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Original article

Association Between ALK Immunohistochemistry and MYCN Gene Amplification in Pediatric Neuroblastoma

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Abstract MYCN amplification is a critical prognostic marker in neuroblastoma requiring molecular diagnostics often unavailable in resource-limited settings. This retrospective study evaluated ALK immunohistochemistry as a potential surrogate marker for MYCN amplification in 49 primary neuroblastomas from patients aged ≤18 years. ALK immunohistochemistry (clone D5F3) was scored as high (≥50% tumor cells) or low (<50%) by two independent pathologists blinded to MYCN status determined by FISH using standard criteria (ratio ≥4.0 or >10 copies/nucleus). MYCN amplification was present in 35% (17/49) of cases, while ALK-high expression occurred in 73% (36/49). ALK-high expression significantly associated with MYCN amplification (p=0.02; OR=9.6, 95%CI = 1.13-81.9), with 94% (16/17) of MYCN-amplified tumors showing ALK-high expression versus 63% (20/32) of non-amplified tumors. Diagnostic performance showed high sensitivity (94%) and negative predictive value (92%), but limited specificity (38%) and positive predictive value (44%). While ALK immunohistochemistry cannot replace molecular MYCN testing, its high sensitivity and NPV support its use as a preliminary screening tool in diagnostic algorithms, particularly in resource-limited settings, helping prioritize cases for molecular testing while identifying candidates for ALK-targeted therapy.

***Keywords*: ALK immunohistochemistry; Diagnostic accuracy; Fluorescence in situ hybridization; MYCN amplification; Neuroblastoma; Pediatric oncology; Prognostic biomarkers; Risk stratification**

Introduction

Neuroblastoma is the most common extracranial solid tumor of childhood and displays striking biological heterogeneity, ranging from spontaneously regressing lesions to highly aggressive metastatic disease. Amplification of the MYCN oncogene—present in roughly 20-25% of cases—remains one of the most powerful adverse prognostic markers, identifying a

high-risk subset with poor event-free survival and treatment resistance[1].

Anaplastic lymphoma kinase (ALK), located on chromosome 2p23 in close proximity to MYCN at 2p24.1, becomes oncogenically activated in neuroblastoma through hotspot mutations, copy-number gain, or focal amplification[2,3]. Constitutive ALK signaling cooperates with MYCN overexpression to drive tumor proliferation and accelerate tumorigenesis, as demonstrated in experimental models harboring the ALK(F1174L) mutation[4]. Targeted ALK inhibitors such as crizotinib have shown antitumor activity in early-phase pediatric trials and are now entering risk-adapted treatment protocols[5].

Several clinicopathological series further indicate that strong ALK protein expression by immunohistochemistry (IHC) or ALK gene amplification occurs predominantly in MYCN-amplified tumors, although published data remain limited by small cohorts and heterogeneous scoring cut-offs[6]. The potential utility of ALK immunohistochemistry as a surrogate marker for MYCN status carries significant clinical implications, particularly in resource-limited settings where molecular diagnostics may be unavailable or delayed. This study therefore aimed to systematically evaluate the diagnostic accuracy of ALK immunohistochemistry for predicting MYCN amplification status in a well-characterized cohort of pediatric neuroblastomas. Given the potential clinical utility of this biomarker, we employed dual independent pathologist evaluation to ensure accuracy of ALK expression interpretation through consensus review, and demonstrate that the scoring system (≥50% cutoff) is straightforward and reproducible, supporting its feasibility for routine diagnostic implementation.

Materials and Methods

Study Design and Case Selection

This retrospective cross-sectional diagnostic accuracy study was conducted at the Institute of Pathology, Department of Medical Services, Ministry of Public Health, Thailand, in accordance with the institutional ethical guidelines. The pathology database was systematically searched to identify all primary neuroblastoma specimens accessioned between 2012 and 2021.

Inclusion criteria: (i) histologically confirmed neuroblastoma according to the International Neuroblastoma Pathology Classification[7]; (ii) patient age ≤18 at diagnosis; and (iii) availability of adequate formalin-fixed, paraffin-embedded (FFPE) tissue with viable tumor content.

Exclusion criteria: (i) post-chemotherapy resection specimens; (ii) recurrent or metastatic lesions without paired primary tumor; and (iii) insufficient tissue for both immunohistochemical and molecular analyses.

Following application of these criteria, 49 cases were eligible for analysis (32 MYCN non-amplified, 17 amplified).

ALK Immunohistochemistry

Four-micrometer sections were cut from representative FFPE blocks and processed on a Ventana BenchMark ULTRA automated immunostainer (Ventana Medical Systems, Tucson, AZ, USA). Heat-induced epitope retrieval was performed using proprietary Cell Conditioning-1 (CC1) solution at 95°C for 64 minutes. Sections were incubated with rabbit monoclonal anti-ALK antibody (clone D5F3, Ventana, ready-to-use formulation) for 16 minutes at 36°C, followed by visualization using the OptiView

DAB IHC Detection Kit according to manufacturer specifications. Each immunohistochemical run included appropriate positive (ALK-rearranged anaplastic large-cell lymphoma) and negative (reactive lymphoid tissue) controls.

Two board-certified pathologists (A.L. and K.W.) independently evaluated all immunostained slides while blinded to MYCN status and clinical outcomes. The third author (P.C.) participated in consensus review for discordant cases only. ALK-high expression was defined as moderate to strong cytoplasmic and/or membranous staining in ≥50% of viable tumor cells, based on established scoring criteria in neuroblastoma literature[6-8]. Specifically, staining intensity was scored as: negative (0), weak (1+), moderate (2+), or strong (3+). Only tumors with moderate (2+) or strong (3+) intensity in ≥50% of cells were classified as ALK-high. Weak staining regardless of percentage, or moderate-strong staining in <50% of cells, were classified as ALK-low. Cases with discordant interpretation underwent consensus review by all three pathologists at a multi-headed microscope to establish final classification.

MYCN Fluorescence *In Situ* Hybridization (FISH)

Serial 4-micrometer sections were subjected to dual-color FISH analysis using the Vysis LSI MYCN (SpectrumOrange)/CEP2 (SpectrumGreen) probe set (Abbott Molecular, Des Plaines, IL, USA). Following deparaffinization and pretreatment, slides underwent protease digestion, probe hybridization at 37°C for 16 hours, and stringent post-hybridization washes. Nuclear counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI).

Signal enumeration was conducted using a fluorescence microscope equipped with appropriate filter sets. A minimum of 100 non-overlapping, intact tumor nuclei were evaluated at 100× oil immersion magnification. MYCN amplification was defined according to International Neuroblastoma Risk Group criteria as either: (i) MYCN:CEP2 signal ratio ≥4.0; or (ii) absolute MYCN copy number >10 signals per nucleus. Borderline cases (ratio 2.0-3.9) underwent extended counting of 200 nuclei with independent verification by a second observer.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA). The primary analysis examined the association between ALK immunohistochemical status (ALK-high/ALK-low) and MYCN amplification status (non-amplified/amplified) using Fisher's exact test (two-tailed) with significance threshold set at $\alpha = 0.05$.

Diagnostic performance indices were calculated including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and overall accuracy. The strength of association was quantified using odds ratio (OR) with 95% confidence intervals (CI) computed via the Woolf logit-interval method.

Post-hoc power analysis using G*Power software (version 3.1.9.7) indicated that the current sample size (17 amplified, 32 non-amplified) achieved approximately 70% statistical power to detect the observed effect size at $\alpha = 0.05$, suggesting adequate but not optimal power for the primary analysis.

Results

Cohort Characteristics

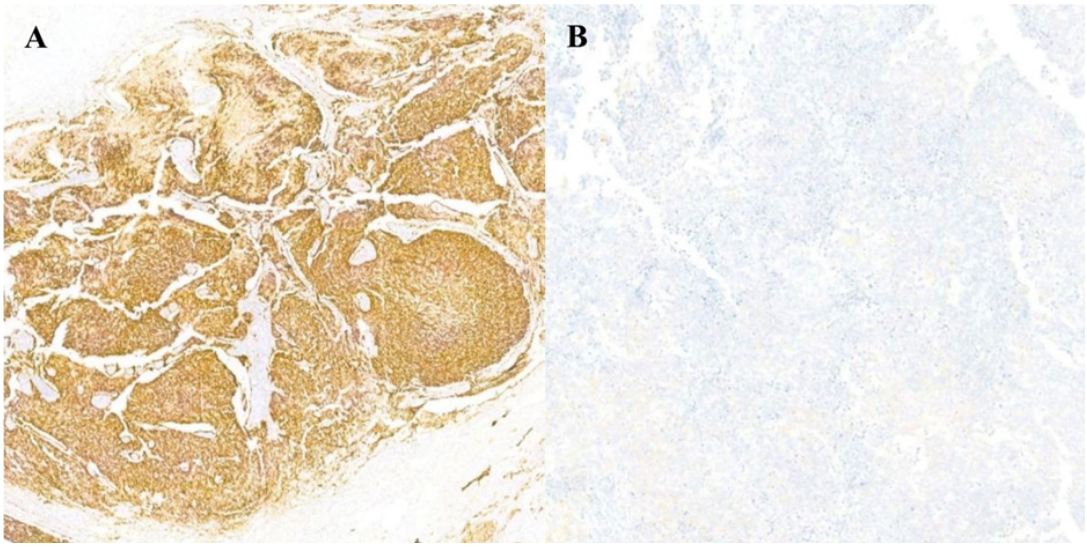
The study cohort comprised 49 primary neuroblastoma specimens meeting all eligibility criteria. MYCN amplification was detected in 17 cases (35%), while 32 cases (65%) demonstrated non-amplified status by FISH analysis. ALK immunohistochemistry revealed high expression (≥50%) in 36 tumors (73%) and low expression (<50%) in 13 tumors (27%). Inter-observer concordance for ALK scoring was excellent, with initial agreement in 47/49 cases (96%); the two discordant cases achieved consensus upon joint review.

The distinction between ALK-high and ALK-low expression patterns was readily apparent. ALK-high tumors characteristically displayed strong, diffuse cytoplasmic and membranous staining involving >90% of tumor cells, producing a distinct pattern clearly visible at 400× magnification (Figure 1A). The staining intensity was consistently strong (3+) with minimal intratumoral heterogeneity. Conversely, ALK-low tumors exhibited either complete absence of staining or weak, focal positivity in <10% of tumor cells, with the majority of the tumor showing no immunoreactivity (Figure 1B). The stark contrast between these patterns facilitated straightforward interpretation and likely contributed to the high inter-observer agreement. Background stromal elements and endothelial cells showed no staining, providing appropriate internal negative controls.

Association Between ALK Immunohistochemistry and MYCN Amplification

Cross-tabulation analysis revealed a statistically significant association between ALK-high expression

Figure 1 Representative ALK immunohistochemical expression patterns in pediatric neuroblastoma.



Remark: (A) ALK-high expression showing strong cytoplasmic and membranous staining in >90% of tumor cells (original magnification ×400).
(B) ALK-low expression demonstrating weak to absent staining in <10% of tumor cells (original magnification ×400).
Immunohistochemistry performed with ALK clone D5F3.

and MYCN amplification (p = 0.02, Fisher's exact test), as detailed in Table 1. ALK-high tumors demonstrated 9.6-fold higher odds of harboring MYCN amplification compared to ALK-low tumors (OR = 9.6; 95%CI = 1.13-81.9).

Table 1 Contingency table of ALK immunohistochemistry versus MYCN Status

	MYCN amplified	MYCN non-amplified	Total
ALK-high	16	20	36
ALK-low	1	12	13
Total	17	32	49

Diagnostic Performance of ALK Immunohistochemistry

ALK-high expression demonstrated high sensitivity of 94% (16/17; 95%CI = 71.3-99.9%) and negative predictive value of 92% (12/13; 95%CI = 64.0-99.8%) for detecting MYCN amplification. Conversely, specificity was modest at 38% (12/32; 95%CI = 21.8-55.6%), with a positive predictive value of 44% (16/36; 95%CI = 27.9-61.9%). Overall diagnostic accuracy was 57% (28/49; 95%CI = 42.2-71.1%). The positive likelihood ratio was 1.51 (95%CI = 1.09-2.09) and negative likelihood ratio was 0.16 (95%CI = 0.02-1.08).

Discussion

This study provides robust evidence for a strong association between ALK-high expression and MYCN gene amplification in pediatric neuroblastoma, with important implications for diagnostic algorithms in both resource-rich and resource-limited settings. The 9.6-fold increased odds of MYCN amplification in ALK-high tumors, coupled with 94% sensitivity and 92% negative predictive value, suggest ALK expression level as a potential screening tool that may help prioritize cases for MYCN testing.

Our findings align with recent series demonstrating consistent ALK-high expression by immunohistochemistry in MYCN-amplified tumors, with sensitivity rates approaching 100%. Phan et al. reported that all MYCN-amplified cases within their Vietnamese cohort of 90 neuroblastoma cases were positive for ALK immunohistochemistry, with 78% of these amplified tumors showing high-level ALK expression[6]. Kim and Kim found that ALK amplification was significantly correlated with MYCN amplification, and all three ALK-amplified neuroblastomas consistently showed ALK immunohistochemistry positivity when tested with all three antibody clones (ALK1, D5F3, and 5A4)[8]. Large genomic surveys demonstrate that ALK amplifications almost invariably arise in MYCN-amplified tumors and are absent from low-risk, non-amplified disease, reinforcing the biological link between the two oncogenes[10]. The biological basis for this association is well-established: both genes reside in close chromosomal proximity (2p23-24), and experimental evidence demonstrates synergistic oncogenic cooperation between activated ALK and MYCN overexpression in neuroblastoma pathogenesis[11,12].

The limited specificity we observed is also consistent with prior reports of frequent ALK expression in MYCN non-amplified neuroblastomas[8,9]. Multiple mechanisms may account for ALK-high expression in the absence of MYCN amplification, including: (i) ALK copy-number gains below the threshold for amplification; (ii) activating point mutations, particularly at hotspot residues F1174, F1245, and

R1275; (iii) transcriptional upregulation through alternative pathways; and (iv) post-translational stabilization of ALK protein. Lee et al. showed that ALK expression increased with advancing stage even in the absence of amplification, underlining the multifactorial regulation of ALK protein levels[9].

The clinical implications of our findings warrant careful consideration. It is crucial to emphasize that ALK immunohistochemistry cannot replace definitive MYCN testing by FISH or molecular methods, which remain the gold standard for risk stratification in neuroblastoma. Rather, ALK IHC should be viewed as a supplementary screening tool that may help optimize resource allocation in specific contexts. Our data demonstrate that while ALK-high expression has limited specificity (44% PPV), requiring molecular confirmation in all cases, ALK-low expression shows more promising negative predictive value (92%). In resource-limited settings where molecular diagnostics may be unavailable or subject to prolonged turnaround times, this performance profile suggests a potential triage strategy: ALK-low cases might be considered for less urgent MYCN testing given their lower probability of amplification, though the 8% false-negative rate mandates eventual molecular confirmation before definitive risk stratification. Conversely, ALK-high cases, with their 44% probability of MYCN amplification, should be prioritized for expedited molecular testing. This approach could help allocate limited molecular testing resources more efficiently while ensuring that all treatment decisions ultimately rest on confirmatory molecular results.

Beyond its potential role as a MYCN surrogate, ALK immunohistochemistry retains independent clinical value for identifying candidates for ALK-targeted therapy. Based on promising results in early-phase trials, ALK inhibitors are under active investigation for treating ALK-altered neuroblastoma, highlighting the growing importance of molecular profiling in identifying patients who may benefit, particularly those with refractory disease[5]. The high prevalence of ALK-high expression in our cohort (73%) underscores the potential therapeutic relevance of this biomarker.

Several limitations merit acknowledgment. The retrospective, single-institution design limits generalizability, while the sample size of 49 cases provides adequate but suboptimal statistical power, as reflected in the wide confidence intervals. Absence of ALK mutation analysis precludes determination of the specific mechanisms underlying ALK-high expression in individual cases. Furthermore, lack of clinical outcome data prevents assessment of the prognostic significance of combined ALK/MYCN status under contemporary treatment regimens.

Future investigations should prioritize: (i) prospective multicenter validation in larger, ethnically diverse cohorts; (ii) comprehensive molecular profiling integrating ALK sequencing, copy-number analysis, and expression profiling to elucidate mechanisms of ALK-high expression; (iii) correlation of integrated biomarker status with event-free and overall survival under modern risk-adapted protocols; and (iv) health economic modeling to evaluate the cost-effectiveness of ALK-guided diagnostic algorithms across different healthcare settings.

In conclusion, ALK expression level demonstrates a strong association with MYCN amplification in pediatric neuroblastoma. The high sensitivity (94%) and negative predictive value (92%) suggest potential

utility as a preliminary screening tool, while the modest specificity (38%) clearly defines its limitations. Given the critical importance of accurate MYCN status for patient management, ALK IHC must serve only as a supplementary tool that complements, rather than replaces, molecular MYCN testing. Our findings support a risk-stratified diagnostic algorithm where ALK immunohistochemistry helps prioritize cases for molecular testing—particularly valuable in resource-constrained environments with limited access to FISH or molecular diagnostics. Beyond its screening role for MYCN status, ALK assessment provides the added benefit of identifying candidates for ALK-targeted therapy, thereby serving a dual purpose in optimizing both diagnostic workflow and therapeutic selection. Ultimately, while ALK IHC offers a practical tool for initial evaluation in this biologically heterogeneous malignancy, definitive risk stratification and treatment decisions must rely on molecular confirmation of MYCN status.

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ความสัมพันธ์ระหว่างการย้อมอิมมูโนฮิสโตเคมีของ ALK และการเพิ่มจำนวนยีน MYCN ในมะเร็งนิวโรบลาสโตมา

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บทคัดย่อ: การเพิ่มจำนวนยีน MYCN เป็นตัวบ่งชี้พยากรณ์โรคที่สำคัญในเนื้องอกนิวโรบลาสโตมา ซึ่งต้องอาศัยการตรวจทางอณูชีววิทยาที่มักไม่สามารถทำได้ในสถานพยาบาลที่มีทรัพยากรจำกัด การศึกษาย้อนหลังนี้ประเมินการย้อมอิมมูโนฮิสโตเคมีของ ALK เพื่อเป็นตัวบ่งชี้ทดแทนสำหรับการเพิ่มจำนวนยีน MYCN ในเนื้องอกนิวโรบลาสโตมา 49 ราย จากผู้ป่วยอายุ ≤18 ปี การย้อม ALK ด้วยแอนติบอดีโคลน D5F3 ถูกแปลผลเป็น ALK-high (ตั้งแต่ร้อยละ 50 ขึ้นไปของเซลล์มะเร็ง) หรือ ALK-low (น้อยกว่าร้อยละ 50) โดยพยาธิแพทย์ 2 ท่านอ่านผลอย่างอิสระโดยไม่ทราบผล MYCN ที่ตรวจด้วยวิธี FISH ผลการศึกษาพบการเพิ่มจำนวนยีน MYCN ในร้อยละ 35 (17/49) ของเคส ขณะที่ ALK-high พบในร้อยละ 73 (36/49) และ ALK-high มีความสัมพันธ์อย่างมีนัยสำคัญกับการเพิ่มจำนวนยีน MYCN (p=0.02; OR=9.6, 95%CI: 1.13-81.9) โดยร้อยละ 94 (16/17) ของเนื้องอกที่มีการเพิ่มจำนวนยีน MYCN พบ ALK-high เทียบกับร้อยละ 63 (20/32) ในกลุ่มที่ไม่มีการเพิ่มจำนวนยีน ค่าประสิทธิภาพการวินิจฉัยแสดงความไวสูง (94%) และค่าทำนายผลลบสูง (92%) แต่มีความจำเพาะจำกัด (38%) และค่าทำนายผลบวกจำกัด (44%) แม้ว่าการย้อม ALK ไม่สามารถทดแทนการตรวจ MYCN ทางอณูชีววิทยาได้ แต่ความไวและค่าทำนายผลลบที่สูงสนับสนุนการใช้เป็นเครื่องมือคัดกรองเบื้องต้นในขั้นตอนการวินิจฉัย โดยเฉพาะในสถานพยาบาลที่มีทรัพยากรจำกัด ซึ่งช่วยจัดลำดับความสำคัญของการตรวจทางอณูชีววิทยา พร้อมทั้งระบุผู้ป่วยที่อาจได้รับประโยชน์จากการรักษาที่มุ่งเป้าที่ ALK

คำสำคัญ: **การย้อมอิมมูโนฮิสโตเคมีของ ALK; ความแม่นยำในการวินิจฉัย; เทคนิค fluorescence in situ hybridization; การเพิ่มจำนวนยีน MYCN; มะเร็งนิวโรบลาสโตมา; มะเร็งในเด็ก; ตัวบ่งชี้การพยากรณ์โรค; การจัดกลุ่มความเสี่ยง**