Original Article

Questioning How to Define the "Ultra-High-Risk" Subgroup of Neuroblastoma Patients

(neuroblastoma / ultra-high risk / *ALK* expression)

A. B. DEMIR[1,3], S. AKTAS[1], Z. ALTUN[1], P. ERCETIN[1], T. C. AKTAS[1], N. OLGUN[2]

[1]Department of Basic Oncology, [2]Department of Clinical Oncology, Institute of Oncology, Dokuz Eylul University, Izmir, Turkey
[3]Department of Basic Medical Sciences, Faculty of Medicine, Izmir University of Economics, Izmir, Turkey

Abstract. Neuroblastic tumours exhibit heterogeneity, which results in different therapeutic outcomes. Neuroblastoma is categorized into three major risk groups (low, intermediate, high risk). Recent identification of new genes raised the possibility of new biomarkers to identify sub-risk groups. In this retrospective cross-sectional study, we aimed to assess new biomarkers defining the ultra-high-risk subgroup within the high-risk group that differ in clinical situation with very bad prognosis. Twenty-five low- and 29 high-risk groups of patients were analysed for their expression of *ALK, ATRX, HIF1a, HIF2a (EPAS), H2AFX*, and *ETV5* genes at the RNA level. Immunohistochemistry was performed to confirm the protein expression level of ALK. The risk group of patients was determined according to the International Neuroblastoma Risk Group Stratification System. Spearman correlation analysis and Mann-Whitney-U nonparametric test were used to assess the importance of expression levels among the groups. $P < 0.05$ was considered as significant. Sensitivity of the results was checked by ROC curve analysis. All analysed genes were found to be highly expressed in the high-risk group compared to the low-risk group, except for *ETV5*. When the ultra-high-risk and high-risk groups were compared, *ALK* was found to be highly expressed in the ultra-high-risk group. Our results show that *ALK* may be a candidate gene whose mRNA expression levels can distinguish the ultra-high-risk subgroup of patients in the high-risk group of patients with non-familial neuroblastoma.

Introduction

Neuroblastoma (NB) is a sympathetic ganglia and adrenal medulla tumour that arises from embryonic neural crest cells (Ratner et al., 2016). NB is seen in every 1/7000 live birth, and approximately 110–120 new NB cases per year are seen in Turkey.

The first NB classification was carried out by the observation of the relation between clinical behaviour and histopathological properties of the tumour (Shimada et al., 1984). Today, the International Neuroblastoma Stratification System uses clinical, radiographic and surgical assessments of the NB patients (Brodeur et al., 1988, 1993), whereas the International Neuroblastoma Risk Group Stratification System (INRGSS) uses age, Shimada histology, tumour differentiation, MYCN status, loss of either 11q23, 1p36 or gain of 17q25, and tumour cell ploidy of the patient in stratification (Monclair et al., 2009).

In addition to *MYCN* amplification (Caren et al., 2008), 1p36 deletion (Fujita et al., 2008; Cohn et al., 2009), 11q23 deletion (Guo et al., 1999; Caren et al., 2008), and 17q25 gain (Gilbert et al., 1984; Abel et al., 1999; Caron et al., 1995) are the most frequently seen genetic changes that play a role in the risk stratification of NB, which shows that different genes that may play a role in NB prognosis can be important to guide the risk stratification and therapy protocol. Different therapeutic outcomes of patients within similar risk groups raise the possibility of the presence of sub-groups within certain risk groups, and expression levels of some genes were shown to change the therapy response in the high-risk group (Azarova et al., 2011; Schonherr et al., 2012). Especially in the high-risk group, some of the cases have very aggressive tumours. Even when using intensive treatment approaches, the patients have very poor

Received April 3, 2020. Accepted November 26, 2020.

This study was funded by the Research Foundation of Dokuz Eylul University/Izmir (Project No: 2014.KB.SAG.16) and Turkish Society of Pediatric Oncology Group (TPOG).

Corresponding author: Safiye Aktas, Dokuz Eylul University, Department of Basic Oncology, Institute of Oncology, 35330, Balcova, Izmir, Turkey. Phone: (+90) 232-4125891; e-mail: safiye.aktas@deu.edu.tr

Abbreviations: ALT – alternative lengthening of telomere pathway, INRGSS – International Neuroblastoma Risk Group Stratification System, NB – neuroblastoma, TERT – telomerase reverse transcriptase.

outcomes. New parameters or new biological markers (biomarkers) might be useful to separate these patients as an ultra-high-risk group.

In this study, *ALK, ATRX, ETV5, HIF1a, HIF1b*, and *H2AX* genes, which are thought to be important for therapeutic outcomes in the high-risk group, were assessed for their expression in the high-risk and low-risk groups. Within the high-risk group, patients who did not respond to the induction therapy and died due to progressive disease within one year were sub-grouped as "ultra-high-risk" group. We hypothesized that the expression levels of some of these genes may differ among patients in the high-risk and the ultra-high-risk group.

Material and Methods

Patients and tissue samples

Fresh tumour tissue samples of 54 NB patients were collected from universities' oncology centres from all over Turkey within the scope of the "Turkish Paediatric Oncology Group Protocol." Samples were cooled to 4 °C in transfer medium and shipped to our centre on ice blocks after signed disclosures and patient consent forms had been confirmed. Direct tissue imprinting was performed for tumour cell confirmation. Samples were stored at −80 °C until analysis. The mean age of 54 cases was 33.12 months (low-risk patients' mean age was 20.40 months while it was 44.10 months for high-risk patients). Twenty-seven of the cases were male, while twenty-seven were female.

Ethical permission was taken from the Local Ethics Commitee of Children's Research Hospital on September 26th, 2013. All cases between 2013–2015 that had sufficient fresh tissue for RNA analysis were included in the study and all these samples were from initial diagnosis. The tumoral areas from samples were chosen by diff quick toluidine blue staining and microscopic evaluation of imprint touch preparation of slides, so that samples included more than 90 % of tumour. All cases were non-familial neuroblastomas. Germline *ALK* mutations for R1275Q, F1174L, or any other were determined negative by next-generation sequencing (Illumina Miniseq, San Diego, CA; Celemics custom design kits, Seoul, Korea). This study included 25 low-risk and 29 high-risk group patients, who were categorized into risk groups and treated with the Turkish Paediatric Oncology Group Neuroblastoma 2009 protocol (see tpog.org.tr/uploads/15_09_2009.pdf). Risk classification was performed according to INRGSS. This classification is based on age, stage, MycN amplification status, any one of 1p36 deletion, 11q23 deletion and 17q25 gain status, Shimada classification of favourable or unfavourable histology status, and DNA ploidy index status. In this protocol, intensive induction chemotherapy including six courses of alternated "dacarbazine + ifosfamide + adriamycin + vincristine" and "cyclophosphamide + cisplatin + etoposide" was used. Within the high-risk group, five patients who did not respond to induction therapy and died due to progressive disease within one year were considered as patients of the "ultra-high-risk" group.

Nucleic acid isolation and real-time PCR analysis

DNA isolation was performed as described by the manufacturer (High Pure PCR Template Preparation Kit/Roche, Mannheim, Germany). Briefly, the tissue samples were lysed mechanically and then incubated at 70 °C for 60 min with the binding buffer in the presence of Proteinase K. One hundred µl isopropanol was added and with further centrifugations (8,000 *g*, 1 min, 3 times), the inhibitors were removed and the samples were washed. At the final step, the DNA was eluted with warm elution buffer. Concentrations of the purified DNA were measured by using fluorometric quantification.

2p24.3 (MYCN) amplification, 1p36 (GNB1) LOH, 11q23 (ARCN1) deletion and 17q25 (Survivin) gain status of DNA samples were assessed by TaqMan real-time PCR (RT-PCR) (Roche LightCycler Nano Real-Time PCR Instrument, Penzberg, Germany) with custom designed TaqMan labelled primers for each corresponding gene region. The reference genes for each genetic region were again selected from the corresponding chromosomes. *NAGK* (2p13.3), *NGFB* (1p13.1), *MYBPC3* (11P11.2), *TP53* (17p13.1) genes were used as reference genes for *MYCN*, 1p36, 11q23, and 17q25, respectively.

RNA isolation was performed as described by the manufacturer (TriPure/Roche, Germany). Briefly, TriPure solution was added to each tissue sample and homogenized with a homogenizer mechanically. Two hundred µl chloroform was added to homogenized samples and incubated for 10 min at room temperature. After centrifugation (12,000 *g*, 15 min, 4 °C), the upper clear supernatant was transferred into a new tube and isopropanol was added. The samples were incubated for 5–10 min at room temperature and centrifuged at 12,000 *g*, 10 min, 4 °C. The supernatants were discarded and cold ethanol (75 %) was added to each pellet. After centrifugation at 7,500 *g*, 5 min, 4 °C, the excess ethanol was removed from the samples and RNA pellets were resuspended in nuclease-free water. Fluorometric quantification was used to determine the RNA concentrations (Qubit 3.0, life technologies, Invitrogen, Singapore)

One µg RNA was used for cDNA synthesis. Briefly, oligo(dT) primers were added to RNA samples along with reaction buffer, RNase inhibitor, nucleotide mix, and reverse transcriptase enzyme and the reaction mix was incubated at 55 °C for 30 min and 85 °C for 5 min. Obtained cDNA samples were kept at −20 °C until their use as templates for determining the expression patterns of *ALK*, *ATRX*, *ETV5, HIF1A*, *HIF2A*, and *H2AFX* assessed by TaqMan RT-PCR (Roche Lightcycler).

DNA index

Tumour cells obtained from the tissues were suspended in freezing medium (95% RPMI complete, 5% DMSO) and stored at -80 °C until analysis. DNA index

was evaluated by a flow cytometer (BD Accuri, San Jose, CA). Samples were prepared for analysis as described by the manufacturer (BD Cycletest Plus DNA Kit, San Jose, CA). Fluorescence-2 (FL2) filter (585/42 nm) was used to detect the propidium iodide fluorescence between 564–606 nm. Mononuclear cells from the blood of a healthy donor was used as a reference. DNA index was determined as the ratio of the G0/G1 cell population of the patient's sample to the G0/G1 cell population of the reference sample.

ALK expression was evaluated by automated immunohistochemistry in tumour sections on polylysine-coated slides according to manufacturer's instructions (Ventana, D5F3, Tucson, AZ).

Next-generation analysis of ALK mutation and copy number variations

In order to obtain patients' germline mutations, we used mononuclear cells separated from patients' fresh blood samples. We used a Roche high pure PCR preparation kit to isolate DNA. After purification and confirmation of DNA concentration, we used Illumina MiniSeq to conduct sequencing according to guidelines.

Statistical analysis

The SPSS 15.0 program was used for statistical analysis. The necessary number of patients for each group (N = 21) was calculated by power analysis. "Mann-Whitney-U" nonparametric test was used to assess the gene expression levels among groups. Sensitivity of the results was checked by ROC curve analysis. Correlation analysis was used to determine the correlations between clinical and genetic features of the patients. P values smaller than 0.05 were considered as significant.

Results

Risk classifications of patients

Twenty-five low-risk and 29 high-risk patients were analysed in this study. Both molecular (*MYCN* amplification, 1p36 LOH, 11q23 loss, 17q25 gain, and DNA index) and clinical (age of the patient, stage and histopathology of the tumour) data of patients were evaluated for the risk classification. Detailed patient data are provided in Supplementary information (see Supplementary Tables 1, 2 and 3).

Expression status of ALK, ATRX, HIF1a, HIF2a, H2AFX, and ETV5 genes

Considering risk classification, the high-risk group of patients showed significantly increased expression of the assessed genes compared to the low-risk group of patients, with the exception of *ETV5*. In the high-risk group, *ALK1, ATRX, HIF1a, HIF2a* (*EPAS*), *H2AFX*, and *ETV5* showed 4.2-fold (P = 0.036), 3.5-fold (P = 0.000), 2.4-fold (P = 0.013), 2.6-fold (P = 0.007), 2.1-fold

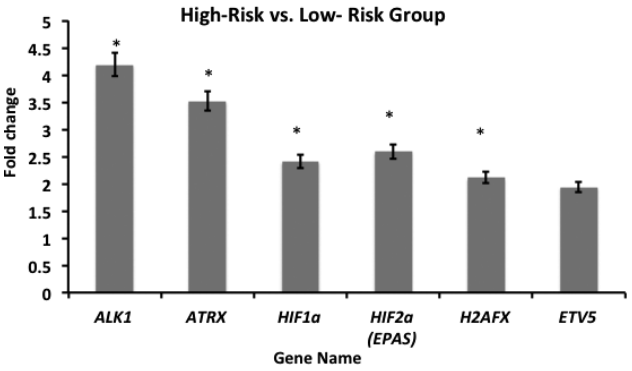

Fig. 1. Bar representation of expression fold change of the high-risk group compared to the low-risk group. Each sample was assayed at least three times and mean values are represented in the graph. For each gene, the cut-off value for Ct (cycle threshold) was considered as 35 and Ct values more than 35 were excluded. The mean values of the remaining patients were grouped for each gene and their mean values are represented. β-Actin was used as a reference gene for normalizations.

(P = 0.005), and 1.94-fold (P = 0.09) increase in their average expression values, respectively (Fig. 1).

For *ALK* expression, 21 low-risk group, 17 high-risk group patients and for *ETV5* expression, 20 low-risk group, 14 high-risk group patients could be evaluated. For *ATRX, HIF1a, HIF2a* (*EPAS*), and *H2AFX* genes, all 29 high-risk and 25 low-risk patients were evaluated.

Within the high-risk group, five patients did not respond to induction therapy and were considered as an "ultra-high-risk" group. *ALK* expression was shown to be significantly higher in the ultra-high-risk group compared to the high-risk group (P = 0.027) (Fig. 2). Al-

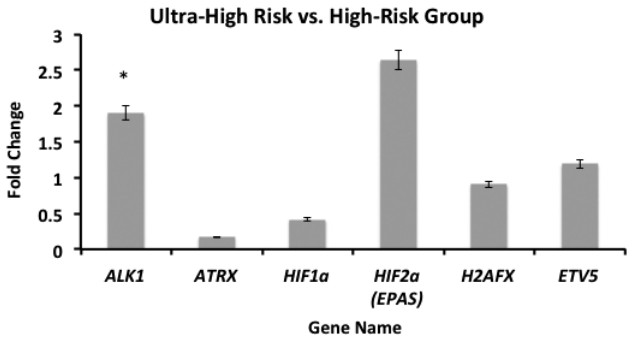

Fig. 2. Bar representation of expression fold change of the ultra-high risk group compared to the high-risk group. Each sample was assayed at least three times and average values are represented in the graph. For each gene, the cut-off value for Ct (cycle threshold) was considered as 35 and Ct values more than 35 were excluded. The mean values of the remaining patients were grouped for each gene and their mean values are represented. β-Actin was used as a reference gene for normalizations.

Supplementary Table 1. Detailed patient data of the "low-risk group" of patients (NA: not available)

											Expression					
Patient Code	**Gender**	**Age (Months)**	**Primary Site of Tumour**	**NMYC Amplification**	**1p.36 LOH**	**11q.23 Deletion**	**17q .25 Gain**	**DNA Index**	**Stage**	**Histo-pathology**	**ALK**	**ATRX**	**HIF1a**	**HIF2a (EPAS)**	**H2AFX**	**ETV**
LR-1	Male	3	Surrenal	Negative	Negative	Negative	Positive	< 1	I	Good	NA	5.49E-02	0.19275	9.88E-03	1.04E-02	NA
LR-2	Female	7	Paraspinal abdominal	Negative	Negative	Negative	Negative	< 1	I	Good	5.53E-04	2.81E-03	7.43E-03	4.90E-04	4.89E-04	1.13E-03
LR-3	Female	1	Abdominal surrenal one-sided	Negative	Negative	Negative	Negative	1	IVS	Bad	3.48E-04	9.78E-02	0.1649	9.01E-03	4.39E-03	4.99E-03
LR-4	Male	14	Paraspinal abdominal	Negative	Positive	Positive	Negative	NA	I	Good	9.05E-05	0.1089	2.75E-02	9.46E-03	7.26E-03	5.70E-04
LR-5	Female	168	Cervical	Negative	Positive	Positive	Negative	< 1	IIB	Bad	7.17E-04	7.51E-03	8.78E-03	4.17E-03	3.07E-03	5.39E-04
LR-6	Male	60	Abdominal surrenal one-sided	Negative	Negative	Negative	Negative	NA	I	Bad	NA	4.27E-02	8.39E-02	7.80E-03	1.31E-02	NA
LR-7	Female	3	Cervical	Negative	Negative	Negative	Negative	< 1	IIA	Bad	NA	0.09955	0.32015	1.40E-02	5.74E-03	5.22E-03
LR-8	Male	42	Thoracal	Negative	Negative	Positive	Positive	1	I	Good	4.64E-05	7.87E-02	6.98E-02	6.30E-03	3.92E-03	2.29E-03
LR-9	Female	18	Thoracal	Negative	Negative	Negative	Negative	NA	I	Bad	1.07E-03	3.76E-03	6.55E-03	2.03E-03	1.53E-03	3.38E-03
LR-10	Male	6	Abdominal surrenal one-sided	Negative	Negative	Negative	Positive	NA	I	Bad	1.21E-03	1.44E-02	3.35E-02	5.82E-03	5.00E-03	5.84E-03
LR-11	Female	16	Thoracal	Negative	Negative	Negative	Negative	NA	I	Bad	1.26E-04	1.51E-03	3.16E-03	1.10E-03	4.38E-04	2.60E-04
LR-12	Female	13	Surrenal	Negative	Negative	Negative	Positive	> 1	I	Good	2.34E-03	4.09E-03	1.79E-02	8.92E-04	3.05E-04	1.26E-03
LR-13	Male	24	Paraspinal (Abdominal)	Negative	Positive	Positive	Negative	1	I	Good	2.25E-03	6.30E-03	4.72E-02	3.79E-03	1.86E-03	3.45E-03
LR-14	Female	5	Abdominal surrenal one-sided	Negative	Negative	Negative	Positive	> 1	IIB	Good	3.86E-05	1.23E-02	2.19E-02	6.61E-03	4.69E-03	1.53E-03
LR-15	Female	6	Cervical	Negative	Positive	Negative	Negative	1	IIA	Good	2.54E-05	2.63E-02	5.95E-02	4.15E-03	5.54E-03	2.82E-04
LR-16	Male	3	Abdominal paraspinal	Negative	Negative	Negative	Positive	1	I	Good	4.22E-04	2.34E-03	8.95E-03	2.80E-03	6.42E-04	2.95E-04
LR-17	Male	4	NA	Negative	Negative	Negative	Positive	1	I	Good	5.42E-05	2.22E-02	4.12E-02	1.97E-02	9.24E-03	3.76E-04
LR-18	Male	15	Abdominal surrenal one-sided	Negative	Positive	Negative	Negative	1	IIB	Good	2.27E-05	4.28E-03	1.46E-02	9.15E-03	6.62E-03	1.20E-04
LR-19	Male	25	Abdominal surrenal one-sided	Negative	Negative	Positive	Positive	NA	I	Bad	5.28E-05	6.30E-03	1.97E-02	9.11E-03	4.73E-03	2.00E-04
LR-20	Male	2	Abdominal surrenal one-sided	Negative	Negative	Negative	Negative	1	I	Bad	3.05E-06	2.99E-03	3.73E-03	8.66E-03	1.47E-02	NA

Supplementary Table 2. Detailed patient data of the "high-risk group" of patients (NA: not available)

											Expression					
Patient Code	**Gender**	**Age (Months)**	**Primary Site of Tumour**	**NMYC Amplification**	**1p.36 LOH**	**11q.23 Deletion**	**17q .25 Gain**	**DNA Index**	**Stage**	**Histo-pathology**	**ALK**	**ATRX**	**HIF1a**	**HIF2a (EPAS)**	**H2AFX**	**ETV**
HR-1	Female	96	Surreneal	Positive	Positive	Negative	Positive	NA	IV	Bad	1.72E-04	0.36645	1.22E-02	3.70E-02	1.01E-02	NA
HR-2	Female	50	Pelvic	Negative	Positive	Negative	Positive	NA	III	Bad	4.05E-04	0.0988	2.28E-01	6.62E-03	1.28E-02	NA
HR-3	Female	66	Surrenal	Negative	Positive	Positive	Positive	> 1	IV	Bad	3.83E-03	7.04E-03	1.12E-02	2.36E-03	5.49E-04	6.31E-04
HR-4	Male	144	Paraspinal abdominal	Negative	Negative	Positive	Negative	NA	III	Good	2.59E-04	4.62E-02	8.00E-02	9.04E-03	7.29E-03	NA
HR-5	Male	18	Surrenal	Positive	Positive	Negative	Positive	1	III	Bad	1.07E-02	2.62E-02	1.51E-01	5.18E-03	1.16E-02	1.41E-02
HR-6	Female	9	Pelvic	Negative	Negative	Negative	Negative	< 1	IV	Bad	1.42E-05	0.14445	7.18E-02	9.97E-03	9.37E-03	3.73E-04
HR-7	Female	84	Surrenal	Negative	Positive	Negative	Positive	< 1	IV	Bad	8.51E-03	1.30E-02	4.43E-02	1.76E-03	1.43E-03	7.11E-03
HR-8	Female	84	Unknown	Negative	Negative	Negative	Positive	< 1	IV	Bad	9.43E-03	3.87E-02	5.26E-01	1.25E-02	2.15E-02	4.10E-03
HR-9	Male	36	Abdominal surrenal	Negative	Positive	Negative	Negative	< 1	IV	Bad	1.00E-03	5.59E-02	1.58E-01	1.36E-02	1.02E-02	5.02E-04
HR-10	Female	30	Thoraco-abdominal	Negative	Negative	Negative	Negative	NA	IV	Good	NA	0.349	7.66E-01	2.42E-02	1.08E-02	NA
HR-11	Female	21	Surrenal	Negative	Negative	Negative	Positive	> 1	IV	Good	NA	0.11505	2.08E-01	1.45E-02	1.11E-02	0.00E+00
HR-12	Male	19	Surrenal	Negative	Positive	Negative	Positive	NA	IV	Bad	NA	8.54E-02	2.66E-01	1.38E-02	3.50E-02	NA
HR-13	Male	36	Surrenal	Negative	NA	Negative	Positive	> 1	IV	Bad	NA	0.1542	4.33E-01	3.50E-02	1.05E-02	NA
HR-14	Female	30	Surrenal	Negative	Positive	Positive	Positive	1	IV	Bad	NA	0.2667	1.82E-01	8.49E-03	1.23E-02	NA
HR-15	Female	51	Thoraco-abdominal	Negative	Negative	Positive	Positive	1	IV	Bad	5.19E-03	6.19E-02	3.21E-01	1.18E-02	1.00E-02	2.17E-03
HR-16	Male	18	Surrenal	Positive	Positive	Negative	Positive	1	IV	Bad	8.34E-04	4.15E-02	1.83E-01	7.56E-03	4.91E-03	1.05E-03
HR-17	Male	24	Surrenal	Negative	Positive	Positive	Positive	1	IV	Bad	1.68E-04	1.27E-02	2.10E-02	5.48E-03	2.44E-03	5.35E-03
HR-18	Female	42	Surrenal	Positive	Positive	Negative	Negative	NA	III	Good	NA	0.227	1.68E-01	2.76E-02	1.33E-02	NA
HR-19	Male	36	Thoraco-abdominal	Negative	Negative	Positive	Positive	NA	IV	Bad	NA	3.31E-03	4.07E-02	0.1588	3.48E-02	NA
0	Female	48	Surrenal	Negative	Negative	Positive	Positive	1	III	Bad	NA	0.1189	1.16E-03	2.12E-02	5.82E-02	NA

Supplementary Table 3. Detailed patient data of the "ultra-high-risk group" of patients (NA: not available)

										Expression					
Patient Code	**Gender**	**Age (Months)**	**Primary Site of Tumour**	**NMYC Amplification**	**1p.36 LOH**	**11q.23 Deletion**	**17q .25 Gain**	**DNA Index**	**Stage**	**Histo-pathology**	**ALK**	**ATRX**	**HIF1a**	**HIF2a (EPAS)**	**H2AFX**	**ETV**
HR-3	Female	66	Surrenal	Negative	Positive	Positive	Positive	> 1	IV	Bad	3.83E-03	7.04E-03	1.12E-02	2.36E-03	5.49E-04	6.31E-04
HR-5	Male	18	Surrenal	Positive	Positive	Negative	Positive	1	III	Bad	1.07E-02	2.62E-02	1.51E-01	5.18E-03	1.16E-02	1.41E-02
HR-9	Male	36	Abdominal surrenal	Negative	Positive	Negative	Negative	< 1	IV	Bad	1.00E-03	5.59E-02	1.58E-01	1.36E-02	1.02E-02	5.02E-04
HR-19	Male	36	Thoraco-abdominal	Negative	Negative	Positive	Positive	NA	IV	Bad	NA	3.31E-03	4.07E-02	0.1588	3.48E-02	NA
HR-29	Male	53	Abdominal surrenal one-sided	Positive	Positive	Negative	Positive	1	IV	Bad	1.24E-03	4.74E-03	4.74E-03	9.65E-04	1.23E-03	9.06E-04

though HIF2α (EPAS) expression was almost 2.5-fold higher in the ultra-high-risk group, this increase was not found to be significant (P = 0.685). Immunohistochemical expression of ALK was correlated with its RNA expression.

Correlation analysis

No significant relation was found between the assessed genes and *MYCN* amplification, 1p36 loss, 11q23 deletion, and 17q25 gain status, except for *ALK*, which was found to be highly expressed in patients with 17q gain (P = 0.018). Gender was shown not to be related with any of the molecular parameters. A significant relation was found between the age and disease stage (P = 0.010). The mean age of stage IIB, III and IV patients was higher than that of stage I, IIA and IVS patients, which was an expected result.

Discussion

Due to different therapeutic outcomes of NB patients, identification of new genes could be important for risk group determination in this disease, especially for the high-risk group. When the expression patterns of *ALK*, *ATRX*, *ETV5*, *HIF1a*, *HIF1b*, and *H2AX* were compared, all showed significantly higher expression in the high-risk group compared to the low-risk group, except for *ETV5*.

ETV5 is a conserved member of the ETS transcription factor family (Jedlicka and Gutierrez-Hartmann, 2008). It is highly expressed in colon carcinomas (Killela et al., 2013), Hec-1A endometrioid carcinoma cell lines, most breast carcinomas, and found to be related with metastatic phenotype and bad prognosis (Firlej et al., 2008). In our study, *ETV5* gene expression showed 1.94-fold increase in the high-risk group compared to the low-risk group; however, this increase was not significant, which may be due to the small number of high-risk group patients that could be evaluated for this gene. Increasing the number of patients would give more significant results for this gene.

HIF1a and *HIF2a* genes were shown to be highly expressed in the high-risk group compared to the low-risk group. High *HIF2a* levels were shown to be related with aggressive phenotype in NB and its reduction negatively affected tumour growth (Helczynska et al., 2008; Hamidian et al., 2015). In our study, increased expression of this gene in the high-risk group seems to support the role of *HIF2a* in bad prognosis of NB. When the ultra-high-risk and high-risk groups were compared, *HIF2a* expression showed a 2.5 fold-increase (Fig. 2). However, this increase was not statistically significant (P = 0.685).

ATRX is a gene that plays a role in chromatin remodelling. Inactivating mutations of *ATRX* were shown to represent a different cluster of patients within the high-risk group (Hertwig et al., 2016), and these mutations were shown to activate the alternative lengthening of telomere pathway (ALT) (Cheung et al., 2012). Within

the high-risk group, patients with *ATRX* mutations showed worse progression (Monge et al., 2007). In the high-risk NB patients, telomerase reverse transcriptase (*TERT*) activation is one of the factors that may play a role in the aggressive disease pattern (Peifer et al., 2015; Valentijn et al., 2015). Telomere lengthening is a frequently seen feature of the high-risk group (Nicolai et al., 2015). *ATRX* mutations were shown to be present in NB cases with unamplified *MYCN* and normal *TERT* levels. Our data support the finding that *ATRX* mutations are not dependent on *MYCN* amplification (Hertwig et al., 2016), since no relation was found between the expression of these two genes. Although increased *ATRX* expression in the high-risk group may act through the *TERT* mechanism, it was previously shown that *TERT* and *ATRX* are mutually exclusive (Mazzocco et al., 2015), meaning that they may activate telomere lengthening independently as well. *ATRX* expression did not show a significant increase in the ultra-high-risk group compared to the high-risk group, which may be an expected result (Cheung et al., 2012). However, the increase in *ATRX* expression in the high-risk group compared to the low-risk group is confusing, since loss of *ATRX* expression was shown to result in worse disease progression. The mutually exclusive nature of *ATRX*, *TERT* and *MYCN* can partially explain the worse progression pattern in the cases where *ATRX* was highly expressed. *ATRX* mutations were thought to play a role in the outcome of different risk-based therapies. Therefore, increasing the number of patients and assessing the *TERT* status in these patients would help to clarify the role of this gene as a marker of a subgroup within the high-risk group.

When ultra-high-risk and high-risk groups were compared, only *ALK* expression was found to be significantly high in the ultra-high-risk group. *ALK* was not found to have any relation with *MYCN* amplification, as well as with 1p36 deletion and 11q23 loss. Therefore, it may be a candidate gene to distinguish the ultra-high-risk subgroup within the high-risk group.

ALK is a receptor tyrosine kinase member of the insulin receptor superfamily (Azarova et al., 2011). Amplified *ALK* induces cell viability and division (Nicolai., 2015) and *ALK*-activating mutations are seen in 8 % of children diagnosed with NB (Mazzocco et al., 2015). Apart from these activating mutations, expression of the ALK protein by immunohistochemistry was shown to have a role in detection of ALK for clinical implications (Molenaar et al., 2012). Increased expression of *ALK* seems to play a role in poor prognosis of NB. *ALK* amplification was previously found in approximately 14 % of *MYCN* amplified cases (George et al., 2008). In our study, we could not find a significant correlation between *ALK* and *MYCN* amplification, which was surprising due to their chromosomal locations.

One of the most important limitations of our study is that we studied a relatively low number of cases. Especially the number of ultra-high-risk group patients was low in our study, since NB is a rare disease. Therefore, evaluation of *ALK* expression in more patients who did not respond to the induction therapy would yield more precise data about the role of this gene in NB. However, due to its increased expression in the ultra-high-risk group, we propose that *ALK* expression has the potential to be used as a marker to determine the ultra-high-risk subgroup within the high-risk group of NB patients, especially for the induction therapy response. The protein expression pattern of this gene could also be important to confirm its RNA expression pattern. However, due to the scant availability of tumour tissue samples from these patients, we could not evaluate the protein expression patterns for all studied RNAs. We studied ALK immunohistochemistry with the antibody clone which is validated for non-small cell lung carcinoma (Uruga and Mino-Kenudson, 2018). This clone D5F3 shows the aberrant protein produced via translocations. None of our cases showed immunohistochemistry positivity more than 2+.

In this study we conclude that although there is no germline (familial) and/or somatic *ALK* gene aberration in neuroblastoma cases, *ALK* expression at the mRNA level might be a biomarker for very bad prognostic high-risk patients who may be categorized as ultra-high-risk patients. These cases did not give any response to induction therapy. Besides, they did not show any *ALK* mutation or aberration, and therefore are not suitable for anti-ALK targeted therapies determined by hybrid capture-based NGS. Although the ALK protein expression pattern in tumour cells was shown previously, to our knowledge, a possible link between *ALK* and the induction therapy outcome has not been shown. Therefore, we propose that the evaluation of *ALK* expression at the mRNA level as a routine NB molecular analysis would help to predict the induction therapy outcomes for these patients and *ALK* may be a marker gene for the ultra-high-risk subgroup of NB patients.

Conflicts of interests

The authors declare no potential conflicts of interest.

Acknowledgement

We would like to thank Prof. Dr. Pembe Keskinoglu for her help in statistical analysis.

References

Abel, F., Ejeskar, K., Kogner, P., Martinson T. (1999) Gain of chromosome arm 17q is associated with unfavourable prognosis in neuroblastoma, but does not involve mutations in the somatostatin receptor 2(SSTR2) gene at 17q24. *Br. J. Cancer* **8**, 1402-1409.

Azarova, A. M., Gautam, G., George, R. E. (2011) Emerging importance of ALK in neuroblastoma. *Semin. Cancer Biol.* **21**, 267-275.

Brodeur, G. M., Seeger, R. C., Barrett, A., Berthold, F., Castleberry, R. P., D'Angio, G., De Bernardi, B., Evans, A. E., Favrot, M., Freeman, A. I. (1988) International criteria for

diagnosis, staging, and response to treatment in patients with neuroblastoma. *J. Clin. Oncol.* **6**, 1874-1881.

Brodeur, G. M., Pritchard, J., Berthold, F., Carlsen, N. L., Castel, V., Castleberry, R. P., De Bernardi, B., Evans, A. E., Favrot, M., Hedborg, F. (1993) Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. *J. Clin. Oncol*. **11**, 1466-1477.

Caren, H., Erichsen, J., Olsson, L., Enerback, C., Sjoberg, R. M., Abrahamsson, J., Kogner, P., Martinsson, T. (2008) High-resolution array copy number analyses for detection of deletion, gain, amplification and copy-neutral LOH in primary neuroblastoma tumors: four cases of homozygous deletions of the CDKN2A gene. *BMC Genomics* **9**, 353.

Caron, H. (1995) Allelic loss of chromosome 1 and additional chromosome 17 material are both unfavourable prognostic markers in neuroblastoma. *Med. Pediatr. Oncol*. **24**, 215-221.

Cheung, N. K., Zhang, J, Lu, C., Parker, M., Bahrami, A., Tickoo, S. K., Heguy, A., Pappo, A. S., Federico, S., Dalton, J., Cheung, I. Y., Ding, L., Fulton, R., Wang, J., Chen, X., Becksfort, J., Wu, J., Billups, C. A., Ellison, D., Mardis, E. R., Wilson, R. K., Downing, J. R., Dyer, M. A. (2012) Association of age at diagnosis and genetic mutations in patients with neuroblastoma. *JAMA* **307**, 1062-1071.

Cohn, S. L., Pearson, A. D., London, W. B., Monclair, T., Ambros, P. F., Brodeur, G. M., Faldum, A., Hero, B., Lehara, T., Machin, D., Mosseri, V., Simon, T., Garaventa, A., Caste, V., Matthay, K. K. (2009) The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report. *J. Clin. Oncol*. **27**, 289-297.

Firlej, V., Ladam, F., Brysbaert, G., Dumont, P., Fuks, F., de Launoit, Y., Benecke, A., Chotteau-Lelievre, A. (2008) Reduced tumorigenesis in mouse mammary cancer cells following inhibition of Pea3- or Erm-dependent transcription. *J. Cell Sci*. **121**, 3393-3402.

Fujita, T., Igarashi, J., Okawa, E.R., Gotoh, T., Manne, J., Kolla, V., Kim, J., Zhao, H., Pawel, B. R., London, W. B., Maris, J. M., White, P. S., Brodeur, G. M. (2008) CHD5, a tumor suppressor gene deleted from 1p36.31 in neuroblastomas. *J. Natl. Cancer Inst.* **100**, 940-949.

George, R. E., Sanda, T., Hanna, M., Frohling, S., Luther, W. 2nd, Zhang, J., Ahn, Y., Zhou, W., London, W. B., McGrady, P., Xue, L., Zozulya, S., Gregor, V. E., Webb, T. R., Gray, N. S., Gilliland, D. G., Diller, L., Greulich, H., Morris, S. W., Meyerson, M., Look, A. T. (2008) Activating mutations in ALK provide a therapeutic target in neuroblastoma. *Nature* **455**, 975-978.

Gilbert, F. Feder, M., Balaban, G., Brangman, D., Lurie, D. K., Podolsky, R., Rinaldt, V., Vinikoor, N., Weisband, J. (1984) Human neuroblastomas and abnormalities of chromosomes 1 and 17. *Cancer Res*. **44**, 5444-5449.

Guo, C., White, P. S., Weiss, M. J., Hogarty, M. D., Thompson, P. M., Stram, D. O, Gerbing, R., Matthay, K. K., Seeger, R. C., Brodeur, G. M., Maris, J. M. (1999) Allelic deletion at 11q23 is common in MYCN single copy neuroblastomas. *Oncogene* **18**, 4948-4957.

Hamidian, A., von Stedingk, K., Munksgaard Thoren, M., Mohlin, S., Pahlman, S. (2015) Differential regulation of HIF-1 α and HIF-2α in neuroblastoma: estrogen-related receptor α (ERRα) regulates HIF2A transcription and correlates to poor outcome. *Biochem. Biophys. Res. Commun.* **461**, 560-567.

Helczynska, K., Larsson, A. M., Holmquist Mengelbier, L., Bridges, E., Fredlund, E., Borgquist, S., Landberg, G., Pahlman, S., Jirstrom, K. (2008) Hypoxia-inducible factor-2α correlates to distant recurrence and poor outcome in invasive breast cancer. *Cancer Res.* **68**, 9212-9220.

Hertwig, F., Peifer, M., Fischer, M. (2016) Telomere maintenance is pivotal for high-risk neuroblastoma. *Cell Cycle* **15**, 311-312.

Jedlicka, P., Gutierrez-Hartmann, A. (2008) Ets transcription factors in intestinal morphogenesis, homeostasis and disease. *Histol. Histopathol*. **23**, 1417-1424.

Killela, P. J., Reitman, Z. J., Jiao, Y., İao, Y., Bettegowda, C., Agrawal, N., Diaz, L. A. Jr., Friedman, A. H., Friedman, H., Gallia, G. L., Giovanella, B. C., Grollman, A. P., He, T. C., He, Y., Hruban, R. H., Jallo, G. I., Mandahl, N., Meeker, A. K., Mertens, F., Netto, G. J., Rasheed, B. A., Riggins, G. J., Rosenquist, T. A., Schiffman, M, Shih, I. M., Theodorescu, D., Torbenson, M. S., Velculescu, V. E., Wang, T. L., Wentzensen, N., Wood, L. D., Zhang, M., McLendon, R. E., Bigner, D. D., Kinzler, K, W., Vogelstein, B., Papadopoulos, N., Yan, H. (2013) TERT promoter mutations occur frequently in gliomas and a subset of tumors derived from cells with low rates of self-renewal. *Proc. Natl. Acad. Sci. USA* **110**, 6021-6026.

Mazzocco, K., Defferrari, R., Sementa, A. R., Garaventa A, Longo L, De Mariano M, Esposito M. R., Negri, F., Ircolò, D., Viscardi, E., Luksch, R., D'Angelo, P., Prete, A., Castellano, A., Massirio, P., Erminio, G., Gigliotti, A. R., Tonini, G. P., Conte, M. (2015) Genetic abnormalities in adolescents and young adults with neuroblastoma: a report from the Italian Neuroblastoma group. *Pediatr. Blood Cancer* **62**, 1725-1732.

Molenaar, J. J., Koster, J., Zwijnenburg, D. A., van Sluis, P., Valentijn, L. J., van der Ploeg, I., Hamdi, M., van Nes, J., Westerman, B. A., van Arkel, J., Ebus, M. E., Haneveld, F., Lakeman, A., Schild, L., Molenaar, P., Stroeken, P., van Noesel, M. M., Ora, I., Santo, E. E., Caron, H. N., Westerhout, E. M., Versteeg, R. (2012) Sequencing of neuroblastoma identifies chromothripsis and defects in neuritogenesis genes. *Nature* **483**, 589-593.

Monge, M., Colas, E., Doll, A., Gonzalez, M., Gil-Moreno, A., Planaguma, J., Quiles, M., Arbos, M. A., Garcia, A., Castellvi, J., Llaurado, M., Rigau, M., Alazzouzi, H., Xercavins, J., Alameda, F., Reventos, J., Abal, M. (2007) ERM/ETV5 up-regulation plays a role during myometrial infiltration through matrix metalloproteinase-2 activation in endometrial cancer. *Cancer Res.* **67**, 6753-6759.

Monclair, T., Brodeur, G. M., Ambros, P. F., Brisse, H. J., Cecchetto, G., Holmes, K., Kaneko, M., London, W. B., Mattha,y K. K., Nuchtern, J. G., von Schweinitz, D., Simon, T., Cohn, S. L., Pearson, A. D.; INRG Task Force. (2009) The International Neuroblastoma Risk Group (INRG) staging system: an INRG Task Force report. *J. Clin. Oncol*. **27**, 298-303.

Nicolai, S., Pieraccioli, M., Peschiaroli, A., Melino, G., Raschellà, G. (2015) Neuroblastoma: oncogenic mechanisms and therapeutic exploitation of necroptosis. *Cell Death Dis*. **6**, e2010.

Peifer, M., Hertwig, F., Roels, F., Dreidax, D., Gartlgruber, M., Menon, R., Krämer, A., Roncaioli, J. L., Sand, F., Heuckmann, J. M., Ikram, F., Schmidt, R., Ackermann, S., Engesser, A., Kahlert, Y., Vogel, W., Altmüller, J., Nürnberg, P., Thierry-Mieg, J., Thierry-Mieg, D., Mariappan, A., Heynck, S., Mariotti, E., Henrich, K. O., Gloeckner, C., Bosco, G., Leuschner, I., Schweiger, M. R., Savelyeva, L., Watkins, S. C., Shao, C., Bell, E., Höfer, T., Achter, V., Lang, U., Theissen, J., Volland, R., Saadati, M., Eggert, A., de Wilde, B., Berthold, F., Peng, Z., Zhao, C., Shi, L., Ortmann, M., Büttner, R., Perner, S., Hero, B., Schramm, A., Schulte, J. H., Herrmann, C., O'Sullivan, R. J, Westermann, F., Thomas, R. K, Fischer, M. (2015) Telomerase activation by genomic rearrangements in high-risk neuroblastoma. *Nature* **526**, 700-704.

Ratner, N., Brodeur, G.M., Dale, R.C., Schor, N. F. (2016) The "neuro" of neuroblastoma: neuroblastoma as a neurodevelopmental disorder. *Ann. Neurol*. **80**,13-23.

Schonherr, C., Ruuth, K., Kamaraj, S., Wang, C. L., Yang, H. L., Combaret, V., Djos, A., Martinsson, T., Christensen, J. G., Palmer, R. H., Hallberg, B. (2012) Anaplastic lymphoma kinase (ALK) regulates initiation of transcription of MYCN in neuroblastoma cells. *Oncogene* **31**, 5193-5200.

Shimada. H., Chatten. J., Newton. W.A., Sachs, N., Hamoudi, A. B., Chiba, T., MArsden, H. B., Misugi, K. (1984) Histopathologic prognostic factors in neuroblastic tumors: definition of subtypes of ganglioneuroblastoma and an age-linked classification of neuroblastomas. *J. Natl. Cancer Inst.* **73**, 405-416.

Uruga, H., Mino-Kenudson, M. (2018) Alk (D5F3) CDx: an immunohistochemistry assay to identify ALK-positive NSCLC patients. *Pharmgenomics Pers. Med*.**11**, 147-155.

Valentijn, L. J., Koster, J., Zwijnenburg, D. A., Hasselt, N. E., van Sluis, P., Volckmann, R., van Noesel, M. M., George, R. E., Tytgat, G. A., Molenaar, J. J., Versteeg, R. (2015) TERT rearrangements are frequent in neuroblastoma and identify aggressive tumors. *Nat. Genet*. **47**, 1411-1414.