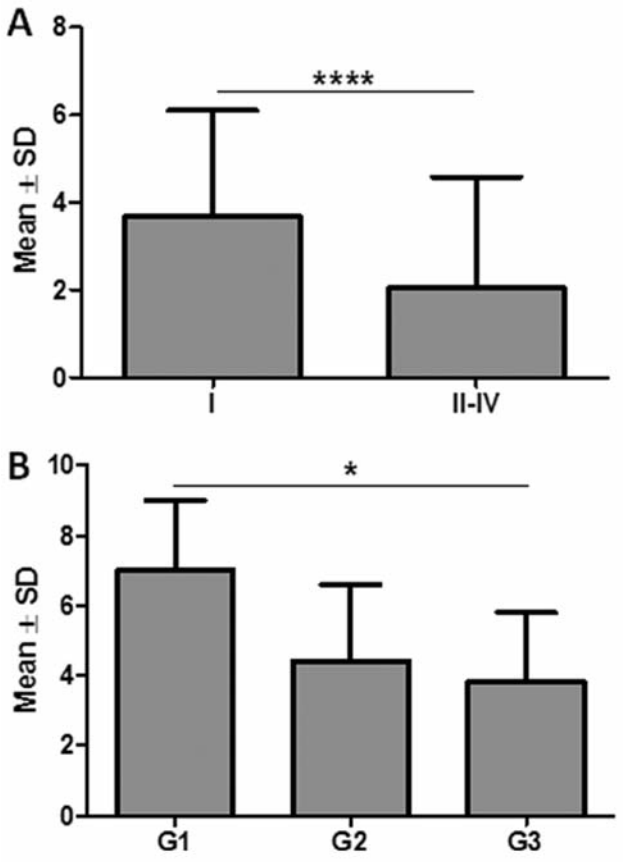

Figure 3. *Expression level of Nogo-A/B in regard to patients clinical advancement stage of the whole study cohort (A; ****p<0.0001; Mann-Whitney test) and malignancy grade of squamous cell carcinomas (B; *p<0.05; Dunn's Multiple Comparison test).*

Table IV. *Immunoreactivity of Nogo-A, Nogo-A/B and Nogo-B receptor (NgBR) in patients with non-small cell lung carcinomas. Data was analyzed using the Mann-Whitney test. Significant p-values are given bold.*

			Nogo-A IRS		Nogo-A/B IRS		NgBR IRS	
Parameter	Number	%	Mean±SD	*p*-Value	Mean±SD	*p*-Value	Mean±SD	*p*-Value
Age								
≤65	112	58.3	0.98±1.86	0.8573	3.58±2.30	0.8336	4.29±2.55	0.6727
>65	79	41.7	0.98±1.92		3.72±2.64		4.48±2.81	
Gender								
Male	147	76.9	0.90±1.84	0.1790	3.65±2.35	0.6536	4.25±2.71	0.1783
Female	44	23.1	1.25±2.01		3.59±2.76		4.77±2.43	
Tumor size								
pT1	55	28.6	1.22±2.23	0.5257	3.55±2.50	0.7069	4.62±2.71	0.5717
pT2-pT4	136	71.4	0.89±1.72		3.68±2.42		4.27±2.63	
Lymph nodes								
Negative	102	53.4	1.04±2.05	0.5784	3.69±2.51	0.9360	4.36±2.92	0.8379
Positive	89	46.6	0.91±1.62		3.58±2.36		4.38±2.32	
Stage								
I	87	45.5	0.96±1.91	0.4524	3.67±2.43	**<0.0001**	4.63±2.91	0.2495
II-IV	104	54.5	1.00±1.86		2.05±2.53		4.15±2.41	

IRS, Immunoreactive score; SD, standard deviation.

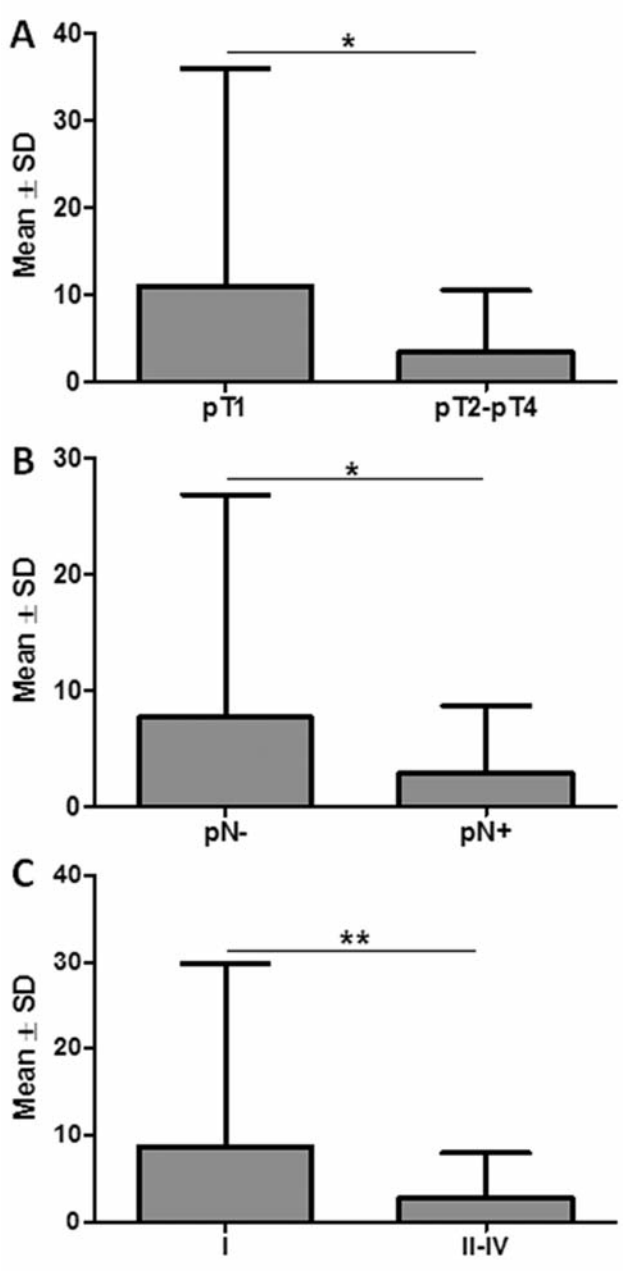

Figure 4. *Relative mRNA expression levels (RQ) of Nogo-B receptor in non-small cell lung carcinomas in regard to primary tumor size (A; *p<0.05), presence of lymph node metastases (B; *p=0.05) and advancement scale (C; **p=0.01); Mann-Whitney test.*

seems that depending on tumor type, Nogo-B may influence several key processes such as apoptosis, invasiveness, migration, as well as their differentiation by inducing EMT. In light of the above facts, we analyzed the expression of Nogo isoforms on protein and mRNA level in a series of NSCLC and NMLT cases, as the previous study of Li *et al*. analyzed only Nogo-B expression on a mRNA level utilizing the *in situ* hybridization technique without determining its impact on patients' clinico-pathological data (23).

Therefore, first we compared the expression levels of particular Nogo isoforms and NgBR between NMLT and NSCLC on a large patient cohort utilizing the IHC technique. The highest immunoreactivity of Nogo-A/B was noted in pneumocytes of NMLT, which was significantly higher in comparison to its expression observed in NSCLC cancer cells of all the analyzed cases and in relation to particular histological types of this malignancy. To note, no differences were seen in its expression between bronchial epithelial cells and cancer cells. Interestingly, an increased immunoreactivity of Nogo-A in NSCLC in cancer cells was noted, although none of its expression was visible in pneumocytes and bronchial epithelial cells of NMLT tissues. Previous investigations showed that this isoform was specific for cells of the central nervous system and tumors of its origin, particularly oligondendrogliomas (8, 32). Our observations may point to the involvement of Nogo-A in the pathogenesis of NSCLC. Although we have noted significant differences with regard to Nogo-A and Nogo-A/B immunoreactivity in immunostained sections, this observations should be interpreted with caution as no antibody is currently capable of recognizing particular Nogo isoforms due to their structure homology (5, 33-35). Noteworthy, the immunoreactivity intensity of Nogo-A was much lower, therefore we concluded that Nogo-B is the main isoform expressed in NSCLC cancer cells and NMLT tissues. In order to overcome the problem of antibody specificity with regard to Nogo isoforms, we analyzed the mRNA expression levels of *Nogo-A*, *Nogo-B1* and *Nogo-B2* splice variants. The *Nogo* gene encodes three major and at least seven minor described transcripts (35). Consistent with our IHC findings we have shown a down-regulation of *Nogo-B1* and *Nogo-B2* in NSCLC cases compared to NMLT tissues. However, down-regualtion of *Nogo-A* mRNA expression in analyzed NSCLC cases was also noted, which when compared to the obtained IHC results may point to the differential expression of this transcript in NSCLC. Although human promoters of the *Nogo* gene were characterized, the precise regulation of this gene and its splice variants expression remains to be further determined (35). We did not observe any differences with regard to NgBR immunoreactivity nor its mRNA expression levels, which may point to its limited role in NSCLC pathogenesis in contrast to breast cancer. Recently, Wang *et al*. showed that NgBR protein levels were increased in cancer cells of ERα breast cancer subtype and correlated with survivin expression, indicating its possible role in the pathogenesis of this malignacy (25).

Nogo-B was proposed to act as an oncosupressor in osteosarcoma (SaOS-2) and tumorigenic HeLa-derived CGL4 cancer cell lines due to their increased apoptosis, not accompanied by growth arrest, following Nogo-B overexpression (23, 36). Furthermore, the *in vitro* results seem

Table V. *mRNA expression levels of Nogo-A, Nogo-B1, Nogo-B2 and Nogo-B receptor (NgBR) in patients with non-small cell lung carcinomas (NSCLC) and corresponding non-malignant lung tissues (NMLT). Differences in expression mRNA levels between NMLT and paired NSCLC samples were analyzed using the Wilcoxon matched-pairs signed rank test, whereas in comparison to whole study group the Mann-Whitney test was utilized. Significant p-values are given bold.*

			NMLT		Paired NSCLC	All NSCLC		
Gene	Mean±SD	Median	Mean±SD	Median	*p*-Value	Mean±SD	Median	*p*-Value
Nogo-A	5.89±11.1	1.725	5.66±14.2	0.6205	0.2494	3.71±9.34	0.6985	**0.0374**
Nogo-B1	3.84±8.74	1.852	1.78±2.88	0.8251	**0.0017**	3.14±9.02	1.089	**0.0273**
Nogo-B2	17.03±58.7	1.546	1.79±2.85	0.8219	**0.0292**	3.67±4.57	2.072	0.6826
NgBR	3.97±5.39	1.930	8.08±22.5	1.469	0.3235	6.01±16.0	1.557	0.4082

SD, Standard derivation.

to be corroborated by the result of studies on human clinical samples which showed its decreased expression in aggressive forms of ATLL and SCLC (23, 24). However, Oertle *et al*. showed that Nogo-B does not play a major role in cancer cells apoptosis, as its stable overexpression did not result in its increase (37). Moreover, recent studies showed that an elevated expression level of Nogo-B in human cervical cancer cell line HeLa may induce its migration and invasiveness *via* induction of fibulin-5 expression leading to EMT (26, 31). Cells undergoing EMT are mostly resistant to apoptotic stimuli, therefore the exact role of Nogo-B is not yet fully clarified (38). Taking into account the specific actions of Nogo isoforms strongly dependent on the cell type, we aimed at analyzing the potential role of Nogo isoforms and NgBR in NSCLC progression by correlating the obtained results with patients clinico-pathological data.

We noted that immunoreactivity of the studied Nogo isoforms differed among particular histological types of NSCLC with the highest Nogo-A levels noted in AC, and the highest Nogo-A/B observed in the SQC cases. Furthermore, statistical analysis revealed the decrease of Nogo-A/B levels with increasing malignancy grade of the SQC tumors, what may point to the involvement of this isoform in SQC pathogenesis and progression. Moreover, advanced stage NSCLC cases presented the lowest Nogo-A/B expression, what may support this thesis. The results of our study seem to contradict the results of Xiao *et al*. and Zhou *et al*., who showed that Nogo-B overexpression in squamous cancer HeLa cells induced its migratory and invasive capabilities (26, 31). In light of the aforementioned studies, our results should be further investigated in *in vitro* experiments on lung cancer cell lines to confirm the findings obtained in tumor immunostained sections. Despite the observed associations of Nogo-A/B immunoreactivity with patients clinico-pathological data, *Nogo-B1* and *Nogo-B2* mRNA showed no impact on the analyzed tumors characteristics. Interestingly, cases with downregulation of *NgBR* mRNA levels were characterized by larger primary tumor size, lymph node involvement, and advanced disease stages. Furthermore, low *NgBR* mRNA expression was associated with patients unfavorable prognosis, pointing to the potential role of NgBR in the progression of NSCLC. Nevertheless, NgBR immunoreactivity in cancer cells had no impact on patients clinico-pathological data, what diminishes the importance of the findings at mRNA level. The potential impact of NgBR expression on NSCLC progression requires further research.

Table VI. *Correlation between the mRNA levels of the studied genes in 87 cases of non-small cell lung carcinomas (Spearman correlation test; significant p-values are given in bold).*

Gene	*Nogo-A*	*Nogo-B1*	*Nogo-B2*	*NgBR*
Nogo-A	x	r=0.37, **p=0.001**	r=0.33, **p=0.002**	r=0.26, **p=0.015**
Nogo-B1		x	r=0.61, **p<0.0001**	r=0.75, **p<0.0001**
Nogo-B2			x	r=0.47, **p<0.0001**

NgBR, Nogo-B receptor.

In this study, for the first time we analyzed Nogo isoforms and NgBR expressions in a large series of NSCLC cases using IHC as well as the real-time PCR techniques. Despite the lack of specific antibodies directed solely against Nogo-B, the combined analysis of Nogo-A and Nogo-A/B antigens expressions may allow to define the Nogo-B as the major isoform of NSCLC. However, it should be noted that Nogo-A, based on the observed elevated expression in AC subtype, may be involved in NSCLC pathogenesis. Our preliminary observations regarding the expression of these proteins may point to the role of Nogo isoforms and NgBR in the progression and pathogenesis of NSCLC, however their effects in this processes seem to be limited. Future studies on larger patients' cohorts and *in vitro* experiments may help to fully elucidate their role in lung cancer biology.

Table VII. *Univariate overall survival analysis (Mantel-Cox test) in the analyzed patients' cohort. Significant p-values are given in bold.*

Clinico-pathological parameter	Immunohistochemistry (191 cases)			Real-time PCR (87 cases)		
	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Nogo-A IRS (0 vs.>0)	0.8083	0.5001-1.306	0.5875	NA	NA	NA
Nogo-A/B IRS (≤4 vs.>4)	0.8830	0.5445-1.432	0.6141	NA	NA	NA
NgBR IRS (≤4 vs.>4)	0.6533	0.3767-1.133	0.1298	NA	NA	NA
Nogo-A RQ (≤median *vs*. >median)	NA	NA	NA	1.648	0.6151-4.413	0.3205
Nogo-B1 RQ (≤median *vs*. >median)	NA	NA	NA	0.9845	0.3667-2.645	0.9759
Nogo-B2 RQ (≤median *vs*. >median)	NA	NA	NA	1.921	0.6761-5.547	0.2205
NgBR RQ (≤median *vs*. >median)	NA	NA	NA	3.025	1.118-8.180	**0.0292**
Age (≤65 years *vs*. >65 years)	1.947	1.242-3.052	**0.0037**	2.138	0.8178-5.558	0.1212
Sex (male *vs*. female)	1.414	0.8551-2.338	0.1769	0.7983	0.2823-2.257	0.6709
Tumor size (pT1 *vs*. pT2-pT4)	2.112	1.325-3.367	**0.0017**	3.514	1.275-9.687	**0.0151**
Lymph nodes (Pos. *vs*. Neg.)	3.140	2.044-4.826	**<0.0001**	10.20	3.300-31.54	**<0.0001**
Stage (I *vs*. II-IV)	2.333	1.526-3.567	**<0.0001**	3.334	1.257-8.844	**0.0156**
Grade (G1,G2 *vs*. G3)	2.107	1.181-3.760	**0.0116**	NA	NA	NA

PCR, Polymerase chain reaction, HR, hazard ratio; CI, confidence interval; IRS, immunoreactive score; RQ, relative expression; Neg., negative; Pos., positive; NA, not analyzed.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

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