Prognostic Significance of NOGO-A/B and NOGO-B Receptor Expression in Malignant Melanoma – A Preliminary Study

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Abstract. *Background: Neurite outgrowth inhibitor type B (NOGO-B) and its receptor (NGBR) were shown to regulate various crucial cellular processes and may be therefore potential factors influencing carcinogenesis. Materials and Methods: Expression of NOGO-A, NOGO-A/B and NGBR was studied in benign melanocytic lesions and primary tumors and metastases of malignant melanoma (MM). Results: Cytoplasmic expression of the studied antigens was detected in melanocytes and MM cells. NOGO-A/B expression was significantly lower in metastatatic MM cases compared to primary MM tumors ($p<0.01$) and bening melanocytic lesions ($p<0.001$). In primary MM tumors, NOGO-A expression intensity positively correlated with NOGO-A/B ($r=0.32$, $p<0.05$) and NGBR expression ($r=0.53$, $p<0.0001$). NOGO-B and NGBR immunoreactivity correlated negatively with depth of primary MM infiltration (both $p<0.01$). Moreover, low NOGO-A/B expression was a factor of poor prognosis of primary MM. Conclusion: NOGO-A/B may be a negative prognostic factor of MM.*

Malignant melanoma (MM) poses a serious healthcare problem worldwide, as in recent years its incidence and mortality rates have been increasing (1). Cutaneous malignant melanoma (CMM) is the most often diagnosed form of this malignancy and despite numerous research projects aimed at discovering potential therapeutic targets, its outcome in the majority of the cases remains fatal (1). Although multiple prognostic factors of MM have been described, the assessment of tumor thickness is the most reliable among them and mandatory in current pathology reports for MM (2). Nevertheless, the clinical course of the disease is often unpredictable; therefore identification of new potential prognostic and predictive factors is of the utmost importance.

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Due to the possible neuroectodermal origin of MM, reticulons (RTN) could be of potential importance in MM pathogenesis and progression. The fourth group of reticulons, denominated as neurite outgrowth inhibitors (NOGO) was shown to be involved in various cellular processes (3, 4). Three main isoforms of NOGO are recognized, NOGO-A, NOGO-B and NOGO-C, which differ in structure and functionality. NOGO-A (200 kDa) and NOGO-C (25 kDa) are potent neurite outgrowth inhibitors predominantly found in the central nervous system (5, 6). NOGO-B (55 kDa), a splice variant of NOGO-A, is expressed ubiquitously in vertebrates and its function strongly varies depending on cellular context (7). NOGO-B expression was found in divergent organs *e.g.* blood vessels, inflammatory cells, liver, lung, and kidney, in which it has been implicated in cell proliferation and apoptosis, as well as in cellular migration and angiogenesis (8-12). The abovementioned effects are mostly mediated by interaction of NOGO-B with its specific NOGO-B receptor (NGBR) (9, 13).

Only few studies concerning the importance of these proteins in tumorigenesis and tumor progression have been conducted but the results were unequivocal. A tumor-suppressor role was ascribed to NOGO-B, as its expression was found to be down-regulated in small cell lung carcinoma (SCLC) and aggressive adult T-cell leukemia/lymphoma (14, 15). Furthermore, it was shown that NOGO-B overexpression induced apoptosis of some cancer cells *via* binding of anti-apoptotic factors *e.g.* B-cell lymphoma (BCL) proteins: BCL-x and BCL2 and sequestering them to the endoplasmatic reticulum membrane (14, 16). On the contrary, increased expression of NOGO-B in the cervical cancer HeLa cell line

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increased its invasiveness by interacting with cytoskeletal protein fibulin-5 (17, 18). Recently conducted studies analyzing NOGO-B expression in invasive ductal breast carcinoma and non-small cell lung cancer showed no impact of its expression on patient outcome (19, 20). Similarly, the results of conducted studies on the prognostic significance of NGBR remains are unequivocal (19, 21).

To our knowledge, expression of NOGO isoforms and NGBR in benign melanocytic naevi and MM have not been determined. Therefore in this study, we aimed to identify the potential role of these proteins in tumorigenesis and progression of MM utilizing immunohistochemical methods.

Materials and Methods

Patients and tumors. The Bioethical Committee at the Medical University of Wroclaw approved this work (decision no. KB/6/2011). The study was performed on paraffin-embedded tissue samples of 41 benign melanocytic naevi and 79 MM, which were collected during surgical procedures at the Lower Silesian Oncology Center in Wroclaw in 1994-2012. Forty-two MM cases were primary tumors, whereas 37 cases were metastatic lesions. The primary tumor samples were excised from 23 (54.8%) male and 19 (46.2%) female patients. The mean patient age was 57.5 years (range=31-77 years, median=56 years). The primary tumors were mostly localized in various parts of the skin, however, one was localized in the mucous membrane of the rectum. Radical resection was performed in 40 (95.2%) of cases. In 20 (47.6%), radical wide excision of the scar after excisional biopsy was undertaken. In 12 (28.6%) patients, lymph-node involvement was found and additional lymphadenectomy was performed. In five patients (11.9%) dacarbazine-based regimen was administered, whereas radiotherapy was applied to eight (19.4%) patients. The mean observation time was 1648 days (range=23-6103 days), in which 28 patients died of the disease. The tissue specimens diagnosed as MM metastases were sampled from lymph nodes (25 cases; 67.6%), skin (11 cases 29.7%) and in one case from the parotid salivary gland (2.7%).
All the tissue specimens were sampled before treatment initiation and fixed in 10% buffered formalin, dehydrated and embedded in paraffin. Hematoxylin and eosin-stained (H&E) 6-μm-thick preparations were made to verify the diagnosis and assess the infiltration depth following the Breslow and Clark guidelines (22).

Immunohistochemistry (IHC). IHC was utilized to assess the expression of NOGO-A, NOGO-A/B, NGBR and the Ki-67 proliferative antigen. The reactions were performed on 4-μm-thick serial paraffin sections. Deparaffinization and antigen retrieval were performed in Target Retrieval Solution pH 6 (in the case of NOGO-A/B and Ki-67) and pH 9 (in case NOGO-A and NGBR) at 97˚C for 20 min in a Pre-Treatment Link Rinse Station (DakoCytomation, Glostrup, Denmark). The sections were then cooled in tris-buffered saline (TBS/0.05%) Tween buffer for 3 min and endogenous peroxidase activity was blocked by 5 min incubation of the sections with H2O2. After rinsing the sections in TBS/0.05% Tween buffer, 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), NGBR (rabbit anti-human, 1:100; Imgenex, San Diego, CA, USA) and Ki-67 antigen (mouse anti-human, MIB1, ready-to-use; DakoCytomation) were applied and incubated at room temperature for 20 min in an automated staining platform (Link48 Autostainer; DakoCytomation) to ensure high reproducibility of the reaction conditions. LSAB+ System-AP based on secondary biotinylated antibodies and streptavidin-conjugated alkaline phosphatase (AP) was applied according to the manufacturer's instructions (DakoCytomation) in order to visualize the studied antigens. The substrate for AP, fuchsin, was applied for 10 min at RT resulting in a red reaction product. In the next steps, sections were counterstained with Mayer's hematoxylin, dehydrated in alcohol (70%, 96%, 99.8%) and xylene, and mounted using SUB-X Mounting Medium (DakoCytomation). Negative controls were performed by incubating without primary antibody, whereas tumor sections known to have high expression of the analyzed markers were used as positive controls. Endothelial cells were regarded as an internal positive control for NOGO-A/B and NGBR expression, whereas brain tissue was used for positive NOGO-A expression.

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, re-evaluation was performed using a double-headed microscope and the staining was discussed until a consensus was achieved. For NOGO-A, NOGO-A/B and NGBR assessment, the semi-quantitative immunoreactive score (IRS) of Remmele and Stegner, also used in our previous work for evaluation of these antigens, was utilized (19, 20, 23). The scale is based on the assessment of the percentage of cancer cells with a positive reaction in relation to all 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 color (0: no reaction, 1: low intensity, 2: moderately intense, 3: intense color).

The Ki-67 antigen was also evaluated semi-quantitatively in whole tissue sections according to tumor cell positivity for nuclear expression of the antigen 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).

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. Univariate survival analysis was performed using Kaplan–Meier approach and the significance levels were determined by the Mantel–Cox log-rank test. For each variable, the hazard ratio (HR) and 95% confidence interval (95% CI) were estimated. The threshold of significance was set at $p<0.05$ in all the analyses.

Results

IHC expression of NOGO-A, NOGO-A/B, NGBR. NOGO-A expression was observed in the cytoplasm of 16 (38.1%) cases of MM primary tumors and seven (18.9%) metastatic lesions. No expression was noted in benign lesions (Figure 1A-C). On the contrary, NOGO-A/B expression in the cytoplasm of melanocytes and melanoma cells was noted in all of the studied benign and malignant lesions (Figure 1D-

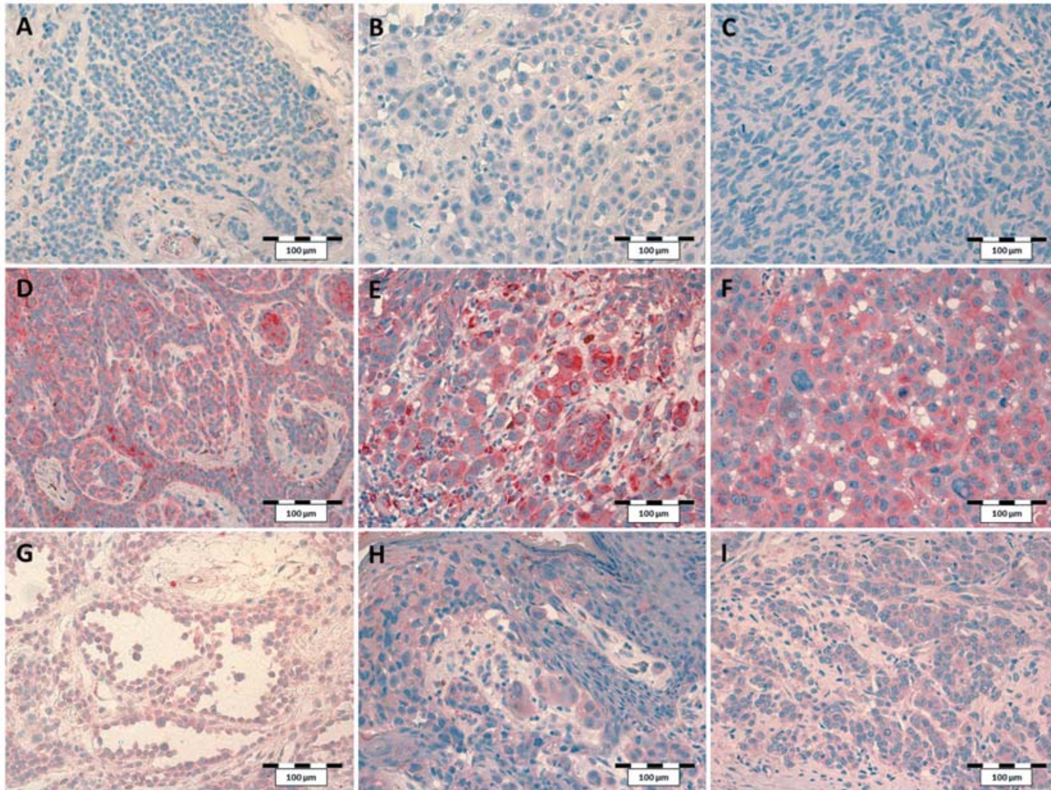

Figure 1. *Expression of neurite outgrowth inhibitor type A (NOGO-A) (A-C), NOGO-A/B (D-F), neurite outgrowth inhibitor receptor (NGBR) (G-I) in benign melanocytic lesions (A, D, G), and in primary (B, E, H) and metastatic (C, F, I) malignant melanoma. Magnification ×200.*

F). NGBR expression was noted in all cases of pigmented lesions and primary MM tumors; however, in the metastatic lesions of MM, its expression was observed in 33 (89.1%) of cases (Figure 1G-H). Statistical analysis revealed that the intensity of NOGO-A expression was significantly higher in primary MM cases as compared to benign lesions (IRS=1.10±1.97 *vs.* 0.35±0.82, $p<0.001$; Figure 2A). NOGO-A/B expression decreased with increasing malignant potential of the studied cases, with its highest levels being noted in benign melonocytic naevi (IRS=7.24±2.83), moderate in primary MM (IRS=6.26±2.67) and the lowest in metastatic MM lesions (IRS=4.42±2.29). *Post hoc* analysis revealed that expression of NOGO-A/B was significantly lower in metastatatic MM cases as compared to primary MM tumors ($p<0.01$) and benign melanocytic lesions ($p<0.001$; Figure 2B). No significant differences were found in NGBR expression intensity in regard to type of the lesion (Figure 2C). In primary MM tumors, NOGO-A expression intensity significantly positivity correlated with NOGO-A/B (r=0.32, $p<0.05$) and NGBR expression (r=0.53, $p<0.0001$).

Correlation of studied antigens with clinicopathological data of primary MM tumors. A negative correlation was found between the expression of NOGO-A/B and the depth of tumor infiltration measured according to Breslow (r=–0.30, $p<0.05$; Figure 3A). Similar negative correlation between the depth of infiltration and NGBR expression was also revealed (r=–0.35, $p=0.05$; Figure 3B). No such relationship was observed with regard to NOGO-A expression. No significant correlations between the studied markers and depth of infiltration according to Clark or Ki-67 antigen expression in melanoma cells, patient age and sex was noted.

Univariate survival analysis in patients with primary MM is summarized in Table I. Among the analyzed parameters a low NOGO-A/B expression ($p<0.05$) and high depth of infiltration according to Breslow and Clark ($p<0.05$ and $p<0.01$, respectively) were associated with significantly shorter overall survival (Figure 4A-C).

Discussion

In this study, we observed cytoplasmic expression of NOGO-A in cells of primary and metastatic MM, as well as cytoplasmic NOGO-A/B expression in melanocytic naevi cells, and primary and metastatic MM cells. The obtained results revealed that NOGO-A expression in these lesions is negligible and that NOGO-B is the main isoform expressed in this tissue. The

Table I. *Univariate overall survival analysis (analyzed by Mantel−Cox test) in 42 patients with primary malignant melanoma. Cut-off points for the analysis were estimated based on the median value.*

Clinicopathological parameter	Cut-off	HR	95% CI	*p*-Value
NOGO-A/B (IRS)	≤6 *vs.* >6	2.213	1.032-4.7545	**0.0412**
NOGO-A (IRS)	≤0 *vs.* >0	0.8422	0.3885-1.826	0.6635
NGBR (IRS)	≤4 *vs.* >4	1.198	0.5585-2.570	0.6428
Ki-67 (%)	≤10% *vs.* >10%	1.186	0.5445-2.583	0.6677
Age (years)	≤57 *vs.* >57	0.7653	0.3624-0.7653	0.4831
Gender	Male *vs*. female	1.254	0.5945-2.644	0.5524
Infiltration by Breslow (mm)	≤2.960 *vs.* >2.960	2.195	1.023-4.710	**0.0437**
Infiltration by Clark (points)	I-III *vs*. IV-V	2.994	1.390-6.449	**0.0051**

NOGO, Neurite outgrowth inhibitor; IRS, immunoreactive score; HR, hazard ratio; CI, confidence interval. Significant p-values are given in bold.

direct analysis of NOGO-B expression is not possible due to the homogeneity of both analyzed NOGO isoforms, NOGO-A and NOGO-B, due to the potential cross-reactivity of the antibodies. The test was supported by high reactivity of the NOGO-A-directed antibody in brain tissue, which was not noted in melanocytic lesions. For this reason, similarly like many other authors, all cases with NOGO-A/B expression were interpreted as being NOGO-B-immunopositive (10, 12, 21).

We also noted cytoplasmatic expression of NGBR in the studied lesions. Our observations are supported by other studies, which showed cytoplasmic expression of NOGO-B and NGBR in normal and tumor cells (14, 19-21, 25, 26). Moreover, we found the localization of these proteins to be in accordance with their described localization in the endoplasmic reticulum (8, 9).

Analysis of IHC reactions showed that NOGO-B expression decreased with increasing malignancy of the tumors. The highest expression of this antigen was noted in benign melanocytic lesions and the lowest was observed in MM metastases. This trend may support the hypothesis that NOGO-B may be a potent oncosupressor, due to its regulatory role in apoptosis (14, 25). Similar down-regulation of NOGO-B expression with increasing malignant potential was observed in adult T-cell leukemias/lymphomas and SCLC (14, 15). Down-regulation of NOGO-B expression could lead to reduced apoptosis which, in turn, could cause an aggressive clinical course. Nevertheless, some reports did not support the proposed ubiquitous suppressor role of NOGO-B in oncogenesis. Previously, we did not observe any decrease in NOGO-B expression in cancer cells of non-small cell lung cancer compared to normal alveolar bronchial cells using IHC (20). Furthermore, Wang *et al.* reported increased NOGO-B and NGBR expression in cancer cells of invasive ductal breast carcinoma in comparison to non-malignant breast epithelial cells (21).

Interestingly, our study showed that NOGO-B and NGBR expression correlated negatively with depth of invasion of primary MM, indicating that both these proteins might be involved in regulating melanoma cell migration and invasiveness, which in turn, could suggest its oncosupressive role in this malignancy. It was already shown that interactions of both these proteins were necessary for chemotaxis of several cell types *e.g.* endothelial cells, vascular smooth muscle cells, immune cells, and the cervical cancer cell line HeLa (8, 9, 17, 18). In the latter cells, overexpression of NOGO-B led to increased migratory capabilities mediated by its interaction with fibulin-5, a cytoskeletal protein, which led ultimately to the induction of the epithelial–mesenchymal transition (17).

Furthermore analysis of survival revealed that lower expression of NOGO-B in MM cases is associated with poor outcome, which might also indirectly confirm the hypothesis on the oncosuppressive role of its protein. Although in breast cancer, NOGO-B expression intensity had no impact on patient prognosis, high NGBR levels were associated with estrogen receptor positivity, advanced clinical stage of the tumors, and their increased proliferative potential (21). On the contrary, in our recent report concerning the same type of cancer, we showed that NGBR expression, determined using IHC, correlated negatively with increasing malignancy of the tumors and the expression of the Ki-67 antigen (19). It is, however, not yet possible to explain the differences between the abovementioned studies. The impact of NOGO-B and NGBR on melanoma cell proliferation determined by the assessment of Ki-67 was also addressed in the present study, however, the statistical analysis did not reveal any significant correlations. Similar observations were reported by Oertle *et al.* who did not show any impact of NOGO-B expression on proliferation of Chinese Hamster Ovary cells and osteosarcoma SaOS-2 cells *in vitro* (26).

In summary, the results obtained in this study suggest the role of NOGO-B as an oncosuppressor in carcinogenesis of MM due to its decreasing expression with increasing malignant potential of melanocytic lesions. Nevertheless,

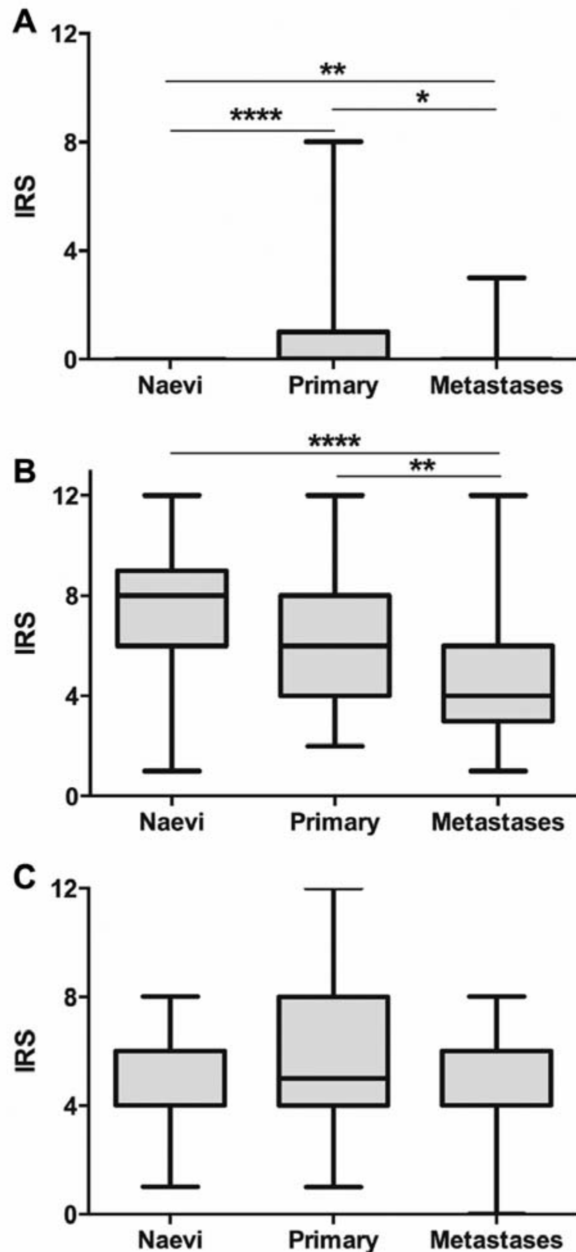

Figure 2. *Expression of neurite outgrowth inhibitor type A (NOGO-A) (A), NOGO-A/B (B) and neurite outgrowth inhibitor receptor (NGBR) (C) in melanocytic naevi, primary tumors and metastases of primary melanoma. *p<0.05, **p<0.01, ****p<0.0001, Dunn multiple comparison test.*

further research utilizing melanoma cell lines and larger patient cohorts could support the prognostic significance of NOGO-B presented in this preliminary study.

Conflicts of Interest

The Authors have no conflict of interest to declare.

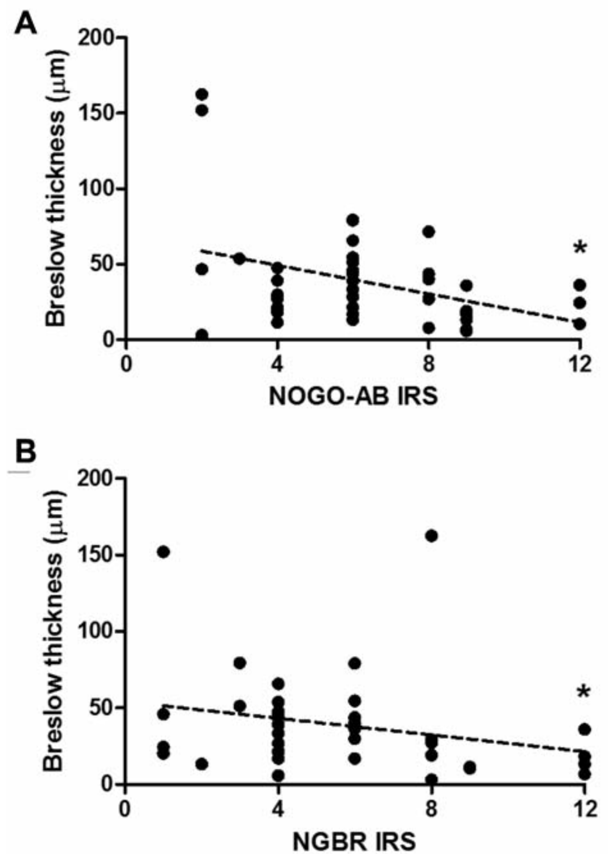

Figure 3. *Negative correlation of depth of infiltration of primary malignant melanomas measured according to Breslow scale and neurite outgrowth inhibitor type A/B (NOGO-A/B) (r=−0.30, p<0.05; A) and neurite outgrowth inhibitor receptor (NGBR) (r=−0.35, p=0.05; B) expression in melanoma cells. *p<0.05, Spearman correlation test.*

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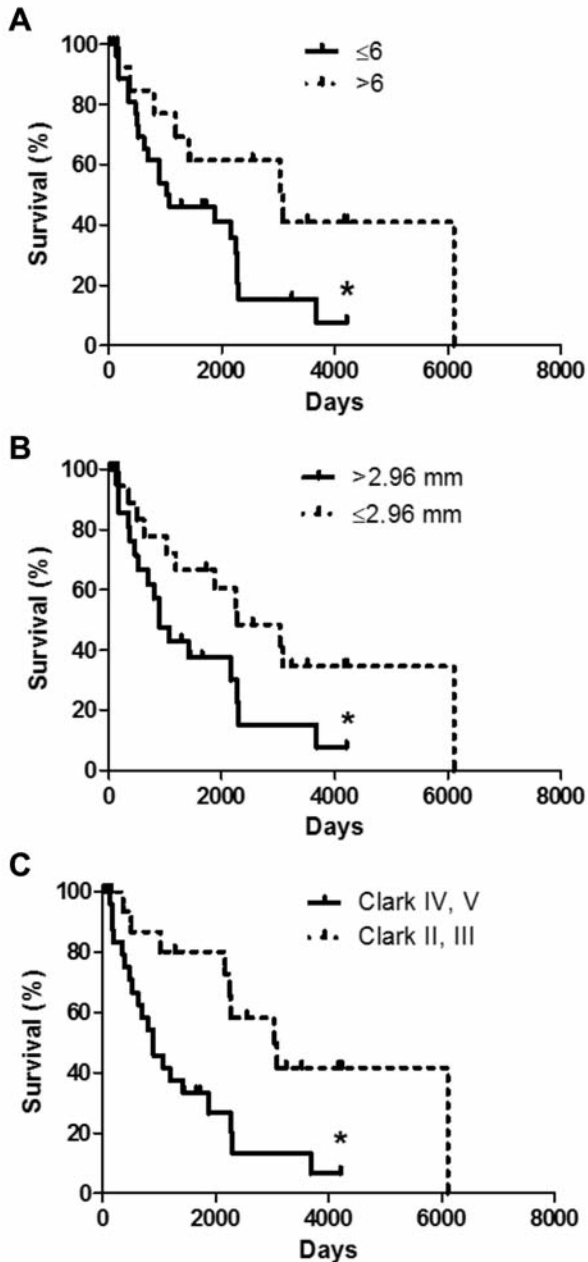

Figure 4. *Kaplan−Meier survival curves of 42 patients with malignant melanoma with regard to neurite outgrowth inhibitor type A/B (NOGO-A/B) expression (A) and depth of infiltration measured according to Breslow scale (B) and Clark method (C). *p<0.05, Mantel−Cox test.*

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