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Original Article

The Role of *ATRX* and *TERT* Expressions in Determining Aggressiveness of Neuroblastoma

ATRX ve TERT Ekspresyonunun Nöroblastom Agresifliğini Belirlemedeki Rolü

Murat Tuncel[1], Selen Kum Özşengezer[1], Gamze Sanlav[1], Deniz Kızmazoğlu[2], Safiye Aktaş[1], Zekiye Altun[1], Tekincan Çağrı Aktaş[1], Erdener Özer[3], Nur Olgun[2]

[1]Dokuz Eylül University Institute of Oncology, Department of Basic Oncology, İzmir, Türkiye
[2]Dokuz Eylül University Institute of Oncology, Department of Pediatric Oncology, İzmir, Türkiye
[3]Dokuz Eylül University Faculty of Medicine, Department of Medical Pathology, İzmir, Türkiye

ABSTRACT

Objective: Neuroblastoma (NB) is a common childhood tumour, affecting telomerase enzyme activity. Expression of telomerase reverse transcriptase (*TERT*) protein is crucial for the fuınctioning of telomerase activity, but its association with risk stratification and prognosis is unclear. The adenosine triphosphate-dependent helicase alpha-thalassemia/mental retardation x-linked (*ATRX*) protein, a chromatin remodeling protein, accumulates H3.3 histone variants. The study aimed to assess the correlation between expression levels of *ATRX* and *TERT* with the NB risk group and its prognosis.

Method: Immunohistochemical expressions of *TERT* and *ATRX* proteins in tumour tissue samples of 54 NB cases at different stages and risk groups were evaluated.

Results: Immunohistochemical expression rates of *TERT* and *ATRX* proteins in tissues were 55.8% and 61.2%, respectively, with ATRX positively expressed at a rate of 50%, and 69% in early and in advanced stages of NB, and rates of 67.7%, and 50% in high and low-risk groups, respectively. *TERT* expression varies in early and advanced stages of NB, with higher levels in high-risk groups. *ATRX* expression is significantly higher in NB patients with *Neuroblastoma myc (NMYC)* gene amplification.

Conclusion: High expression of *ATRX* in NB patients with *NMYC* gene amplification suggests that *ATRX* may be used as a potential immunohistochemical prognostic marker in NB patients.

Keywords: Neuroblastoma, *TERT*, *ATRX*, immunohistochemistry

ÖZ

Amaç: Nöroblastom (NB), telomeraz enzim aktivitesini etkileyen yaygın bir çocukluk çağı tümörüdür. *TERT* ekspresyonu telomeraz aktivitesi için çok önemlidir, ancak risk sınıflandırması ve prognoz ile ilişkisi belirsizdir. Bir kromatin yeniden şekillendirme proteini olan adenozin trifosfat bağımlı helikaz alfa-talesemi/zeka geriliği, X'e bağlı (*ATRX*), H3.3 histon varyantlarını biriktirir. Bu çalışmada *ATRX* ve *TERT* ekspresyon düzeyleri ile NB risk grubu ve prognoz arasındaki ilişkinin değerlendirilmesi amaçlanmıştır.

Yöntem: Farklı evre ve risk gruplarındaki 54 NB olgusunun tümör dokusu örneklerinde immünohistokimyasal telomeraz ters transkriptaz (*TERT*) ve *ATRX* protein ekspresyonları değerlendirildi.

Bulgular: Dokularda *TERT* ve *ATRX* ekspresyonu sırasıyla %55,8 ve %61,2 idi ve *ATRX* erken evrelerde %50 ve yüksek evrelerde %69 oranında pozitif eksprese edildi. Yüksek riskli gruplarda %67,7 ve düşük riskli gruplarda %50 ekspresyon göstermiştir. *TERT* ekspresyonu erken ve yüksek evrelerde değişkenlik gösterirken, yüksek riskli gruplarda daha yüksek seviyelerdedir. *ATRX* ekspresyonu *NMYC* amplifikasyonu olan NB hastalarında anlamlı derecede yüksektir.

Sonuç: *NMYC* amplifikasyonu olan NB hastalarında *ATRX*'in yüksek ekspresyonu, *ATRX*'in NB hastalarında potansiyel bir immünohistokimyasal prognostik belirteç olarak kullanılabileceğini düşündürmektedir.

Anahtar kelimeler: Nöroblastom, *TERT*, *ATRX*, immünohistokimya

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Corresponding Author
Selen Kum Özşengezer,
Dokuz Eylül University Institute of Oncology, Department of Basic Oncology, İzmir, Türkiye
E-mail: selenkum@gmail.com
ORCID: 0000-0002-7068-5979

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INTRODUCTION

Neuroblastoma (NB) is the most common extracranial solid tumour in children. This tumour, which is frequently seen in children younger than two years of age, is defined in 90% of children younger than five years of age[1]. NB may show spontaneous regression or benign transformation to ganglinouroma especially in patients under 1 year of age. In patients over 1 year of age, the disease has a more aggressive course[1]. Genetic changes play an important role in the prognosis and treatment of the disease[2]. In the risk classification made for the treatment of the disease, genetic mutations and chromosomal changes in the patient are also examined in addition to the patient's age, tumour stage and histology[3,4]. MYCN oncogene amplification is an amplification observed in 20-30% of NB patients and is associated with poor prognosis[5] In advanced NB patients with *MYCN* amplification, event-free and overall survival (OS) rates are reported to be considerably lower compared to the patients without[6]. According to the The Turkish Pediatric Oncology Group (TPOG) - NB 2009 Protocol study conducted between 2009 and 2020, 70% of the patients diagnosed in Türkiye had advanced disease and 59% of these patients were in the high risk group according to the NB diagnosis and treatment protocol[4]. The tumour suppressor gene alpha-thalassemia/mental retardation X-linked (*ATRX*) blocks DNA replication and transcription by taking part in a chromatin remodelling whose main function is the accumulation of histone variant H3.3[7]. *ATRX* mutations are commonly found in glioma and are associated with the development of alternative telomere lengthening (ALT), a non-telomerase-dependent telomere lengthening mechanism[8]. In addition to its known roles, it influences various cellular processes associated with epigenetic regulation[9]. The loss of *ATRX* can arise through gene mutations, deletions, or chromosomal rearrangements such as gene fusions. The ALT phenotype is frequently linked to distinct molecular changes, including amplification of the platelet-derived growth factor receptor-alpha and mutations in the tumour suppressor gene tumor tumor protein p53. In most NB cells, upregulation of telomerase activity or activation of the ALT pathway results in activation of telomere maintenance mechanisms. Activation of the ALT pathway is mostly caused by mutations in the *ATRX* gene. As a result of this activation, telomeres elongate leading to carcinogenesis. *ATRX* mutations are observed especially in NB patients older than 18 months and show a positive correlation with age[7]. Telomerase is a key enzyme involved in regulating cell proliferation and tumorigenesis. It maintains chromosomal integrity by adding hexameric sequences to the ends of chromosomes, thereby preventing telomere shortening and the onset of cellular senescence. The catalytic subunit of human telomerase, known as *hTERT*, serves as the primary rate-limiting component for telomerase activity[10]. This enzyme is active in over 90% of human malignancies. In cancer, telomerase reverse transcriptase (*TERT*) expression can be elevated through multiple mechanisms, such as gene amplifications, promoter mutations, and chromosomal rearrangements. Notably, promoter rearrangements of *TERT* have been identified in high-risk NB cases[11]. In NB, determination of prognostic markers that can provide information about the risk groups and prognosis of patients is very important both for the development of targeted therapies and for obtaining better treatment results. Some molecules that may play a role in the prognosis of NB or treatment response continue to be revealed[12-17]. In this study, we investigated *TERT* and *ATRX* protein expressions in tumour tissues of early-and late- stage NB patients from different NB risk groups and evaluated whether or not their expression levels could be differentiating markers in terms of aggressiveness in early- and late- stages of NB by revealing their relationship with NB risk groups and prognosis.

MATERIALS and METHODS

Experimental Groups

Permission was obtained from Dokuz Eylül University (DEU) Non-Interventional Research Ethics Committee for the conduction of this research study (decision no: 2023/14-10, dated: 03.05.2023). Signed consent forms were obtained from the patients during the sample collection and storage stages. The study was carried out using tumour paraffin tissue samples of 53 NB patients submitted to DEU Institute of Oncology Department of Basic Oncology within the scope of TPOG-2020 protocol. Tissue samples of NB patients whose molecular analyses were performed and known to be in low and advanced stages of NB were used in the study[12,18].

Study population of 53 people consisted of 22 girls and 31 boys (Table 1). In NB, stage L1 refers to localised tumours confined within a single anatomical compartment without any image-defined risk factors (IDRFs). Stage L2 is characterised by regional tumours with the presence of IDRFs. The tumour may extend into different compartments on the ipsilateral side of the body. Stage M includes cases with distant metastatic spread. Involvement of the bone, liver, distant lymph nodes, or

Table 1. Characteristic features of patients with neuroblastoma	
Characteristic	**Total (n=53)**
Age (months) (mean ± SD)	22.95±27.21
Gender, n (%)	
Female	22 (41.5)
Male	31 (58.5)
Risk groups, n (%)	
Low	18 (34.0)
Intermediate	9 (17.0)
High	22 (41.5)
***MYCN* AMP, n (%)**	
Positive	15 (28.3)
Negative	37 (69.8)
EFS (months) (mean ± SD)	21.39±27.01 (n=44)
OS (months) (mean ±SD)	24.70±26.62 (n=44)

Note: The International Neuroblastoma Risk Group Staging System (INRGSS) classification is used to determine the risk groups, and characteristic features of 53 study participants were stratified based on the criteria established by the Turkish Pediatric Oncology Group-2020 and INSS.
AMP: Amplification, EFS: Event-free survival, OS: Overall survival

pleural/abdominal effusion containing malignant cells outside the primary site indicates metastatic disease. Finally, stage MS represents a special form of metastatic disease that occurs only in patients younger than 18 months. It is defined by limited involvement of the skin, liver, and/or bone marrow (less than 10% of total marrow cellularity)[1]. In NB molecular analyses, real-time polymerase chain reaction (RT-PCR) analyses were performed to detect *NMYC*, 11q, 1p and 17q. Out of 53 patients, 22 were in the high-risk, 9 in the intermediate-risk and 18 in the low-risk group.

MYCN amplification status was defined based on RT-PCR results, with samples showing greater than a 10-fold increase were classified as positive, and those below this threshold as negative. As part of the TPOG study, NB Formalin-Fixed Paraffin-Embedded tissue samples at various disease stages were randomly selected from the archival collection of the Departments of Paediatric Oncology and Basic Oncology at Dokuz Eylül University. Tumour sections were cut from paraffin-embedded blocks and mounted on adhesive slides for immunohistochemical (IHC) analysis of ATRX and *TERT* protein expressions. According to the TPOG-2020 classification system, the patients were categorised into high-risk (n=22), intermediate-risk (n=9), and low-risk (n=18) groups[4,19].

Antibodies

In this study polyclonal human *ATRX* and *TERT* antibodies (Bioss, Inc. 500 West Cummings ParkSuite 6500 Woburn, MA, USA) were used, along with a secondary antibody obtained from Ventana. All antibodies were stored in accordance with the manufacturers' recommendations, and appropriate dilution ratios were observed during application. Optimal dilutions were determined through control staining, with both *ATRX* and *TERT* antibodies used at a 1:100 dilution. Paraffin-embedded tissue blocks of tumour specimens of NB patients were selected to represent all three risk categories to ensure comprehensive analysis across the disease spectrum.

IHC Staining

Tissue sections were incubated overnight at 60 °C in an oven prior to application of the staining procedure[18]. Following incubation, the slides underwent deparaffinization in xylene for one hour, and were subsequently rehydrated using a graded series of alcohol solutions. Antigen retrieval was achieved by heating the slides in citrate buffer using a microwave. After treatment with hydrogen peroxide and appropriate washing steps, human-specific primary Immunoglobulin G antibodies were applied, based on prior optimization. Subsequently, a multimer Horseradish Peroxidase-conjugated secondary antibody was added and allowed to incubate. In the final staining step, diaminobenzidine combined with hydrogen peroxide was used to catalyze the chromogenic reaction. The nuclei of the tumour cells were then counterstained with haematoxylin, and the slides were passed through an ascending alcohol series before being cleared in xylene. The prepared slides were examined under a light microscope (Olympus BX50). *ATRX* expression was detected in the nuclei, whereas *TERT* protein exhibited both nuclear and cytoplasmic localization. To evaluate staining intensities of *ATRX* and *TERT*, five randomly selected fields per section were analyzed for each sample. Tumour cell staining intensities were classified as follows: 0= no staining; 1= weak; 2= moderate; 3= strong. Additionally, the proportion of stained cells for *ATRX* and *TERT* was scored as 0= none; 1=0-20%; 2=21-50%; 3=51-80%; 4=81-100%. Given the strong correlation between staining intensities and area scores, the intensity score was utilized for statistical analysis in this study[20,21].

Determination of *NMYC* and 11q by RT-PCR and Flow Cytometry in Molecular Evaluation

Paraffin-embedded NB tissue samples were used for DNA isolation, and concentrations were quantified fluorimetrically using a Qubit® fluorometer. RT-PCR was performed to assess *N-MYCN* amplification and 11q23 deletion. Threshold cycle values were determined for target and reference genes in patient and control DNA samples. Copy number alterations were detected using specific primers and TaqMan probes:

for *N-MYCN*, primers 5'-GTGCTCTTCCAATTCTCGCCT-3' and 5'-GATGGCCTAGAGGAGGGCT-3' with a 6-carboxyfluorescein(FAM)-labeledTaqManprobe;for11q23 (*ARCN1* gene), primers 5'-ATCTGGAGGCAGCACAGCT-3' and 5' TACACTGGATTATACCCTGGCTGG-3' with a FAM-labeled probe. PCR reactions were performed in eight replicates on a Roche Nano RT-PCR system. Relative quantification was calculated using the ΔΔCT method with healthy reference DNA as a calibrator, and findings were further validated via absolute quantitation using reference DNA standards (Table 2)[12,18].

Multivariate Survival Analysis

The patients included in the study were clinically categorised according to disease stage and risk classification. Patients were also categorised according to the molecular, and histological characteristics of the tumour, and age at diagnosis. The risk assessment and disease stage of the patients were considered to be confounding variables. Therefore, multivariate Cox regression survival analysis including the previously mentioned confounding factors was performed to confirm our findings. Cox regression analysis using different models showed that *ATRX* and *TERT* did not differ in univariate survival analysis. Again, no statistically significant difference was found between these parameters in multivariate Cox regression analysis. However, in the models created in Cox regression analysis, a significant relationship was detected between N-MYC Amp in NB patients and event-free survival (EFS) and OS rates (Table 3).

Statistical Analysis

We used Fisher's exact test to assess the relationship between categorical variables and *ATRX* and *TERT* expression patterns. The Independent Samples t-test was used to compare mean EFS and OS times between groups. Data were presented as mean ± standard deviation and the number of observations. EFS was defined as the time to the emergence of recurrence, secondary malignancy, or death. OS analysis included the interval from study registration to death or last follow-up. Survival curves were generated using the Kaplan-Meier method, and differences were tested using the log-rank test. All statistical analyses were conducted using IBM SPSS Statistics Version 29 (IBM Corp., USA), with p-values <0.05 considered statistically significant.

RESULTS

Data on the Clinicopathology of NB Patients

In this study, tissue samples from 22 female, and 31 male patients diagnosed with NB were analysed. The patients' ages ranged from 1 month to 11 years. Staging was performed in accordance with the criteria established by the Turkish Paediatric Oncology Group (2020) and the International Neuroblastoma Staging System. Risk stratification was based on the International Neuroblastoma Risk Group Staging System (INRGSS). According to the applied classification system, patients were categorised in the high (n=22), intermediate (n=9), and low-risk (n=18) groups, respectively (Table 1).

Risk Cassifications of Patients

Twenty-two-high, nine intermediate, and eighteen low-risk patients were analysed in this study. Both molecular (*MYCN* amplification, 11q23 loss and DNA index) and clinical (age of the patient, stage and histopathology of the tumour) data of patients were evaluated for the risk classification (Table 4).

Association of Age with Molecular Alterations and Survival Outcomes

To assess the prognostic impact of age, patients were stratified into two groups based on the well-established prognostic cut-off of 5 years of age (<5 years, n=38; ≥5 years, n=15). Loss of ATRX nuclear expression (negative staining) was significantly more frequently detected in patients aged 5 years or older compared to younger patients (46.7% vs. 15.8%, p=0.021). Similarly, a non-significant trend towards higher *TERT* expression was observed in the older age group (73.3% vs. 50%, p=0.13). Kaplan-Meier survival analysis revealed that patients ≥5 years of age had significantly worse OS compared to those <5 years (5-year OS: 40% vs. 78%, p=0.009 by log-rank test). EFS also followed a similar though non-significant, trend (5-year EFS: 33% vs. 63%, p=0.058). In a multivariate Cox regression model adjusted for *MYCN* amplification status and INRGSS risk groups, age ≥5 years remained an independent predictor of poorer OS [hazard ratio (HR): 3.2, 95%, confidence interval (CI): 1.1-9.3, p=0.032].

Expression of *TERT* and *ATRX* in Tissue Samples of Patients

In tissue samples, *TERT* and *ATRX* were expressed in 55.8% and 61.2% of cases, respectively. In NB, *ATRX* expression increased with disease stage, and observed in 50% of early-stage and 69% of advanced-stage tumours.

Similarly, *ATRX* expression was higher in high-risk patients (67.7%) compared to low-risk patients (50%). *TERT* was expressed at a rate of 55% in the early stage and 62.1% in the advanced stage. *TERT* was expressed at a rate of 58.1% in high-risk groups and 55.6% in low-risk groups. *ATRX* and *TERT* alterations were common in *MYCN*-amplified tumours (*ATRX* 86.7%, 13/15; 95% CI: 62.1-96.3; *TERT* 73.3%, 11/15; 95% CI: 48.0-89.1). In *MYCN*-non-amplified cases, the corresponding frequencies were 61.2% (23/38; 95% CI: 44.7-74.4) and 55.8% (21/38; 95% CI: 39.7-69.9), respectively. No significant differences were observed between groups in terms of expression rates of these risk predictors (*ATRX*: p=0.10; *TERT*: p=0.35). *ATRX* expression was found to be significantly higher in NB patients with *MYCN* amplification compared to patients without (p=0.027) (Figure 1 and 2).

Association Between ATRX and *TERT* Expressions and EFS and OS in High and Low Risk Groups in NB

The prognostic value of *ATRX* and *TERT* expressions in NB was assessed by stratifying patients into "high" and "low" expression groups. Associations between gene expression and OS, EFS, event occurrence, and risk classification were evaluated using Kaplan-Meier analysis,

Table 2. RT-PCR results for *MYCN* amplification and 11q values

Patient	*MYCN* Amp	11q	Patient	*MYCN* Amp	11q
No: 1	933.254	6879228.0000	No: 16	13.798	1.1745
No: 2	34.134	0.3140	No: 17	7.458	0.3290
No: 3	0.926	0.6250	No: 18	5.827	0.0040
No: 4	216.665	0.0002	No: 19	0.297	0.1540
No: 5	0.742	1.9250	No: 20	6.207	0.3890
No: 6	0.055	0.0630	No: 21	7.299	0.1740
No: 7	10.849	1.6280	No: 22	61.206	0.9450
No: 8	5.003	17.0170	No: 23	3.079	3.8390
No: 9	0.948	1.8970	No: 24	0.011	7.9510
No: 10	0.545	1.5900	No: 25	1.376	0.8820
No: 11	2.071	4.6410	No: 26	1.713	0.2800
No: 12	107.733	3.5030	No: 27	1.190	0.0380
No: 13	2.825	5.4140	No: 28	0.774	10.7502
No: 14	2.025	0.5110	No: 29	2.134	0.0700
No: 15	32.314	1.1850	No: 30	59.923	11.5720
No: 31	0.856	0.3240	No: 46	25.654	5.7430
No: 32	4.535	0.1540	No: 47	0.578	0.3800
No: 33	47.583	0.2440	No: 48	3.598	1.2000
No: 34	3.200	11.5950	No: 49	0.683	0.2450
No: 35	2.376	0.3370	No: 50	12.792	0.7170
No: 36	4.322	1.1850	No:51	10.792	1.1060
No: 37	0.981	0.4210	No: 52	0.591	0.0900
No: 38	0.385	0.8780	No: 53	3.338	1.2340
No: 39	23.065	0.5170			
No: 40	54.221	0.4190			
No: 41	0.922	0.4350			
No: 42	5.688	0.0080			
No: 43	2.604	0.2630			
No: 44	1.023	22702.5490			
No: 45	1.508	0.3226			

2p24.3 (*MYCN*) amplification and 11q23 (*ARCN1*) deletion of DNA samples were evaluated by TaqMan real-time PCR (RT-PCR) (Roche LightCycler Nano) with TaqMan labelled primers specifically designed for each relevant gene region. *N-MYC* amplicons and 11q aberrations of the patients are given in Table 3.
RT-PCR: Real-time PCR: Polymerase chain reaction

Table 3. Modeling of the results of Cox regression analysis

Model 1								
	B	**SE**	**Wald**	**df**	**Sig.**	**Exp(B)**	**95.0% CI for Exp(B)**	
							Lower	**Upper**
TERT	-0.695	0.850	0.669	1	0.414	0.499	0.094	2.642
ATRX2	0.865	0.770	1.261	1	0.261	2.374	0.525	10.741
N-MYC Amp	1.930	0.817	5.584	1	0.018	6.893	1.390	34.177
Model 2								
	B	**SE**	**Wald**	**df**	**Sig.**	**Exp(B)**	**95.0% CI for Exp(B)**	
							Lower	**Upper**
TERT	-1.045	0.917	1.299	1	0.254	0.352	0.058	2.122
ATRX2	0.655	0.798	0.674	1	0.412	1.925	0.403	9.194
N-MYC Amp	2.055	0.885	5.390	1	0.020	7.803	1.377	44.213
Risk 2 factor	-0.129	1.138	0.013	1	0.910	0.879	0.095	8.170

Model 1 comprises three independent variables. Model 2 was employed to generate a new model with four independent variables by incorporating an additional independent variable. The values obtained are statistically significant at $p<0.05$.
B: Beta coefficient, SE: Standard error, CI: Confidence interval

Table 4. Summary of molecular characteristics and risk stratifications

a) Distribution of risk groups by *MYCN* amplification and 11q status

	High risk (n=22) n (%)	**Intermediate risk (n=9) n (%)**	**Low risk (n=18) n (%)**	**Total (n=49)**
***MYCN* amplified (n=15)**	13 (86.7)	0 (0)	2 (13.3%)	15
***MYCN* non-amplified (n=38)**	9 (23.7)	9 (23.7)	16 (42.1)	34*
11q deletion (n=25)	13 (52.0)	6 (24.0)	6 (24.0)	25
No 11q deletion (n=28)	9 (32.1)	3 (10.7)	12 (42.9)	24**

*As the risk groups of 4 out of 53 patients were not specified, relevant data of the remaining 49 patients are presented
**Data of 24 out of 34 patients.

b) Correlations between *MYCN* amplification and 11q deletion

	11q deletion (+)	**11q deletion (-)**	**Total**
***MYCN* (+)**	4	11	15
***MYCN* (-)**	21	17	38
Total	25	28	53

*Fisher's exact test p-value=0.145
Molecular analysis revealed the distribution of risk groups, *MYCN* amplification, and 11q23 deletion.

with group comparisons conducted via the log-rank test. No significant differences were observed in terms of OS or EFS between *ATRX*-defined groups (OS: p=0.294; EFS: p=0.337) and *TERT*-defined groups (OS: p=0.693; EFS: p=0.740). Additionally, expressions of *ATRX* and *TERT* showed no significant correlation with event occurrence or risk classification (all p>0.05). Kaplan-Meier curves indicated substantial overlap in survival probabilities across expression-defined groups.

These results suggest that expressions of *ATRX* and *TERT* per se are not independent prognostic indicators in this cohort and are insufficient to predict survival outcomes. Their potential clinical utility may require their integration with additional molecular markers in a multivariate analytical framework. The corresponding survival curves are shown in Figure 3.

DISCUSSION

Ongoing advancements in NB research have contributed to a modest improvement in patient prognosis over recent years, with a corresponding increase in the 5-year survival rates. While patients with low-risk NB have shown relatively high cure rates, outcomes for patients with high-risk NB have remained

largely unchanged. As such, elucidating the molecular characteristics and genetic alterations underlying NB is critical for developing strategies that enable early diagnosis and implementation of targeted therapeutic approaches[22]. Notably, *ATRX* mutations and the loss of nuclear protein expression have been observed more commonly in patients over the age of 12 who present with stage 4 disease. In a study by Cheung et al.[23], *ATRX* gene deletions were identified in 43% of older adolescents with advanced NB (>12 years) and in 11% of paediatric patients aged 5-12 years. Similarly, in our study, OS rates were significantly worse in the ≥5 age group, and age was found to be an independent risk factor in multivariate analysis, independent of other prognostic parameters such as *MYCN* status and risk group. These findings suggest that age is associated not only with clinical stage and molecular alterations, but also with tumour biology, including loss of *ATRX* mutations and telomere mechanisms. Therefore, age is a strong prognostic marker in NB that reflects molecular subgroups beyond classical risk classifications.

According to Clusters of Orthologous Genes database data, survival was significantly preserved in patients assigned to less intensive treatment due to the change in the age threshold from 12 to 18 months. This finding supports that biologically "more favourable" tumours in younger age groups do not always require intensive treatment and that age can be safely used as a variable in intensification of treatment. In this study, the better prognosis of the <5 age group also demonstrates that the younger age group has biologically more "favourable" tumours and can achieve better survival without requiring intensive treatment[24].

Data from a separate study related to the ≥5 year- and adolescent/young adult categories have shown a progressive, and significant drop in survival rates with advancing age. adolescent/young and adult patients

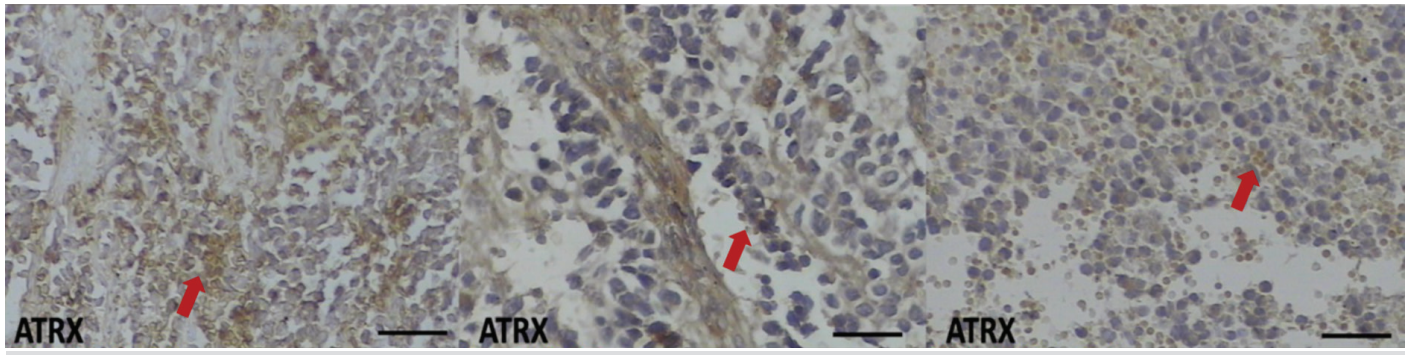

Figure 1. Immunohistochemical staining for ATRX in a neuroblatoma patient

This tissue sample obtained from neuroblastoma patient demonstrates a high rate of positive nuclear staining for ATRX as shown with red arrows (20X). ATRX was diluted 1:100.

ATRX: Alpha-thalassemia/mental retardation x-linked

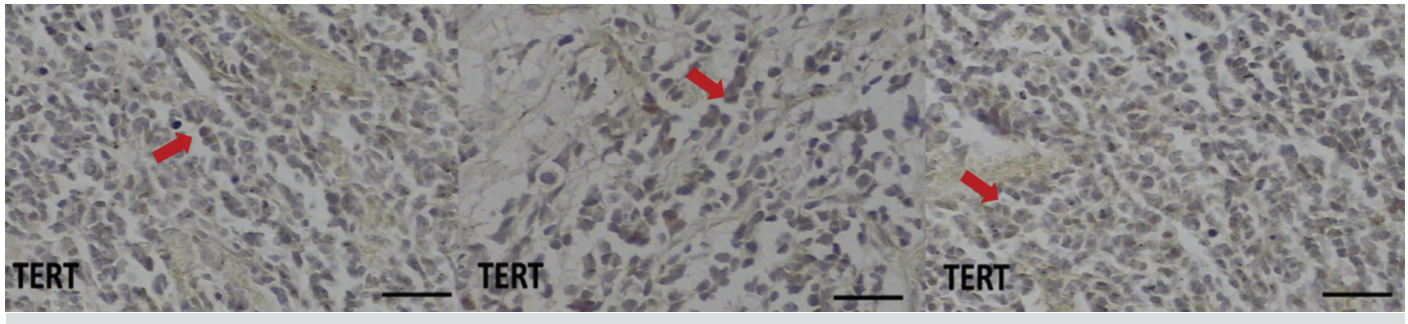

Figure 2. Immunohistochemical staining for TERT in a neuroblatoma patient

This tissue sample obtained from neuroblastoma patient demonstrates both positive nuclear and cytoplasmic staining for TERT as shown with red arrows (20X). TERT was diluted 1:100.

TERT: Telomerase reverse transcriptase

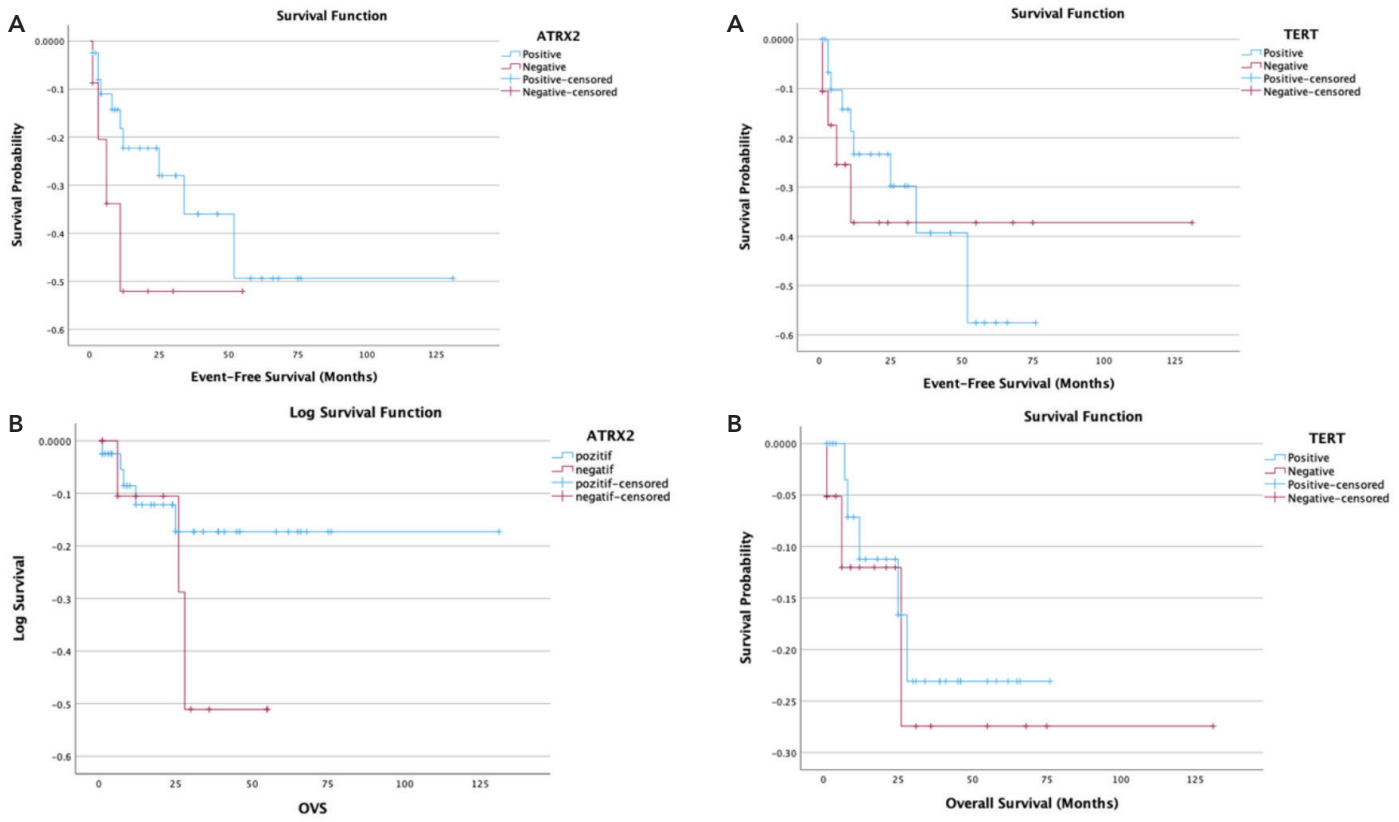

Figure 3. ATRX and TERT case plots showing overall survival (OS) and event-free survival (EFS) rates for neuroblastoma (NB)

ATRX- and TERT- positive and negative case plots for OS and EFS in NB patients. Kaplan-Meier curves showing ATRX- and TERT-positive and negative NB cases. OS (A) and EFS (B) of NB patients with ATRX and TERT- negative (red line) and ATRX- and TERT-positive (blue line) tumours are shown. Log-rank test was used to compare ATRX- and TERT- positive and negative NB cases.

ATRX: Alpha-thalassemia/mental retardation x-linked, TERT: Telomerase reverse transcriptase

have demonstrated poorer OS compared to children in Surveillance, Epidemiology, and End Results -based studies, even when detected earlier which suggests both stage of NB and biological differences as determinants of survival. Ten-year OS has been observed to decrease to around 19% in adult-onset NB series. In keeping with previous research, our findings in this study have also demonstrated a notable drop of 40% in OS rates in the group of children aged ≥5 years. The trend is similar, even though this figure is better than the rates indicated for adults and adolescents in the literature[25].

In NB, telomere length has been investigated as a significant prognostic indicator, with evidence indicating that shorter telomeres are associated with a more favourable clinical outcome, whereas longer or unchanged telomere lengths correlate with poorer prognoses[20]. More recently, activation of telomerase due to genomic rearrangements near the *TERT* gene locus has been identified in NB, delineating a subgroup of high-risk tumours characterized by exceptionally poor survival outcomes[13,26]. These investigations have demonstrated that *TERT* rearrangements are linked to elevated levels of *TERT* mRNA and enhanced telomerase enzymatic activity. In the study conducted by Lee et al.[20], *TERT* expression was assessed through IHC staining, revealing an inverse, but statistically insignificant association between *TERT* expression and patient survival. Similarly, in the current study, although a marked increase in *TERT* expression was observed in tissue samples of patients, still the inverse relationship with survival lacked statistical significance. Additionally, one of the earliest reports identifying *ATRX* involvement in NB revealed *ATRX* mutations in 44% of metastatic NB cases among adolescents and young adults, whereas such mutations were absent in tumour tissues obtained from infants with metastatic disease[23]. Children whose tumours harbored *ATRX* mutations were generally older than five years or exhibited a more indolent or chronic disease progression. In the same investigation, *ATRX* mutations were found to occur independently of *MYCN* amplification and were linked

with nuclear loss of *ATRX* protein, telomere lengthening, and the activation of the alternative lengthening of telomeres (ALT) pathway[23]. Our findings also support this trend. Indeed OS was significantly worse in the ≥5 age group and remained an independent risk factor in the multivariate model, independent of age, *MYCN* status, and INRGSS risk group (HR: 3.2; 95% CI: 1.1-9.3; p=0.032). This result suggests that age acts as a higher-level marker that captures biological differences on the *ATRX*/*TERT* axis in addition to the classic risk classification.

Another study indicated that the majority of high-risk NB tumours exhibit either *TERT* rearrangements, *MYCN* amplification, or *ATRX* mutations —alterations that collectively promote telomere elongation and provide a molecular basis for defining this subtype of NB[26]. Conversely, low-risk tumours are typically devoid of these genomic alterations and exhibit low *TERT* expression, which may reflect an inability to achieve unlimited proliferative capacity. The most aggressive subtype of NB has been associated with telomerase activation resulting from either *TERT* rearrangements or *MYCN* amplification. With ongoing advances in telomerase inhibitor development, these findings may offer a promising therapeutic avenue for treating the most lethal forms of this paediatric malignancy. Furthermore, three recent reports have shown that *ATRX* mutations frequently cause loss of protein expression in NB and are more prevalent in older patients and those diagnosed with stage IV disease[23,27,28]. In our cohort, *MYCN* amplification was also found to be significantly associated with EFS and OS in multivariate analysis, consistent with the rekevant literature data. Since *ATRX* and *TERT* expressions per se fail to predict survival these biomarkers should be evaluated together with multiple parameters rather than independently in clinical practice.

Only 4 out of 53 patients (7.5%) in our dataset had both *MYCN* amplification and 11q deletion, indicating a trend of reciprocal exclusivity; however, this negative correlation was not statistically significant (p=0.145). This finding is directionally consistent with the well-established genomic landscape of NB, where these two alterations are known to rarely coincide and define distinct molecular subtypes demonstrating aggressive clinical behavior[29,30]. For instance, a comprehensive genomic study by Molenaar et al.[28] in 2012 on 87 NB cases found a strong pattern of mutual exclusivity between *MYCN* amplification and 11q loss, a hallmark that helps stratify high-risk disease. Similarly, the large-scale study performed by Pugh et al.[27] in 2013, which analyzed the genetic landscape of 240 high-risk NBs, confirmed that 11q deletion and *MYCN* amplification are significantly and mutually exclusive events (p<0.001), underscoring their roles in alternative pathways of oncogenesis. Limited sample size of our study compared to large cohort studies may reduce statistical power of our conclusions. Nevertheless, the very low frequency of co-occurrence observed among these parameters reinforces the concept that these are separate oncogenic drivers.

CONCLUSION

The present study revealed a relationship between expression levels of *ATRX* and *TERT* and both the NB risk classification and clinical prognosis. Interestingly, *ATRX* expression was found to be higher in NB patients, especially in those with *MYCN* amplification, suggesting its potential utility as an IHC prognostic marker. Considering their biological significance, *ATRX* and *TERT* emerge as important molecular candidates that may enhance our understanding of NB pathology. Nonetheless, their exact mechanisms of action in NB are yet to be fully elucidated. To clarify their roles, future research should be performed with larger patient cohorts and should focus on in-depth mechanistic investigations, supported by validation in clinical tissue samples.

Ethics

Ethics Committee Approval: Permission was obtained from Dokuz Eylül University (DEU) Non-Interventional Research Ethics Committee for the conduction of this research study (decision no: 2023/14-10, dated: 03.05.2023).

Informed Consent: Signed consent forms were obtained from the patients during the sample collection and storage stages.

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Footnotes

Author Contributions

Surgical and Medical Practices: M.T., S.K.Ö., D.K., S.A., Z.A., N.O., Concept: D.K., S.A., Z.A., T.Ç.A., E.Ö., N.O., Design: D.K., S.A., Z.A., T.Ç.A., E.Ö., N.O., Data Collection or Processing: M.T., S.K.Ö., G.S., D.K., S.A., Z.A., N.O., Analysis or Interpretation: M.T., S.K.Ö., G.S., S.A., Z.A., Literature Search: M.T., S.K.Ö., G.S., S.A., Z.A., Writing: M.T., S.K.Ö., G.S., N.O.

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