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Expression Pattern of Tumor-associated Antigens in Neuroblastoma: Association With Cytogenetic Features and Survival

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Abstract. *Background/Aim: The prognosis of high-risk and relapsed neuroblastoma (NB) patients remains poor. The identification of tumor-associated markers is important for differential diagnosis, prognosis, and the development of targeted therapies. The aim of the study was to determine the expression profile of nine most common NB antigens and assess their association with clinicopathological characteristics and patient survival. Patients and Methods: Tumor samples from 86 patients with NB were evaluated for the expression of tumor-associated antigen (TAA) using quantitative PCR. Twenty-one patients with benign tumors and 17 healthy donors were assigned as controls. Results: Overexpression of tyrosine hydroxylase (TH), PHOX2B, PRAME, GPC2, B7-H3, and Survivin is the most typical for NB. Positive expression of MAGEA3, MAGEA1, and NY-ESO-1 at low levels was detected in 54%, 48%, and 52%, respectively, and was not NB specific. Higher TH expression was observed in samples without MYCN-amplification, while higher expression of Survivin, PHOX2B, and GPC2 was significantly associated with the presence of 1p.36 deletion. Overexpression of TH, PHOX2B, and MAGEA1 was associated with better event-free (EFS) and overall survival (OS). Survivin overexpression was associated with poor EFS but had no impact on OS. Multivariate analysis confirmed Survivin as independent marker for poor survival, and PHOX2B and MAGEA1 for better survival. Conclusion: High expression of TH, PHOX2B, and MAGEA1 genes are favorable prognostic factors for OS and EFS, whereas high expression of Survivin is associated with an increased risk of relapse or progression.*

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Neuroblastoma (NB) is a tumor that develops from immature cells of the sympathetic nervous system. It is the most common cancer in infants and the third-most common cancer in children, following leukemia and brain cancer. Despite advances in screening, diagnosis, and treatment, outcomes for high-risk and relapsed patients are still poor. While traditional methods, such as surgery, chemotherapy, and radiation therapy are reaching their limit of effectiveness, new possibilities are emerging through immunotherapy and cell therapy. Identification of new tumor-specific antigens and features of their expression is a crucial step in the development of targeted immunotherapy for NB.

The most well-known marker for NB is disialoganglioside GD2, which is targeted by the monoclonal antibody dinutuximab (1). However, GD2 is not a protein and therefore cannot serve as an antigen for T-cell immune response or anti-tumor vaccines. Additionally, GD2 is also expressed on peripheral nerves, resulting in adverse reactions, such as pain and neurotoxicity. There are other antigens associated with NB, including tissue-specific proteins such as tyrosine hydroxylase (TH), transcription factors, such as PHOX2B and N-myc, glypican surface proteins (GPC), and a wide range of cancer-testis antigens. These antigens can be targeted for the development of immunotherapies and anti-tumor vaccines against NB.

Tyrosine hydroxylase is a key enzyme involved in the biosynthesis of catecholamines, such as dopamine, noradrenaline, and epinephrine from tyrosine. The activity of TH is closely related to the functioning of the sympathetic

nervous system, and its expression is limited to certain areas in the brain and adrenal glands (2). Due to its high expression in NB, TH has been identified as a potential tumor-associated antigen (TAA) (3-5).

Paired-like homeobox 2b (PHOX2B) is a homeodomain transcription factor essential for the differentiation and survival of sympathetic neurons and is also known as a highly specific marker of NB cells (6).

Glypicans belong to the family of proteoglycans, which are attached to the cell membrane by their C-terminal domain *via* the glycosylphosphatidylinositol (GPI) anchor. Most of the studies that have examined the expression of glypicans on tumor cells have focused only on GPC1 and GPC3, however, there is evidence that GPC2 is in fact the most typical glypican for NB cells (7, 8).

Cancer/testis antigens (CTAs) are a group of proteins that are normally expressed in normal male germ cells and diverse types of cancer, but not in normal somatic cells (9). The expression and immunogenicity of several CTAs, such as MAGEA1, MAGEA3, PRAME and NY-ESO-1 (CTAG1B) have been reported for NB (10-12).

B7-H3 (CD276) is another well-described NB marker (13, 14). B7-H3 is an immune checkpoint ligand that plays a predominantly inhibitory role in adaptive immunity by suppressing T-cell activation and proliferation. It is over-expressed among several types of human cancer and associated with disease progression (15).

Many TAAs are involved in tumor pathogenesis and are associated with progression and metastasis. Survivin (also known as BIRC5) plays a major role in both cell division and the inhibition of apoptosis, allowing tumor cells to survive and proliferate (16, 17). The meta-analysis of Survivin expression in various types of cancer identified it as an unfavorable prognostic marker in terms of overall survival (18). Although data on Survivin expression in NB are limited, the study by W. Kim and colleagues demonstrated a significantly poorer prognosis for patients with stage 3 NB who had high levels of Survivin expression (19).

The Glypican 2 (GPC2) expression was found to be of diagnostic value in 16 different types of tumors and was positively or negatively associated with the prognosis of various cancers (20). Reduced overall (OS) and event-free survival (EFS) have been reported in NB patients with high GPC2 expression (21).

Another TAA, the cancer-testis antigen PRAME, is expressed and associated with poor prognosis in various types of cancer (22). High PRAME expression has been reported in patients with high-stage NB and is associated with worse OS and EFS (23).

In this study, we aimed to determine the expression profile of the most common NB antigens and assess their association with clinicopathological characteristics and patient survival.

Patients and Methods

Patients and specimens. Tumor samples (79 tissues and seven bone marrow metastases) were obtained from 86 patients with NB. Tissue samples from 21 patients with typical childhood benign tumors [ganglioneuroma (n=11), giant cell tumor (n=5), osteoma (n=2), and chondroblastoma (n=3)], obtained surgically at Belarusian Research Center for Pediatric Oncology, Hematology and Immunology (BRCPOHI) between 2007 and 2022, were included in the comparison group. All patients had a histologically confirmed diagnosis. To determine the expression profiles of the studied antigens in normal cells, samples of peripheral blood (n=7) and bone marrow (n=10) from healthy donors were used. Our study was approved by the Ethics Committee of BRCPOHI and written informed consent was obtained from each patient. The tumor stage was classified based on INSS classification. From April 2008 to January 2018, all patients received treatment according to the NB 2004m protocol. From January 2018, high-risk patients received therapy according to the NB 2018 protocol. Both protocols applied tandem autologous bone marrow transplantation as a consolidation therapy.

Cell lines. Cell lines K-562 (CCL-243) and HEK293T (CRL-3216) were purchased from the ATCC collection (Gaithersburg, MD, USA). IMR-32 cell line was purchased from the Russian Cell Culture Collection. All experiments were performed using mycoplasma-free cells.

RNA isolation and cDNA synthesis. Fresh or frozen samples of tumor tissue were cut into pieces with a scalpel and homogenized with a mortar and pestle. Mononuclear cells from bone marrow and peripheral blood samples were isolated using density gradient centrifugation with Histopaque. Total RNA was extracted using TRIzol reagent (Invitrogen, Waltham, MA, USA), according to the manufacturer's recommendation. RNA quantity and quality were determined using DS11 Spectrophotometer (DeNovix, Wilmington, DE, USA). One microgram of RNA was annealed with 200 ng of random primers by incubation at 65˚C for 5 min and cooling at 4˚C. Synthesis of cDNA was performed in a reaction mix with 0.5 mM dNTP Mix, 1 μl RNAsin (Promega, Madison, WI, USA), and 1 μl SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) in a total volume of 20 μl.

Real-time PCR. Nine neuroblastoma antigens (tyrosine hydroxylase (*TH*), *Survivin*, *PHOX2B*, *GPC2*, *B7-H3*, *MAGEA1*, *MAGEA3* and *NY-ESO-1*) were selected to examine their expression at the mRNA level. Quantitative assessment of cDNA expression was performed using real-time PCR on CFX96 instrument (Bio-Rad, Hercules, CA, USA). Amplification was performed in a volume of 20 μl with TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), using 5 μl of cDNA, 500 ng of each primer, and 150 ng of TaqMan-probe. Cycling conditions were as follows: pre-heating at 50˚C for 5 min, followed by 95˚C for 10 min, then 60 cycles at 95˚C for 15 s followed by 60˚C for 30 s. Amplification of each sample was in duplicate. The *GUS* gene was chosen as a control housekeeping gene to normalize the expression of the studied antigens (24). The sequences of primers and TaqMan-probes are shown in Table I. The relative expression of the studied antigens was calculated using the Pfaffl's method (25). Briefly, various cell lines were analyzed for the expression of the studied antigens. For each antigen, the cell line with

Table I. *Primers and TaqMan-probes for RQ-PCR measurement of antigens expression.*

Primer/probe	Sequence, 5' → 3'
hTH_F	ATTGCTGAGATCGCCTTCCA
hTH_R	AATCTCCTCGGCGGTGTACTC
hTH_TM	**FAM**-ACAGGCACGGCGACCCGATTC-**BHQ1**
hSurviv_F	CCACCGCATCTCTACATTCA
hSurviv_R	TGTTCCTCTATGGGGTCGTC
hSurviv_TM	**FAM**-GAGCGGATGGCCGAGGCTG-**BHQ1**
PHOX2B_F	TGCTGACTTCAGTTCCTGCA
PHOX2B_R	CCGTGGTCCGTGAAGAGTTT
PHOX2B_TM	**FAM**-AGCAGTCCGTACGCCGCAGTTCCT-**BHQ1**
hMAGE1_F	TCTGTGAGGAGGCAAGGTTT
hMAGE1_R	CAGGAGTGTGGGCAGGAG
hMAGE1_TM	**FAM**-ACCAAGGAGAAGATCTGCC TGTGGGT-**BHQ1**
hMAGE3_F	GTGAGGAGGCAAGGTTCTGA
hMAGE3_R	ATGACTCTGGTCAGGGCAAC
hMAGE3_TM	**FAM**-TCCATTGCCCAGCTCCTGCC-**BHQ1**
NYESO1_F	AGGGGTGCTTCTGAAGGAGT
NYESO1_R	GGAGACAGGAGCTGATGGAG
NYESO1_TM	**FAM**-GCAACATACTGACTATCCGA CTGACTGCTG-**BHQ1**
hPRAME_F	CGCCTGGATCAGTTGCTCA
hPRAME_R	AGGGGCTCGGGACTTACAT
hPRAME_TM	**FAM**-ATGCATCTGTCCCAGAGTCC-**BHQ1**
hGPC2_F	GGCCTGGAGACTGGAAGAAAT
hGPC2_R	CCAGATAGTTGCCCCAGTCA
hGPC2_TM	**FAM**-TTAAGGTGCCGGTGTCTGAAGG-**BHQ1**
B7-H3_F	CACCATCACACCCCAGAGAAG
B7-H3_R	GGCCTTCGGTGAAACTGTG
B7-H3_TM	**FAM**-GAGGTCCAGGTCCCTGAGGA-**BHQ1**
GUS_F	GAAAATATGTGGTTGGAGAGCTCATT
GUS_R	CCGAGTGAAGATCCCCTTTTTA
GUS_TM	**FAM**-CCAGCACTCTCGTCGGTGA CTGTTCA-**BHQ1**

the highest level of expression was considered as a positive control. The cDNA from every control cell line was serially diluted in water with a step of 10 in order to construct a PCR calibration curve and calculate the efficiency of the respective primer pair. The level of the antigen expression was calculated as a relative value to the expression of GUS adjusted for the amplification efficiency. K-562 cells were used as a positive control for *MAGEA1*; IMR-32 for *TH*, *Survivin*, *PHOX2B*, *B7-H3*, and *GPC2*; HEK293T for *MAGEA3*; positive patient sample for *NY-ESO-1*.

Immunohistochemistry. Tumor tissue obtained during surgery was fixed in 10% buffered-formalin, routinely processed, paraffin-embedded, sectioned at 5 mm, and stained with hematoxylin and eosin. Representative paraffin blocks were selected for immunohistochemistry (IHC) studies. Immunohistochemistry was performed on 2.5-μm-thick paraffin sections following heat-induced epitope retrieval using CC1 (Ventana, Narcastet, France), then staining with polyclonal rabbit antibodies (Elabscience, Hubei, PR China) to PHOX2B (1/500), Tyrosine hydroxylase (1/100), MAGE1(1/100), MAGEA3(1/200) Glypican 2 (1/100) Survivin (1/100,), PRAME (1/100), CD276 (1/200), L1CAM (1/300) and ALK (clone ALK01, RTU, Ventana) on a Ventana Benchmark XT automated stainer, using Ventana UltraView Universal DAB Detection kit.

Cytogenetics. Analysis of cytogenetic alterations, including MYCN gene amplification, 17q gain, 1p36 and 11q deletions were performed in interphase nuclei by fluorescent *in situ* hybridization (FISH); 200 nuclei were analyzed for each chromosomal region. The Vysis LSI N-MYC (2p24) SpectrumGreen/CEP2 SpectrumOrange Probe was used for the MYCN gene copy number study. Vysis LSI 1p36/LSI 1q25 Dual Colour Probe was used to detect 1p36 deletions. Vysis ATM SpectrumOrange/CEP11 SpectrumGreen Probe was used to study 11q deletions. Vysis LSI TOP2A SpectrumOrange/CEP17 SpectrumGreen Probe was used to detect 17q gain. All probes manufactured by Abbott Molecular (Abbott Park, IL, USA). Karyotypes were recorded according to the International System for Human Cytogenetic Nomenclature (ISCN) recommendations.

Statistical analysis. Sensitivity and specificity of antigens expression in NB compared to benign tumors were determined by the receiver operating characteristic (ROC) curve analysis using GraphPad Prism 8 software (San Diego, CA, USA). Youden index was calculated as sensitivity + specificity−1. The optimal cut-off value was determined as antigen expression with the largest Youden index. OS and EFS curves were plotted using the Kaplan–Meier method and were compared with the log-rank test using GraphPad Prism 8 software. For EFS, an event was defined as a relapse or disease progression. The associations between CTAs expression and clinicopathological features were assessed using the Mann–Whitney *U*-test (for two independent groups) or the Kruskal–Wallis test (for three and more independent groups). For post-hoc analysis of three or more independent groups the Dunn's test for multiple comparisons was used. Cox regression was performed with IBM SPSS Statistics 23 (IBM, Armonk, NY, USA). The level of significance was 5% for all statistical tests.

Results

Expression profile of the studied antigens. All tumor specimens were confirmed as pathological tissues with at least 50% of tumor cells according to histological, cytogenetic, or flow cytometry analysis. Thus, the obtained quantitative values reflect the levels of gene expression in the tumor, but not the content of tumor cells in the sample. The distribution of expression levels of the NB-associated antigens is shown in Figure 1.

Immunohistochemical staining of tissue samples for the antigens tested was performed for 24 patients. IHC results were used to qualitatively assess antigen expression and verify quantitative PCR data. Neuroblasts were uniformly positive for PHOX2B and TH. Staining for MAGEA1, MAGEA3, Glypican 2, Survivin, PRAME, and ALK demonstrated diffuse positivity for neuroblasts. CD276 showed focal labeling (Figure 2).

Based on the results of real-time PCR, all samples were revealed to express *PHOX2B*, and 85/86 (99%) samples were *TH*, *Survivin*, *PRAME*, *B7-H3,* and *GPC2* positive. One

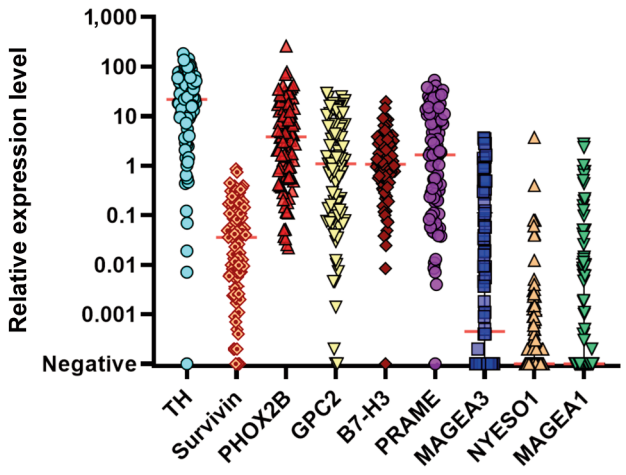

Figure 1. *Antigen expression levels in NB tumors (middle line – median).*

negative value for each of these antigens belongs to different patients, which is presumably due to occasional mutational damage of these genes. However, the level of antigen expression varied significantly. The highest expression levels were observed for the *TH*, *PHOX2B*, and *PRAME* genes, with medians 21.8, 3.8, and 1.7, respectively, and the lowest for Survivin with median 0.035. About half of the samples expressed the antigens *MAGEA3* (46/86, 53.5%), *MAGEA1* (41/86, 47.7%), and *NY-ESO-1* (45/86, 52.3%) but the expression levels of these genes were extremely low, with median less than 0.0005.

In bone marrow and peripheral blood samples obtained from healthy donors, expression of *TH*, *PHOX2B*, *PRAME*, *MAGEA1*, and *MAGEA3* was not detected, except for an occasional expression of less than 0.001 in one blood sample for *TH*.

Minor *B7-H3* expression was found both in bone marrow and peripheral blood samples from healthy donors but did not overlap with the expression in NB. Most of the healthy samples were positive for *Survivin* and *GPC2* with significantly lower levels of expression compared to NB, with the exception for *Survivin* expression in bone marrow samples (Figure 3).

Antigen expression in benign tumors and ROC analysis. To assess whether the expression of selected antigens has diagnostic significance for malignant NB tumors, we performed a similar expression measurement in a group of benign tumors followed by ROC analysis.

Ganglioneuromas had reduced expression of all antigens, but some of them were positive for *TH* (9/11, 81.8%, median 5.36) and *PHOX2B* (7/11, 63.6%, median 0.0024). Therefore, the nerve-specific markers *TH* and *PHOX2B* were excluded from further ROC analysis. Samples of giant cell tumor, osteoma, and chondroblastoma were negative for *PHOX2B* but still have sporadic low-level *TH* expression. The frequency of occurrence and expression levels of other antigens were similar among all benign tumors. The comparative distribution of antigen expression in neuroblastoma and benign tumors with ROC analysis is shown in Figure 4.

Overexpression of *PRAME*, *GPC2*, *B7-H3*, and *Survivin* is the most typical for NB and allows to determine a statistically significant threshold for distinguishing NB from benign solid tumors ($p<0.0001$). CTA antigens *MAGEA3*, *NY-ESO-1*, and *MAGEA1* demonstrated low or sporadic expression in both NB and benign tumors.

Association of antigen expression with clinicopathological characteristics. The median level of antigen expression in primary tumor samples (n=76) was determined and compared for groups of patients with different values of common clinicopathological characteristics, including sex, age, stage of the disease, risk group, cytogenetic features of tumor cells, and survival (Table II).

Expression of *MAGEA3* was higher in girls than boys ($p=0.01$), others did not correlate with sex. There was no statistically significant association between age and the level of expression of any antigen. The expression levels of *TH*, *PHOX2B*, and *MAGEA1* tended to decrease from low-risk to high-risk groups with PHOX2B expression being significantly higher in the low-risk group compared to the high-risk group (Table II).

Higher *TH* expression was observed in samples without *MYCN*-amplification ($p=0.006$) and higher expression of *Survivin*, *PHOX2B*, and *GPC2* was associated with the presence of 1p36 deletion ($p=0.0008$, $p=0.018$, and $p=0.024$, respectively). Surprisingly, 17q gain which results in amplification of *Survivin* locus located at 17q25, was not associated with increased expression of this gene. TH expression had an inverse association with the MKI level. Upon dividing the stages of the disease into local and metastatic, *TH*, *PHOX2B*, GPiC2, and *MAGEA1* showed significant association of higher expression level and local nature of the tumor (Table II). After comparing expression levels of these antigens among particular disease stages there was no significant difference for *TH* and *GPC2* but for *PHOX2B* and *MAGEA1* there was a tendency to a decrease in the level of expression at later stages.

Association between CTA/TAAs and clinical outcome. To analyze whether high expression of the studied antigens affects EFS and OS, patients were divided into two groups with high and low expression of the studied antigens in primary tumor samples (n=76) based on different cut-offs. These cut-off levels were determined as percentile values, specifically the 10th percentile, 25th percentile, median, 75th percentile, or 90th percentile.

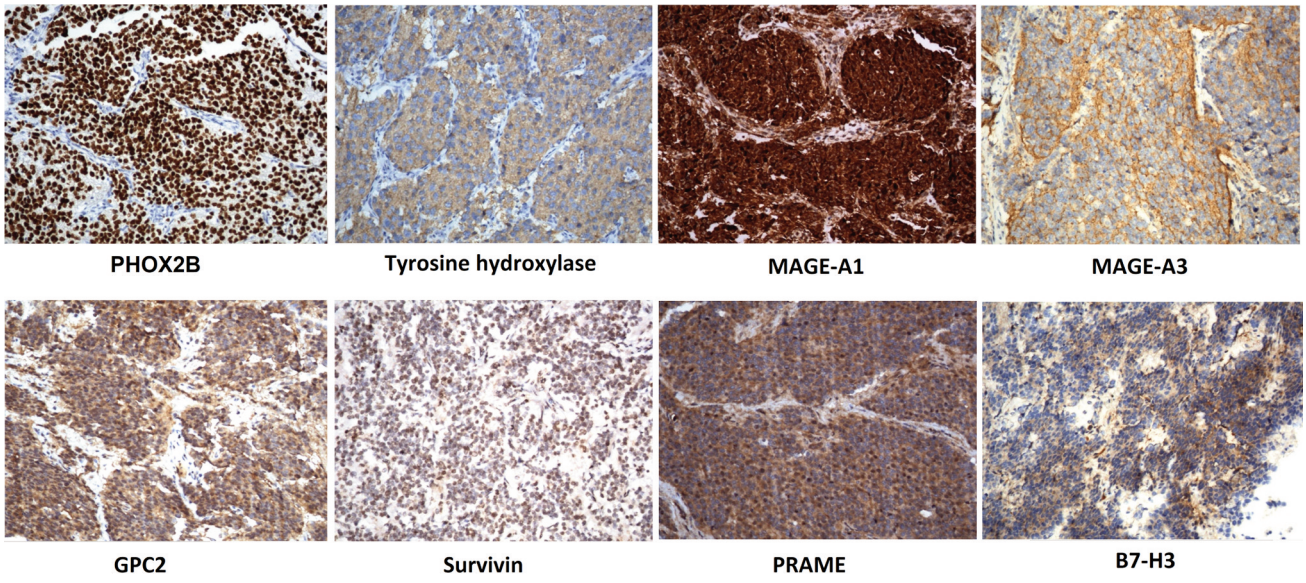

Figure 2. *Immunohistochemical features of tissue samples taken from patients with poorly differentiated neuroblastoma. Positive samples are shown. Counterstaining Bluing reagent –Hematoxylin II. Original magnification ×200.*

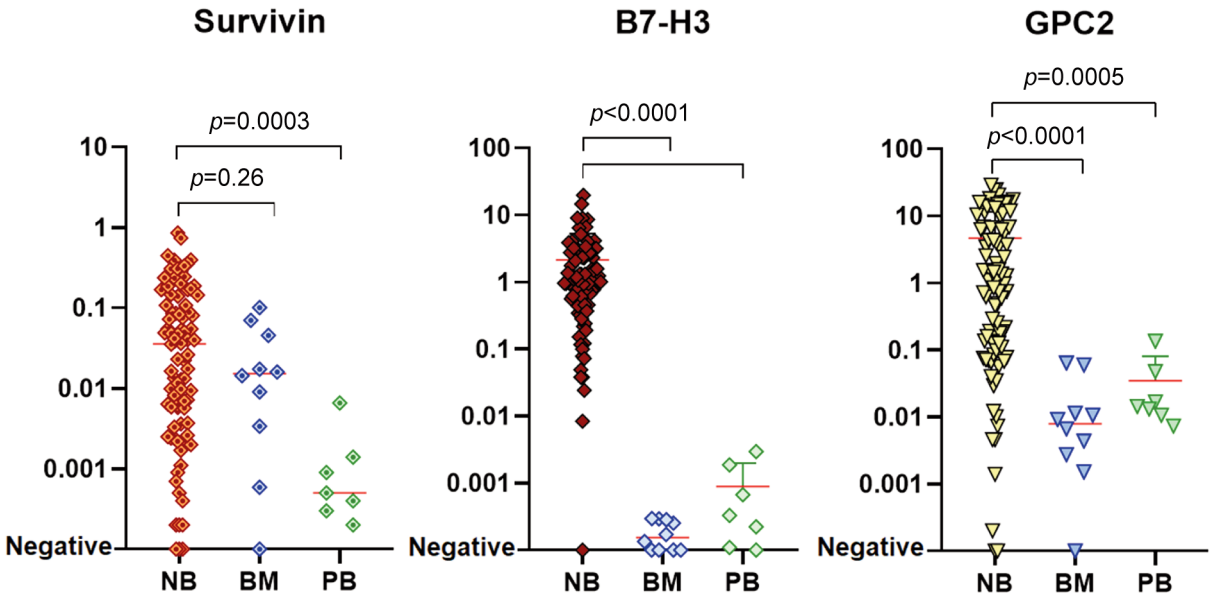

Figure 3. *Distribution of antigen expression in NB, normal bone marrow (BM) and peripheral blood (PB) (Mann–Whitney U-Test).*

No statistically significant thresholds were identified for *GPC2*, *B7*-H3, *PRAME*, and *MAGEA3*. The median (3.038) was chosen as a threshold of high expression for *PHOX2B*, the 75th percentile (53.93) for *TH*, and the 90th percentile (0.308) for *Survivin*. For *MAGEA1*, all expression values above zero were considered as an appropriate threshold for dividing patients into two groups with a statistically significant difference in EFS and OS.

High expression of *TH*, *PHOX2B*, and *MAGEA1* was associated with better EFS and OS. High *Survivin* expression was associated with poor EFS but had no impact on OS (p=0.59, not shown) (Figure 5).

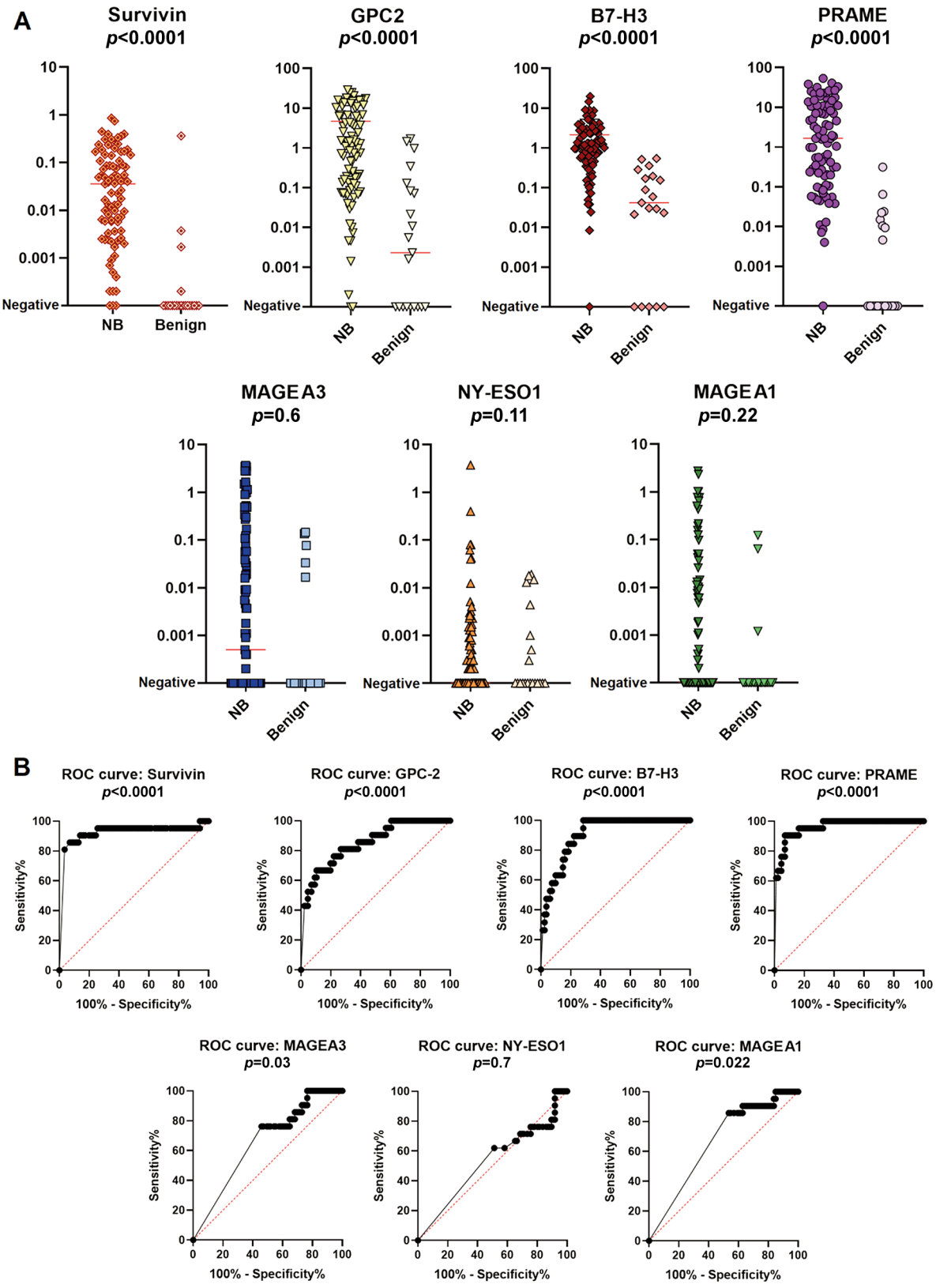

Figure 4. *Specificity of tumor-associated antigens expression in neuroblastoma. A) Distribution of antigen expression in neuroblastoma and benign tumors (Mann–Whitney U-Test. Black middle line – median). B) ROC analysis.*

Table II. *Association of TAA expression and clinicopathological characteristics of patients.*

Variable	N	*TH*		*Survivin*		*PHOX2B*		*GPC2*		*B7-H3*		*PRAME*		*MAGEA3*		*NY-ESO-1*		*MAGEA1*	
		M	*p*-Value	M	*p*-Value	M	*p*-Value	M	*p*-Value	M	*p*-Value	M	*p*-Value	M	*p*-Value	M	*p*-Value	M	*p*-Value
Age																			
<18 months	15	43.27	0.06	0.023	0.94	10.18	0.13	1.50	0.18	1.32	0.41	0.28	0.06	0	0.08	0.00004	0.55	0.00007	0.52
≥18 months	61	18.22		0.016		2.06		0.62		0.96		1.85		0.005		0.0002		0.00005	
Sex																			
Female	39	21.58	0.73	0.038	0.50	3.91	0.81	0.74	0.91	0.97	0.47	0.98	0.60	0.018	**0.01**	0.0003	0.28	0.0001	0.71
Male	37	22.06		0.01		2.75		0.75		1.07		1.88		0		0.0001		0.00005	
Risk group																			
Low	31	43.27	0.058	0.01	0.46	7.24	**0.039**	1.37	0.13	1.24	0.38	1.25	0.83	0.004	0.74	0.0002	0.40	0.0018	**0.028**
Intermediate	9	18.60		0.04		5.11		0.29		0.75		0.98		0.0001		0.0001		0.001	
High	35	18.22		0.05		1.28		0.49		0.85		1.88		0.0002		0.0001		0	
EFS																			
CR/PR	53	28.14	0.08	0.013	0.53	4.85	0.06	1.1	0.14	1.13	0.46	1.64	0.36	0.004	0.08	0.0002	0.71	0.001	**0.003**
PD	23	16.27		0.04		0.92		0.49		0.75		0.54		0		0.0001		0	
OS																			
Alive	56	33.35	**0.01**	0.02	0.94	6.72	**0.001**	1.13	**0.02**	1.21	**0.04**	1.86	0.10	0.003	0.42	0.0002	0.83	0.0004	**0.04**
Dead	20	10.65		0.02		0.53		0.14		0.59		0.43		0		0.0002		0	
MYCN																			
Non-amplified	55	32.74	**0.006**	0.016	0.29	3.72	0.74	0.75	0.66	1.06	0.72	1.59	0.90	0.004	0.29	0.0002	0.18	0.0002	0.21
Amplified	21	9.36		0.036		1.63		0.74		0.86		1.64		0.00001		0.00004		0	
1p																			
No	45	22.06	0.79	0.007	**0.0008**	2.05	**0.018**	0.46	**0.024**	0.94	0.07	1.25	0.25	0.001	0.73	0.0002	1.00	0.0004	0.61
Yes	29	20.45		0.055		11.54		1.58		1.11		1.64		0.001		0.0001		0	
17q																			
No	28	25.99	0.48	0.041	0.35	4.81	0.61	1.07	0.84	1.06	0.69	1.44	0.77	0	0.07	0.0002	0.69	0	0.098
Yes	35	21.58		0.013		5.11		1.28		1.13		1.66		0.019		0.0002		0.001	
imb11q																			
No	44	19.99	0.98	0.019	0.91	4.29	0.95	1.19	0.69	1.07	0.74	0.54	0.12	0.004	0.22	0.0003	0.26	0.0008	0.17
Yes	12	14.33		0.007		0.48		0.068		0.48		1.92		0.0007		0		0	
MKI																			
Low	39	42.21	**0.0096**	0.022	0.12	6.89	0.39	1.28	0.74	1.13	0.70	0.98	0.13	0.002	0.64	0.0002	0.06	0.0004	0.44
Intermediate	5	21.58		0.089		4.50		0.85		0.37		11.21		0.12		0		0.0002	
High	9	2.47		0.053		0.92		0.74		1.31		3.43		0.006		0.0001		0	
Stage*																			
Local	34	38.85	**0.087**	0.019	0.95	7.31	**0.004**	1.33	**0.028**	1.27	0.12	1.61	0.79	0.003	0.54	0.0002	0.12	0.0019	**0.018**
Metastatic	40	18.88		0.026		1.10		0.18		0.75		1.34		0.0001		0.00004		0	

CR: Complete response; PR: partial response; PD: progressive disease; MKI: mitosis-karyorrhexis index; M: median; *p*: *p*-value, *local: stages 1, 2, and 3; metastatic: stage 4 according to the INSS classification system. Bold values indicate statistical significance.

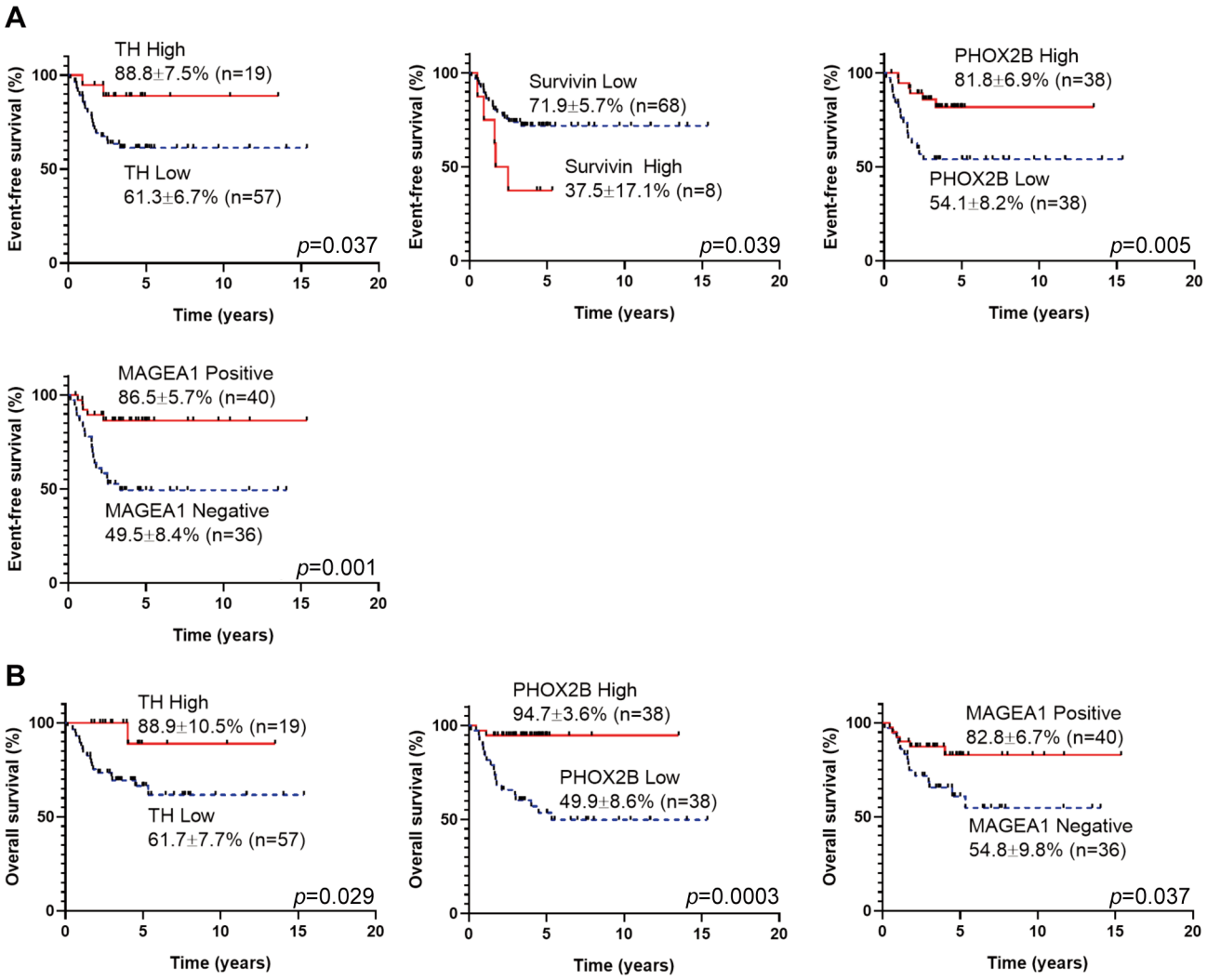

Figure 5. *Survival of patients with neuroblastoma divided into groups with high and low expression of the studied antigens: A) Event-free survival (EFS±SEM%); B) Overall survival (OS±SEM%).*

The prognostic effect of high and low expression of the studied antigens was analyzed using Cox regression in univariable and multivariable models. Age as a quantitative variable and *MYCN* amplification status as categorical variable were included into Cox regression analysis as predictors with well-defined prognostic value. The cut-off levels of antigen expression in the Cox regression analysis were also applied in the survival analysis. Only those factors that showed a statistically significant predictive impact on survival in univariable analysis were included into multivariable analysis.

It was found in the univariable analysis that high expression of *Survivin* had a significant negative impact on EFS. On the other hand, high expression of *PHOX2B* and *MAGEA1* appeared to be favorable prognostic factors. In multivariable analysis all three antigens remained statistically significant predictors of EFS, even in the presence of such factors as age and *MYCN*-amplification (Table III).

These results suggest that high expression of *PHOX2B* and *MAGEA1* in primary neuroblastoma tumors can be considered as independent favorable prognostic factors, while high *Survivin* expression is an independent unfavorable factor for EFS. Additionally, high *PHOX2B* expression showed a significant impact on improved OS in both univariable and multivariable analysis (Table III).

Discussion

In oncology, there is a growing emphasis on immunotherapy and targeted therapy as alternatives to high-dose chemotherapy and radiation therapy, which are often accompanied by severe

Table III. *Univariable and multivariable Cox Regression analysis on event-free and overall survival.*

Event-free survival				
Factor	HR	Lower 95%CI	Upper 95%CI	*p*-Value
Univariable analysis				
Age	1.220	1.041	1.430	**0.014**
MYCN	3.155	1.384	7.189	**0.006**
TH	0.241	0.057	1.030	0.055
Survivin	2.729	1.011	7.365	**0.047**
PHOX2B	0.288	0.113	0.730	**0.009**
MAGEA1	0.221	0.082	0.595	**0.003**
Multivariable analysis				
Age	1.354	1.116	1.641	**0.002**
MYCN	1.849	0.788	4.338	0.158
Survivin	3.625	1.090	12.051	**0.036**
PHOX2B	0.216	0.074	0.631	**0.005**
MAGEA1	0.272	0.097	0.764	**0.013**
Chi-square=29.17, df=5, $p<0.001$				
Overall survival				
Factor	HR	Lower 95%CI	Upper 95%CI	*p*-Value
Univariable analysis				
Age	1.143	0.952	1.374	0.152
MYCN	2.023	0.825	4.958	0.124
TH	0.145	0.019	1.084	0.060
PHOX2B	0.107	0.025	0.462	**0.003**
MAGEA1	0.375	0.144	0.977	**0.045**
Multivariable analysis				
PHOX2B	0.114	0.026	0.492	**0.004**
MAGEA1	0.420	0.161	1.095	0.076
Chi-square=16.74, df=2, $p<0.001$				

Bold values indicate statistical significance.

toxic effects. Immunotherapy strategies vary depending on the target molecule. Certain cancer-testis antigens, such as MAGEA3, NY-ESO-1, and PRAME, are well-known targets for anti-cancer vaccines, which can be formulated using proteins, peptides, RNA, or DNA. The protein Survivin is also being investigated in various anti-cancer vaccine trials and is a target for low molecular weight inhibitors (26, 27). Surface proteins like B7-H3 and GPC2 are of great interest as they can serve as targets for monoclonal antibodies, immunoconjugates, and CAR-T therapy (14, 28). Thereby, the identification of an appropriate tumor-specific antigen is the most important part of the development of any immunotherapy approach.

In addition, studying the expression profiles of TAA and CTA antigens in various types of cancer can reveal some important features of tumor biology, which may have a practical impact on prognosis. This study was focused on describing the expression profile of the most characteristic NB antigens and evaluating their association with outcome.

The traditional approach for the diagnosis of solid tumors involves histomorphological analysis combined with IHC staining of key diagnostic markers. However, IHC has limitations in terms of sensitivity and provides only an approximate quantitative assessment of protein expression. In this study, we relied on quantitative data obtained through real-time PCR. The results of PCR were analyzed using the Pfaffl method, which is more accurate than the delta-delta Ct method and allows to compare the expression of different targets (primer pairs). However, we admit that the measurement of gene expression at the RNA/cDNA level remains a surrogate estimation that does not reflect the exact amount of protein in the cell.

The main features of TAA expression in solid tumors are high heterogeneity and wide variation among patients. According to our results, relatively high expression of the *TH*, *PHOX2B*, *B7-H3*, and *PRAME* genes is typical for most NB tumors. The expression of the selected antigens is specific for neuroblastoma to varying degrees.

GPC2 expression varies widely from very high to extremely low, which is consistent with the data on the low density of Glypican 2 molecules on the tumor cell membrane in some patients (29). The expression of *Survivin* was relatively low but still markedly positive in nearly all cases.

TH and PHOX2B are well-known NB markers, which are widely used for minimal residual disease (MRD) monitoring in the bone marrow and are highly specific for NB cells (30). However, TH and PHOX2B are tissue-specific markers of the sympathetic nervous system and cannot be used to differentiate neuroblastoma from its benign ganglioneuroma counterpart. Increased expression of *PRAME*, *GPC2*, *B7-H3*, and *Survivin* genes have been shown to be highly reliable in distinguishing NB from benign tumors. It should be noted that the diagnostic expression levels of the antigens determined by ROC analysis were significantly lower than the prognostic levels equal to or greater than the median. In the analysis of OS and EFS, patients were categorized into groups based on different thresholds. The most significant differences in outcomes were observed when using relatively high thresholds, such as the median, 75th percentile, and 90th percentile.

The results of survival analysis unexpectedly indicated favorable outcome for patients with overexpression of *TH* and *PHOX2B* genes in the primary tumor. This finding should not be confused with the positive value of MRD in the bone marrow, which is often determined by *TH* and *PHOX2B* expression and is associated with poor outcome. To our knowledge, such a positive effect of high TH and PHOX2B expression on prognosis has not been previously described. We hypothesize that high expression of these

genes is a marker of neuroblastoma maturation, which is associated with improved outcome (31-33).

We also found a favorable effect of the presence of *MAGEA1* expression in the primary tumor on OS and EFS, which is consistent with previously published studies (34). On the contrary, increased expression of the *Survivin* gene showed a strong tendency toward worse EFS, as previously shown in one of the studies (19, 35). Based on our results, we found no evidence to support the previously shown negative impact of increased *PRAME* and *GPC2* expression on outcome (21, 23). Further studies are needed to fully understand the relationship between *PRAME* and *GPC2* expression and disease prognosis in the context of our study.

Conclusion

In conclusion, we can summarize that NB shows high expression of *TH*, *PHOX2B*, *PRAME*, and *B7-H3* genes. The expression of *GPC2* and *Survivin* in NB is heterogeneous, and the expression of CTA antigens *MAGEA1*, *MAGEA3*, and *NY-ESO-1* in NB is sporadic. High expression of *TH*, *PHOX2B*, and *MAGEA1* genes are favorable prognostic factors for OS and EFS, whereas high expression of *Survivin* is associated with an increased risk of relapse or progression.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

AM designed the study and wrote the manuscript. LK and VS performed RQ-PCR analysis. TM performed ICH and histology examinations. IP contributed to patients' recruitment and collection, analysis, and interpretation of data. AM and LK performed the statistical analysis. All Authors read and approved the final manuscript.

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