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RESEARCH ARTICLE

Clinicopathological Study of N MYC Immunohistochemical Expression in Neuroblastoma in a Cohort of Egyptian Children

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Abstract

Introduction: Neuroblastoma is marked by its heterogenous clinical behavior, and MYCN oncogene is a key molecular marker associated with high-risk neuroblastoma. Although, MYCN status can be assessed using various methods, few studies have explored MYCN expression using immunohistochemistry (IHC). This study aimed to evaluate the N MYC protein expression using IHC in formalin-fixed, paraffin-embedded (FFPE) neuroblastoma tissue sections and correlate expression levels with different clinicopathological parameters. **Materials and Methods:** N MYC IHC was performed on forty neuroblastoma cases and scored based on staining intensity and the percentage of positive tumor cells. Expression levels were categorized as negative, low expression, or high expression. **Results:** Negative, low, and high immune expression were observed in 22.5%, 50%, and 27.5% of cases respectively. High immune expression was seen in neuroblastoma with undifferentiated or poorly differentiated morphology, a high mitosis-karyorrhexis index (MKI), and unfavorable histology. **Conclusions:** N MYC high immune expression correlates with poor histopathological prognostic factors and may serve as a potential screening tool for MYCN amplification in neuroblastoma, though further validation is required.

Keywords: MYCN- N MYC- neuroblastoma- immunohistochemistry- children- Egypt

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Introduction

Neuroblastoma, an embryonal tumor originating from neural crest cells, is regarded as the most common solid extracranial childhood neoplasm, accounting for approximately 8-10 % of all pediatric neoplasms and contributing to about 15% of pediatric cancer-related deaths. In the United States, there are roughly 700 new patients are diagnosed each year. Neuroblastoma incidence, in a study included Egypt, in children under 14 years old and in adolescents aged 15-19 years per one million persons was 10.4 and 0.9 respectively. However, limited data are available regarding the incidence of neuroblastoma in Middle East, including Egypt [1-4].

A hallmark of neuroblastoma that characterizes it and sets it apart from other solid tumors of childhood, is its wide heterogeneous variations regarding the clinical presentation, course as well as overall prognosis, ranging from tumor spontaneous regression, tumor maturation, and aggressive tumor progression with metastatic spread which is resistant to intensive multimodality treatment regimens. Because of this difficulty in predicting the clinical outcomes of the patients, neuroblastoma was described as an enigmatic disease for many years and still remains a major complex medical challenge in pediatric oncology [5-7].

Advances in research over the last decades have proved that the distinctive diverse neuroblastoma's clinical behavior was closely correlated with the genetic/molecular characteristics of the individual tumors and highlighted the importance of intra- and inter-tumoral genetic variations in the development of therapeutic resistance [8, 9].

MYCN was the first amplified oncogene which was recognized to be of high clinical importance owing to its high correlation with the most aggressive neuroblastoma form and is regarded as the most reliable genomic hallmark

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indicative of aggressive clinical behavior. MYCN amplification occurs in about 50% of high-risk tumors. N-Myc, the expression product of the MYCN gene is a transcription factor which is involved in cellular processes such as proliferation, cell growth, metabolism, protein synthesis, apoptosis and differentiation. Disruptions in the regulation of these processes can result in excessive and unrestricted cell proliferation [10-15].

Several methods have been used to determine MYCN oncogene amplification, such as Southern or dot blot methods, PCR, CISH and FISH techniques. These techniques detect MYCN amplification status at the nucleic acid level. On the other hand, immunohistochemistry uses specific antibodies to stain proteins in situ and is considered much less expensive, easier and faster than FISH and PCR. However, no reliable N MYC antibody is used in IHC with no uniform evaluation standard and few data are available regarding the feasibility of N MYC IHC in neuroblastoma as a screening tool for MYCN amplification [16-20].

In the present study, IHC for N MYC was assessed in 40 formalin-fixed paraffin-embedded (FFPE) sections of neuroblastoma by three observers to detect N MYC expression. The results of immunohistochemistry were correlated with different clinicopathological parameters.

Materials and Methods

Patients

After ethical approval by the Faculty of Medicine Ethics Committee (IRB NO: 00012098, FWA NO: 00018699) has been granted, the current study comprised 40 retrospective cases of newly diagnosed neuroblastoma. Cases were obtained from the archives of the Pathology Department, Borg Alarab Hospital, Alexandria University. All cases which were enrolled in the study had paraffin blocks with sufficient viable tumor tissue with no history of neoadjuvant therapy. Immunostaining for synaptophysin and chromogranin was used when indicated to confirm the diagnosis.

Immunohistochemical staining for N MYC

Three microns thick tissue sections of each paraffin block were cut and mounted on positively charged slides. Immunohistochemical staining was done according to the instruction manual. Antigen retrieval was performed by immersing the slides in sodium citrate buffer in a microwave oven for 10 minutes. The primary antibody used was (Polyclonal Rabbit Anti-Human, N MYC Antigen, Catalogue Number: PA5-105270, Invitrogen, ThermoFisher Scientific) and was diluted to 1:200 with PBS. DAB chromogen was applied, and the slides were counterstained with Mayer's hematoxylin. Positive and negative controls were included in all runs in the form of brain tissue and sections without primary antibody incubation respectively.

Cases were considered positive when a brown nuclear staining was seen. The expression was assessed according to the following scoring system which depended on staining intensity (0=negative,1=weak, 2=moderate, 3=strong) and percentage of positive cells (0 = 0% positive cells, 1 = less than 25 % positive cells, 2 = 25–50% positive cells, 3 = 50–75% positive cells, 4 = 75-100% positive cells). The final IHC score was calculated based on the formula that final score = staining intensity * positive proportion. Cases with scores of 0 were defined as "negative. Cases with scores of < 9 (1–8) were classified into the "low expression" group, while cases with scores ≥ 9 (9-12) were classified into the "high expression" group [21].

Statistical analysis

Data analysis was done using SPSS version 25 to evaluate the correlation between N MYC immune expression and different clinicopathological parameters in neuroblastoma. In all statistical tests, a level of significance of 0.05 was used, below which the results were considered to be statistically significant.

Results

Clinical data results

Among the included 40 neuroblastoma cases, 22 cases were males while 18 cases were females with male to female ratio of 1.2:1. Their ages (in months) ranged from 0.25 month (7 days) to 132 months (11 years) with a mean age of 29.51 months.

24 cases were presented with suprarenal (adrenal) gland tumors while 16 cases presented with non-adrenal tumors. The stage according to the International Neuroblastoma Risk Group Staging System (INRGSS) was assessed in each case [22].

Stage L1, L2, MS and M disease were seen in 8 cases, 11 cases, 7 cases, and 14 cases, respectively.

Histopathological results

The 40 cases in the current study included 38 cases of neuroblastoma, 1 case of ganglioneuroblastoma, nodular and another case of ganglioneuroblastoma, intermixed.

Among the 38 cases of neuroblastoma, according to the degree of differentiation, 5 cases were subtyped as undifferentiated neuroblastoma, 26 were subtyped as poorly differentiated neuroblastoma while 7 cases were subtyped as differentiating neuroblastoma. Among the same cases, low MKI was seen in 25 cases and intermediate MKI was seen in 8 cases. On the other hand, high MKI was seen in 5 cases.

According to the International Neuroblastoma Pathology Classification (INPC) histology group, the favorable histology group comprised 23 cases including the case of ganglioneuroblastoma, intermixed while the unfavorable histology group comprised 17 cases including the case of ganglioneuroblastoma, nodular.

Immunohistochemical results

Out of the 40 cases enrolled in this study, 9 cases showed N MYC negative immune expression, 20 cases showed low immune expression while 11 cases showed high immune expression. Different immunohistochemical pattern are shown in Figure 1.

Table 1. Relationship between N MYC Immune Expression and Clinicopathological Parameters

	IHC						Test of significance (p)
	Negative		Low expression		High expression		
	No.	%	No.	%	No.	%	
Age (Months)	(No=9)		(No=20)		(No=11)		
Mean ± SD.	38.6±44.99		17.1±15.9		44.6±38.44		(H=5.42, P=.07)
Median (Min-Max)	18(3-123)		15(1-72)		12(12-120)		
Sex	(No=9)		(No=20)		(No=11)		
· Male	7	77.8	8	40	7	63.6	(^{M}CP=0.135)
· Female	2	22.2	12	60	4	36.4	
Site	(No=9)		(No=20)		(No=11)		
·Adrenal	7	77.8	9	45	8	72.7	(^{M}CP=0.17)
· Non adrenal	2	22.2	11	55	3	27.3	
Stage	(No=9)		(No=20)		(No=11)		
· L1	2	22.2	4	20	2	18.2	
· L2	2	22.2	6	30	3	27.3	(^{M}CP=0.62)
· MS	2	22.2	5	25	0	0	
· M	3	33.3	5	25	6	54.5	
Degree of differentiation in neuroblastoma	(No=8)		(No=19)		(No=11)		
· Undifferentiated	0	0	0	0	5	45.5	
· Poorly differentiated	6	75	14	73.7	6	54.5	(^{M}CP=0.002*)
· Differentiating	2	25	5	26.3	0	0	
MKI	(No=8)		(No=19)		(No=11)		
· Low	7	87.5	15	78.9	3	27.27	(^{M}CP=0.018*)
· Intermediate	1	12.5	3	15.8	4	36.36	
· High	0	0	1	5.3	4	36.36	
Histology group	(No=9)		(No=20)		(No=11)		
· Favorable histology	6	66.67	16	80	1	9.09	(^{M}CP<0.001*)
· Unfavorable histology	3	33.33	4	20	10	90.91	

H,Kruskal. Wallis Test; MCP, Monte Carlo Test

In neuroblastoma cases (38 cases), negative immune expression was seen in 8 cases, low immune expression was seen in 19 cases, while high immune expression was seen in 11 cases. The case of ganglioneuroblastoma, nodular showed low immune expression while the case of ganglioneuroblastoma, intermixed showed negative immune expression.

In undifferentiated neuroblastoma cases, all cases showed high immune expression. In poorly differentiated neuroblastoma cases, negative immune expression was seen in 6 cases, low immune expression was seen in 14 cases, and high immune expression was seen in another 6 cases. In differentiating neuroblastoma cases, negative immune expression was seen in 2 cases and low immune expression was seen in 5 cases. No cases showed high immune expression in this subtype.

High N MYC immune expression was found to be associated with poor neuroblastic differentiation, high MKI and unfavorable histology and that was found to be statistically significant. The correlation between N MYC immune expression and different clinicopathological parameters is summarized in Table 1.

The data regarding MYCN amplification status using FISH technique was recorded in 20 cases, and the correlation between MYCN amplification status by FISH and N MYC protein expression by immunohistochemistry was of a strong statistical significance (data not shown) and revealed a sensitivity and specificity (95% CI) of [71.43% (29.04% to 96.33%) & 100% (75.29% to 100%) respectively], while PPV and NPV (95% CI) was [100% (47.82% to 100%) & 86.67% (59.54% to 98.34%) respectively].

Discussion

MYCN oncogene is an identified major oncogenic driver in the development of neuroblastoma and MYCN-amplified tumors are correlated with a more aggressive clinical behavior. Most MYCN- amplified tumors are also known to show N MYC protein overexpression. Moreover, some studies showed that the overexpression of the N MYC protein, rather than the amplification of the gene is critical for determining the aggressive tumor behavior and here comes the value of immunohistochemistry [21, 23, 24].

Based on immunohistochemistry, proposed subgrouping of neuroblastoma with unfavorable histology have been suggested by Ikegaki et al and Shimada et al, and tumors that are characterized by the overexpression of MYCN or MYC proteins were referred as MYC group,

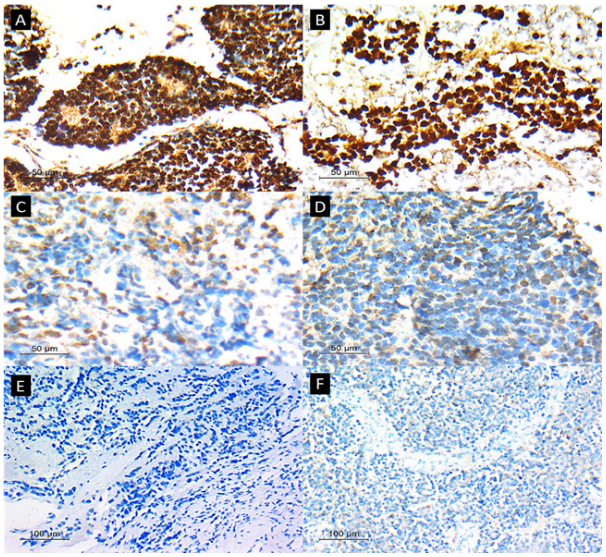

Figure1. N MYC Immunohistochemical Staining in Neuroblastoma. A and B, Two different neuroblastoma cases showing high immune expression (x50); C and D, Two different neuroblastoma cases showing low immune expression (x50); E and F, Another two neuroblastoma cases showing negative immune expression (x100).

and this group accounts for more than half of high-risk neuroblastoma cases [25].

In the present work, N MYC protein expression was assessed using immunohistochemistry. The correlation between N MYC immune expression and the known MYCN amplification status by FISH was of a statistical significance. This is in agreement with what has been reported in the few studies conducted in this prospect [20, 21, 24].

There was a variation of immune expression among neuroblastoma cases with different grades of differentiation, different MKI and among the two INPC histology groups as it was highly expressed in tumors with undifferentiated/poorly differentiated morphology, high MKI, and tumors with unfavorable histology and this relationship was of a statistical significance. These findings are in agreement with Wang et al and Zhao et al who reported strong association between augmented N MYC protein expression and the same parameters [23, 24].

Indeed, the results of the present study are in accordance with the published data that reported MYCN amplification to be a significant driving factor that prevents tumor maturation and enhances the mitotic and karyorrhectic activities. As a result, MYCN-amplified tumors usually exhibit the typical histological features of undifferentiated/poorly differentiated subtype with high MKI and in turn categorized into the unfavorable histology group [26, 27].

Although most of the included cases of M stage showed high N MYC immune expression and least of cases of stage L1 showed high N MYC immune expression, the relationship between N MYC immune expression and the stage was of no statistical significance. This finding is opposite to those reported by Wang et al and Zhao et al who have demonstrated a positive relationship between the stage and N MYC immune expression. This discrepancy might be attributed to the use of a different staging system (The International Neuroblastoma Staging System (INSS)) [23, 24].

In conclusions, despite being FISH is the gold standard for detection of MYCN amplification, the results of this study indicate that immunohistochemistry may be used as a screening tool for MYCN amplification in neuroblastoma and its high immune expression is associated with poor histopathological factors which may indicate that it could serve as a prognostic factor. However, there is no universally accepted antibody with no immune evaluation standard, an issue that mandates further research to reach an accepted immunohistochemistry method that can be used especially in resource-limited facilities.

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Approval

The research is approved by the Faculty of Medicine, Alexandria university.

Conflict of interest

The authors declare no conflict of interest.

Ethical declaration

Approval by the Faculty of Medicine Ethics Committee, Alexandria University was granted (IRB NO: 00012098, FWA NO: 00018699) before clinical data collection.

Authors contribution

Yasmin H. Ali, Dina M.Abdallah, Doaa A. Abdelmonsif and Galila El Taweel participated in the study design and coordination. Yasmin H. Ali, Dina M. Abdallah and Galila El Taweel performed the immunohistochemical analysis. Doaa A. Abdelmonsif provided the FISH results. Yasmin H. Ali and Dina M. Abdallah wrote the main manuscript text. Yasmin H. Ali and Dina M. Abdallah prepared Figure 1. All authors have read and approved the final manuscript.

Data Availability

Raw data sharing does not apply to Immunohistochemistry as no datasets were generated.

Originality Declaration for Figures

All figures included in this manuscript are original

and have been created by the authors specifically for the purposes of this study. No previously published or copyrighted images have been used. The authors confirm that all graphical elements, illustrations, and visual materials were generated from the data obtained in the course of this research or designed uniquely for this manuscript.

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