

# ANALYSIS OF PI3K/AKT/mTOR SIGNALLING PATHWAY IN HIGH RISK NEUROBLASTIC TUMOURS

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Neuroblastoma (NB) represents one of the most common paediatric tumours. Despite advance in NB research and treatment, the outcome of the patients from the high-risk group remains poor. PI3K/AKT/mTOR signalling pathway which is involved in oncogenesis and cancer progression of many tumours, in parallel constitutes the target for the biologically based oncological therapy.

In this study we analyzed the status of PI3K/AKT/mTOR signalling route in the primary tumour tissue samples from a group of 39 high-risk NB. The pathway activation state was assessed immunohistochemically using antibodies with specificity towards PI3Kp85, PI3Kp110, phospho-AKT, phospho-mTOR, phospho-p70S6K and phospho-4EBP1. Moreover, expression of PTEN, bcl2 and cyclin D1 was examined.

We found that most of tumours were positive for PI3Kp85 and PI3Kp110, as well as for p-AKT, p-mTOR and its downstream effectors p-p70S6K and p-4EBP1. PTEN was expressed in all cases, bcl2 and cyclin D1 staining was found in more than 90% of examined NB. Statistical analysis revealed that p-AKT expression was correlated with p-mTOR and strong cyclin D1 labelling. Furthermore, high expression of p-4EBP1 was significantly associated with p-p70S6K expression, high cyclin D1 and lower differentiation of the tumour.

PI3K/AKT/mTOR signalling pathway activation is a common event in high-risk NB and it seems that this group of patients may benefit from targeted therapy with kinase inhibitors.

**Key words:** neuroblastoma, high risk, PI3K/AKT/mTOR pathway, intracellular signaling.

## Introduction

Neuroblastic tumours (NB) are the most common solid infantile tumours, the second most frequent paediatric solid tumours and the most common fatal paediatric cancers [1, 2]. NB create a complex group of embryonal neural crest-derived neoplasms, presenting heterogeneity in pathological, biological, molecular and clinical picture. Several patho-clinical and molecular factors allow to optimize the therapeutic approach. Treatment of NB patients ranges from the “wait and see” strategy to the multimodal

aggressive therapy protocols that include intensive induction chemotherapy, surgery, high-dose chemotherapy with stem cell transplantation, irradiation and maintenance therapy of minimal residual disease [1-3]. The newest International Neuroblastoma Risk Group (INRG) classification is based on established prognostic factors, including patient's age, tumour stage, tumour histology, risk group in Shimada system, *NMYC* status, 1p and 11q status [4-6]. INRG divides tumours into very low-, low-, intermediate- and high-risk categories. The high-risk group includes all *NMYC*-amplified neuroblastic

tumours, independently of the stage of disease. Patients older than 18 months with metastatic tumours (stage M) and younger patients with metastatic disease (stage MS) and 11q genetic aberration, irrespectively of *NMYC* status, form the rest of patients in the high-risk group [5, 6]. The treatment results in this group of patients, according to multimodal treatment protocols are still unsatisfactory and 5-year EFS (event-free survival) for those patients is lower than 50% [1-3]. The development of new biologically based therapies that would target the pathways responsible for malignant transformation and progression is necessary for high-risk NB.

One of the promising targets for the tailored oncological therapy with specific kinase inhibitors is phosphatidylinositol-3'-kinase (PI3K)/AKT/mTOR pathway [7-9]. PI3K signalling, called a survival pathway, plays a pivotal role in the intracellular signalling in cancer, being connected with oncogenic transformation, cancer progression and metastasis [7-9]. Activation of PI3K by growth factors, cytokines and growth factor receptors results in phosphorylation of the key effector protein kinase AKT. AKT has functional connections with TSC1/2 and the other transduction pathways and proteins, including GSK3, FOXO and MDM2. In addition, AKT directly regulates several members of the apoptotic pathway and influences cellular proliferation by inactivating cell cycle inhibitors [7-10]. Through effects on tuberous sclerosis complex (TSC1/2), AKT activates mTOR (mammalian target of rapamycin), which plays a central regulating role of protein synthesis and cell

growth [10-12]. The main effectors of mTOR are p70S6K and 4E-BP1, which profoundly affect mRNA translation [8, 11, 13].

Currently, the understanding of the PI3K/AKT/mTOR signalling events in NB derives primarily from *in vitro* models and only few *in vivo* analyses [14-18]. The aim of the present study was to determine the status of PI3K/AKT/mTOR pathway in a series of high-risk NB tumour samples.

## Materials and methods

The study was performed on routinely processed paraffin-embedded tumour samples taken at the primary surgical biopsy or tumour resection. Tumour tissue was collected from 39 children with high-risk neuroblastic tumours, treated in two paediatric oncology centres: clinics of Medical University of Gdansk and Children's Health Memorial Institute in Warsaw. The patients were classified to a high-risk group NB according to the criteria of INRG 2009 [4-6].

The patho-clinical data included: patients' age, tumour stage, primary tumour localization, tumour histology and *NMYC* status. Patients' age ranged from 1 to 160 months (median 40, mean 49.5 months). The group consisted of three *NMYC*-amplified tumours in stage III (L2) and 36 tumours in stage IV (M) including 21 *NMYC*-amplified cases. Primary tumour localization was adrenal in 27, abdominal extra-adrenal in 8 and other in four cases. There were 34 cases of neuroblastoma Schwannian stroma-poor (5 undifferentiated, 16 poorly differen-

Table I. Antibodies and basic data on immunohistochemical procedures used in the study

ANTIBODY/CLONE	SOURCE	ANTIGEN RETRIEVAL	DILUTION	INCUBATION TIME AND TEMPERATURE
PI3K85 (p85 $\alpha$ (B-9) SC-1637, pAb)	Santa Cruz Biotechnology, CA, USA	microwaves, 2 min, citrate buffer	1 : 100	30 min at 37°C
PI3K110 (PI3Kp110 sc-7177, pAb)	Santa Cruz Biotechnology, CA, USA Novocastra, UK	microwaves, 2 min, citrate buffer	1 : 100	30 min at 37°C
PTEN (NCL-PTEN, mAb)	Cell Signaling Technology, MA, USA	microwaves, 2 min, citrate buffer	1 : 100	30 min at 37°C
p-Akt (Ser473, 736E11; mAb)	Cell Signaling Technology, MA, USA	pressure cooker, 5 min, citrate buffer	1 : 25	overnight incubation 4°C
p-mTOR (Ser 2448 clone 49F9 mAb)	Cell Signaling Technology, MA, USA	pressure cooker, 5 min, citrate buffer	1 : 50	overnight incubation 4°C
p-p70S6K (Thr 389, mAb)	Cell Signaling Technology, MA, USA	pressure cooker, 5 min, citrate buffer	1 : 100	overnight incubation 4°C
p-4EBP1 (Thr 37/ 46, 236B4 Mab)	Cell Signaling Technology, MA, USA	pressure cooker, 5 min, citrate buffer	1 : 100	30 min at 37°C
Cyclin D1 (NCL-Cyclin D1, mAb)	Novocastra, UK	pressure cooker, 2 min, citrate buffer	1 : 40	30 min at 37°C
Bcl2 (M0887 Mab)	DAKO, Denmark	microwaves, 2 min, citrate buffer	1 : 50	30 min at 37°C

**Table II.** The expression levels frequency of examined proteins

PROTEIN	κ-VALUE	EXPRESSION LEVEL		
		NEGATIVE CASES (%)	LOW CASES (%)	HIGH CASES (%)
PI3K 110	0.78	2 (5)	16 (41)	21 (54)
PI3K 85	0.76	2 (5)	16 (41)	21 (54)
p-AKT	0.88	1 (2.5)	16 (41)	22 (56.5)
p-mTOR	0.82	9 (23)	14 (36)	16 (41)
p-70S6K	0.9	1 (2.5)	11 (28)	27 (69)
p-4EBP1	0.92	4 (10)	17 (46)	18 (44)
PTEN	0.88	0	14 (36)	25 (64)
Bcl2	0.92	2 (5)	16 (41)	20 (54)
Cyclin D1	0.95	3 (7)	10 (26)	26 (67)

tiated and 13 differentiated) and five cases of ganglioneuroblastoma nodular unfavourable subtype. Thirty six tumours were from unfavourable Shimada group.

### Immunohistochemical analysis

Details of the antibodies used and conditions of the immunohistochemistry are presented in Table I. The slides were stained manually with DAKO and Novocastra reagents. Appropriate positive and negative controls (omission of the primary antibody) were carried out for every antibody. Diaminobenzidine was the chromogen in all reactions, En Vision (DAKO) was used for visualization.

The expression of examined proteins was assessed in neuroblastic cell component of the tumours in a descriptive and semiquantitative method, based on the percentage of positive cells and staining intensity. Two pathologists performed analysis independently (EIS and GKG or WG). Each subcellular localization of immunolabelling was taken into consideration. The labelling in less than 5% of neoplastic cells was considered negative (score 0), immunopositivity of 5-50% of cells was considered low expression scored as 1 and high expression concerned cases with more than 50% positive cells (scored as 2). The labelling intensity was graded as follows: negative (0), low (1), intermediate (2) and strong (3). The final expression level was counted as the sum of the above mentioned parameters: 0 and 1 was considered immunonegative, 2 and 3 reflected low and 4, 5 – high expression.

### Statistical analysis

For univariate analysis, the  $\chi^2$  test and Mann-Whitney test were used to compare categorical and continuous variables, respectively. The relation

between continuous and ordinal variables was analyzed with the Spearman's rank correlation. The inter-observer (EIS vs. GKG or EIS vs. WG) agreement in immunohistochemistry assessment was measured with the  $\kappa$  statistics. Data were expressed as median and range. The value of  $p < 0.05$  was interpreted as statistically significant. Analyses were performed with the software package STATISTICA (StatSoft Inc, 2007, USA).

### Results

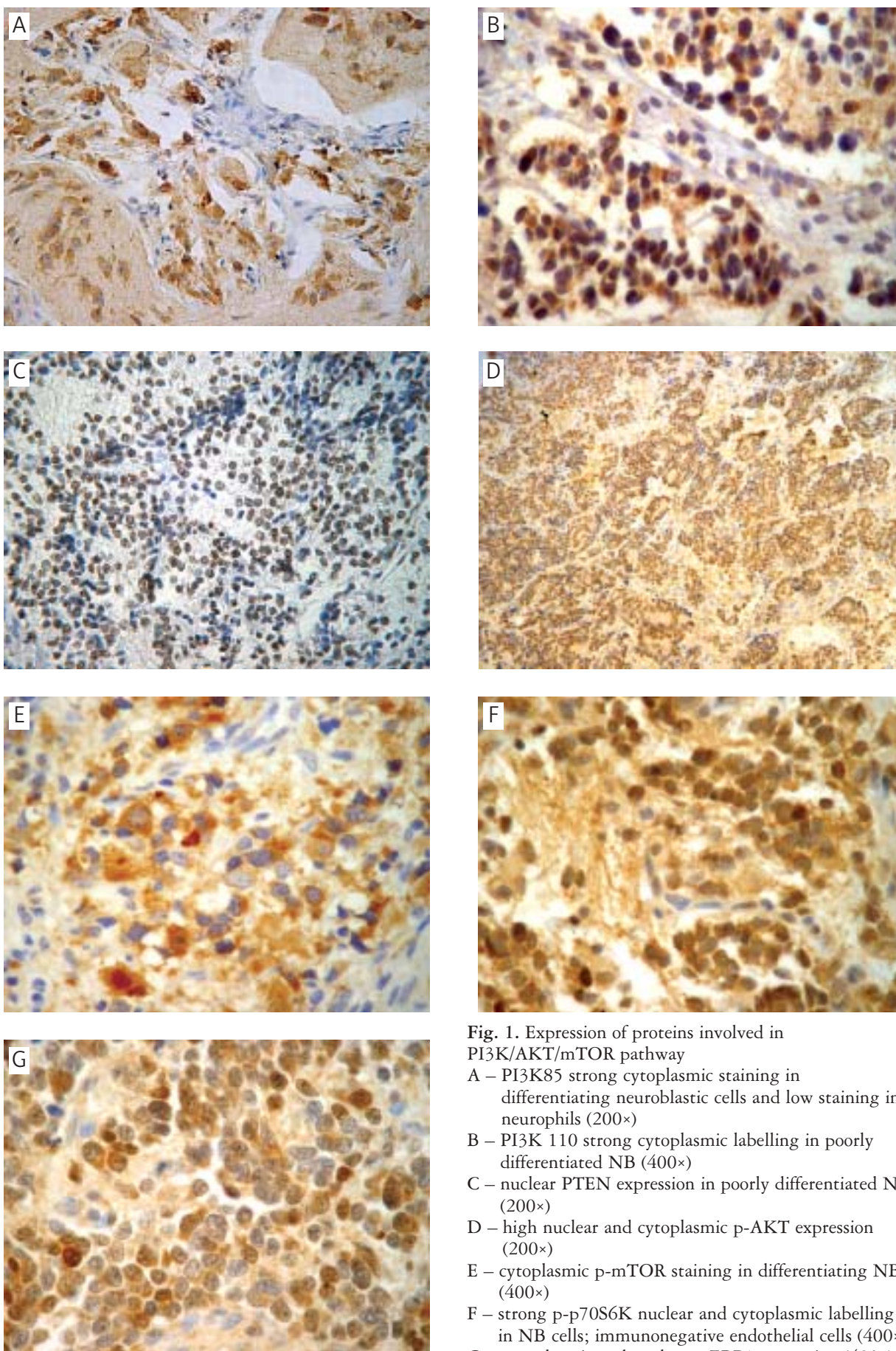
The results of immunohistochemical analysis are presented in Table II. The inter-observer agreement of PI3K 110 and PI3K 85 expression level, measured with  $\kappa$  statistics was substantial. For the next examined proteins, the inter-observer agreement was very good (almost perfect).

PI3K 110 and PI3K 85 expression was found in 95% of cases as diffuse cytoplasmic labelling (Fig. 1a, 1b). The tendency for more intense staining parallel to the level of neuroblastic cells differentiation was observed. Although PI3K 110 and PI3K 85 expression characterized the same number of the cases, these results were not correlated ( $p = 0.46$ ).

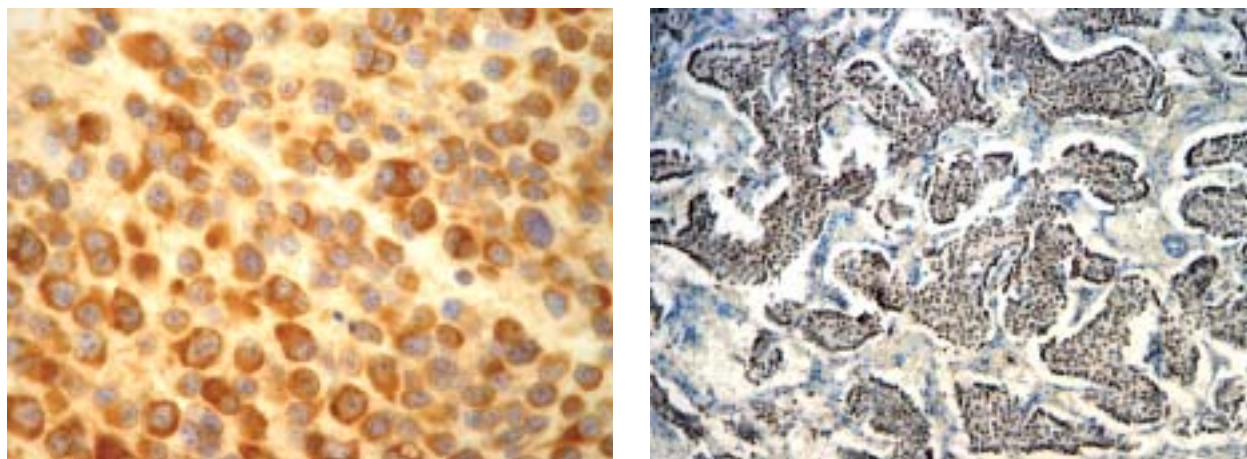
PTEN expression was nuclear. It was found in all tumours, including high expression in 2/3 of cases. No cytoplasmic staining was observed (Fig. 1C). Phospho-AKT expression characterized almost all examined cases, with high intensity in 57% of tumours. It was mainly cytoplasmic and in some cases parallel nuclear labelling was visible (Fig. 1D). In undifferentiated and poorly differentiated and differentiating neuroblasts the staining was intense, in ganglion cells p-AKT expression was low or absent. Phospho-mTOR cytoplasmic staining concerned 77% of cases (Fig. 1E). In some samples, nuclear labelling of neuroblastic cells was also detected. Phospho-p70S6K cytoplasmic and/or nuclear immunopositivity characterized almost all tumours (Fig. 1F). Phospho-4EBP1 expression was cytoplasmic and in some cases – nuclear (Fig. 1G), being more prominent in less differentiated cells.

Most examined NB cases presented cytoplasmic bcl2 staining (Fig. 2A). The expression was higher in less differentiated cells. Cyclin D1 expression was nuclear. It characterized almost all cases, including high expression in 67% of tumours (Fig. 2B).

The statistical analysis for possible reciprocal correlations of all expression results and basic pathological data was performed, showing several significant associations. PI3K110 expression was positively correlated with PTEN ( $p = 0.005$ ). Tumours with high p-AKT expression showed usually p-mTOR positivity ( $p = 0.02$ ). Low p-AKT expression was significantly more frequent in p-mTOR-negative tumours than in p-mTOR-positive NB ( $p = 0.018$ ;



**Fig. 1.** Expression of proteins involved in PI3K/AKT/mTOR pathway  
 A – PI3K85 strong cytoplasmic staining in differentiating neuroblastic cells and low staining in neurophils (200×)  
 B – PI3K 110 strong cytoplasmic labelling in poorly differentiated NB (400×)  
 C – nuclear PTEN expression in poorly differentiated NB (200×)  
 D – high nuclear and cytoplasmic p-AKT expression (200×)  
 E – cytoplasmic p-mTOR staining in differentiating NB (400×)  
 F – strong p-p70S6K nuclear and cytoplasmic labelling in NB cells; immunonegative endothelial cells (400×)  
 G – cytoplasmic and nuclear p-EBP1 expression (400×)



**Fig. 2.** Expression of bcl2 and cyclin D1. A – high cytoplasmic bcl2 labelling (400×). B – high nuclear labelling of cyclin D1 in NB cells, immunonegative endothelial cells (200×)

with Yates correction  $p = 0.048$ ). High expression of p-AKT was also significantly correlated with high cyclin D1 labelling ( $p = 0.01$ ). Furthermore, high expression of p-4EBP1 was significantly associated with high p70S6K positivity ( $p = 0.04$ ), high cyclin D1 expression ( $p = 0.04$ ) and borderline with NMYC amplification ( $p = 0.052$ ). Finally, NB with high p-4EBP1 presented lower differentiation (undifferentiated and poorly differentiated vs. differentiating and ganglioneuroblastoma) ( $p = 0.007$ ).

## Discussion

High-risk NB constitute a substantial subgroup of NB patients, as more than 50% of all NB are in metastatic stage at the diagnosis and about 20% of all NB are NMYC-amplified [1, 2, 5, 6]. This group creates a large clinical problem, because majority of these tumours are refractory to the treatment and have a poor outcome [1, 2, 3, 19]. Novel treatment modalities are intensively investigated in NB, including PI3K/AKT/mTOR pathway targeted therapy [3, 20, 21]. Several inhibitors of this pathway and blockers of related growth factor receptors, show *in vitro* and *in vivo* cytotoxic or cytostatic effects on NB cells [14-16, 18, 20]. In tumours, PI3K/AKT/mTOR pathway is often activated by genetic alterations of PIK3CA gene and loss of tumour suppressor PTEN function [7, 9, 10, 22]. These genetic changes are very rarely present in NB [23-25]. However, NB cells may express several growth factor receptors (TRK, HER family, IGFR), which trigger diverse signalling routes, driving proliferation, survival and differentiation [24, 26-28]. In addition, neoplastic cells may generate survival and proliferative signals by up-regulation of receptor activity or by alterations in the signalling pathways themselves,

also with auto and paracrine signalling loops [8, 10, 13, 29].

Phosphorylation site-specific antibodies allow for detection of activated signalling molecules of PI3K/AKT/mTOR pathway in paraffin-embedded biopsy tissues and enable complex profiling of tumours [30, 31]. Such pharmacogenomic profiling might provide predictive characteristics of tumours, selecting patients for the tailored targeted treatment [16, 30-33].

The data on PI3K/AKT/mTOR pathway status in clinical samples of high-risk NB are sparse. Previously, Brown *et al.* as the first performed morphoproteomic study of three high-risk NB cases, finding activation of mTOR/p70S6K, NF $\kappa$ B and ERK1/2 pathways [34]. Opel *et al.* [14] who first reported AKT activation as a poor prognostic factor in NB, did not use the risk group system, but specified the number of patients in Shimada classification. These authors used anti-phospho-AKT(S473 and T308), anti-phospho-S6 ribosomal protein and anti ERK(T202/Y204). In their study, AKT activation was related with variables of aggressive disease, including NMYC amplification, advanced stage and unfavourable histology [14]. Johnsen *et al.* [16] described immunopositivity of p-AKT and p-mTOR in all 30 tested NB (including seven high-risk) and four ganglioneuromas, but not in Schwannian stroma and surrounding adrenal gland. In this study, mTOR inhibitors administered to NB cell lines and xenografts, caused increased apoptosis, decreased proliferation, inhibited angiogenesis, as well as downregulated VEGF, cyclin D1 and NMYC expression [16].

In the present study, we analyzed PI3K/AKT/mTOR pathway to assess its activation state in view of modern therapeutic perspectives. PI3Kp85 and p110 expression was examined for the first time in NB, taking into consideration that one of PI3K

regulation mechanisms undergoes the inhibitory action of the regulatory p85 on the p110 catalytic subunit [9, 10]. In our series, PI3Kp110 and PI3Kp85 expression was diffuse cytoplasmic, high in 54% of cases. The expression levels of both PI3K subunits were not correlated. It can suggest a kind of activating disequilibrium between PI3K subunits expression, however this finding needs confirmation with direct proteins analysis. There is an opinion that no validated, reliable antibodies against PI3K subunits are available [9]. In our experience, both tested antibodies allowed for detection of PI3Kp85 and p110 specifically within the neuroblastic cells lineage.

Activated PI3K generates a second messenger phosphatidylinositol(3,4,5)-triphosphate (PIP3) that triggers AKT phosphorylation and further cascade signals [7, 12, 13]. A basic negative regulator of PI3K/AKT pathway transduction is PTEN. PTEN function leads to converting PIP3 back to PIP2, resulting in inhibition of PI3K in the basal conditions [10, 22]. PTEN expression can be cytoplasmic or nuclear, depending in part on cell cycle stage and differentiation status [22, 24]. The nuclear import of this protein is regulated by PI3K/AKT/mTOR cascade [22]. In our NB series, no cytoplasmic staining was observed. PTEN nuclear expression was detected in all cases, including 30% with low intensity. Qiao *et al.* reported a decreased level of PTEN protein expression in undifferentiated tumours, proposing the role of lowered PTEN in NB progression [35]. We do not have such observations, but we revealed a strong significant positive correlation between PTEN and PI3Kp110 expression. In fact, p110 catalyzes the conversion of PIP2 to PIP3, which reaction is negatively regulated by PTEN [10, 22].

AKT phosphorylation inhibits formation of the TSC complex, which activates downstream mTOR kinase and increases  $\beta$ -catenin-dependent transcription of genes encoding different cell cycle-regulating proteins such as CMYC, NMYC or cyclin D1 [8, 10, 16, 36]. MTOR is a central controller of cell growth, a master switch of cellular catabolism and anabolism and a target for oncological treatment with rapamycin and its derivatives [11, 12, 16, 17]. In our series, almost all NB cases expressed phosphorylated AKT in cytoplasm, with high levels in 56% of tumours. In some cases, coexistent nuclear labelling was visible. The highest staining characterized undifferentiated and poorly differentiated neuroblasts, whilst ganglion cells presented low expression or immunonegativity. The downstream effector of p-AKT-mTOR was activated in 77% of cases. Statistical analysis proved the correlation between high p – AKT expression and p-mTOR positivity. In addition, low p – AKT expression was significantly more frequent in p-mTOR-negative tumours than in

p-mTOR-positive NB. These statistical findings confirm tight functional associations between AKT and mTOR. Both kinases are linked to each other via positive and negative feedback loops, however, AKT-independent mechanisms of mTOR activation also exist [8, 10, 11, 16, 29]. The next association of p-AKT expression in our study, was a significant correlation between high p-AKT and high cyclin D1 labelling ( $p = 0.01$ ). High nuclear cyclin D1 expression characterized 67% of tumours, similarly to the study of Molenaar *et al.* [36]. In addition, concurrent cytoplasmic bcl2 expression was found almost in all cases. Cyclin D1 plays a basic role in cell cycle progression, controlling G1 entry checkpoint [34, 36]. Cyclin D1 gene transcription and translation is activated by PI3K/AKT signalling [18, 36]. Overexpression of cyclin D1 is important in NB pathogenesis, upregulating proliferation and downregulating neuronal differentiation [36].

MTOR has two main downstream effector proteins, regulating translation: 4EBP1 and p70S6K (S6K1) [11, 12]. The constant nuclear entry and exit of mTOR is necessary for activation of above kinases [11, 12, 31] and we observed concurrent cytoplasmic and nuclear p-mTOR expression in some cases. 4E-BP1 in a phosphorylated form releases subunit eIF4F stimulating translation [11, 12]. 4E-BP1 is considered as a funnelling factor through which the transforming signals converge, channelling the oncogenic proliferative signals regardless of the upstream-specific oncogenic alteration [13]. We examined this protein for the first time in NB, finding its expression in most of the cases in the cytoplasm and often within the nucleus. High expression of p-4EBP1 was significantly associated with high cyclin D1 expression and lower NB differentiation. Phospho-p70S6K cytoplasmic and often nuclear immunopositivity characterized almost all tumours. Phospho-p70S6K stimulates ribosome rearrangement into active polysomes and increases the capacity of the translational events essential for the G1/S transition of the cell cycle [11, 31]. Expression of p-4EBP1 was correlated with that of p-p70S6K. An additional observation was immunopositivity of p-4EBP1 and p-p70S6K in eight p-mTOR-negative tumours. The explanation for this may be parallel activation of ERK1/2 pathway or by crosstalk with other cellular signalling networks in these NB cases [8, 11, 14, 29].

We conclude that constitutive activation of PI3K/AKT/mTOR pathway is a frequent event in the biology of high-risk NB. Biomarkers of PI3K/AKT/mTOR pathway still do not have a defined role in the clinic, but emerging data are promising [8, 14, 16, 30-33]. Study of functional expression profiles of these biomarkers in NB can be helpful in selection of patients for targeted treatment and in further understanding of NB biology.

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