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MYCN Gene Copy Number Status Detected by FISH Method and Its Correlation with Outcome and Clinicopathological Variables in Childhood Neuroblastoma

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Abstract

Background: Neuroblastoma is the most common extracranial solid tumor in children. MYCN gene amplification (MNA) is an independent prognostic factor for rapid tumor progression and poor prognosis, regardless of age and clinical stage. Gain of the MYCN gene locus on the short arm of chromosome 2 can also be found in neuroblastoma.

Method: In this retrospective descriptive analysis of genetic alterations in neuroblastoma tumor samples, both before and after standard chemotherapy, we examined the MYCN gene copy number status in 20 neuroblastic tumor samples using the fluorescence in situ hybridization (FISH) method. We also evaluated its relationship with clinical variables and tumor maturation after standard chemotherapy treatment. Additionally, we compared disease outcomes among different MYCN copy number categories.

Results: Among the tumor samples, four (25%) exhibited increased MYCN copy numbers, 20% showed MYCN amplification, and 5% displayed MYCN gain. We observed decreased survival rates in advanced stages of neuroblastoma. Furthermore, in male patients, we noted an association between increased MYCN copy number and metastatic tumors.

Conclusion: We found that increased MYCN copy number is moderately associated with an immature phenotype and correlated with lower event-free survival. However, we did not detect a statistically significant difference.

Keywords: Neuroblastoma, MYCN amplification, Chemotherapy, Refractory, Progression-free survival

Introduction

Neuroblastoma is the most common extracranial solid tumor in children in the USA and Europe. It

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originates from the postganglionic sympathetic nerves and accounts for 8%-10% of childhood cancers.[1] Neuroblastic tumors with favorable genetic features exhibit whole chromosome gain with few segmental chromosomal aberrations, without gene amplifications, and near-triploid DNA content. However, if near-triploid tumors also have MYCN amplifications or segmental chromosomal aberrations, they tend to exhibit clinically aggressive behavior.[2] On the other hand, neuroblastic tumors with unfavorable genetic features show unbalanced gain of the long arm of chromosome 17, as well as segmental chromosomal abnormalities such as deletion at the chromosomal region 1p36.3 or 11q23.[3] The MYCN gene is a cellular proto-oncogene of the MYC family of transcription factors. It plays a role in multiple functions and interactions, including proliferation, migration, stem cell hemostasis, cell cycle progression, and increased sensitivity to apoptosis.[2-4] The MYCN gene is located on the short arm of chromosome 2 at band 2p24.[5-8] Its involvement in oncogenesis is thought to occur through extrachromosomal elements called double minutes (DMs), as well as repeated amplicons within a chromosome as a homogeneously staining region (HSR). MYCN gene amplification (MNA) is an independent prognostic factor for poor prognosis in tumors, regardless of age and clinical stage of the disease. MNA occurs in approximately 16% of neuroblastic tumors.[9] MYCN amplification is widely accepted as being related to prognosis, and most current treatment protocols assess the MYCN gene status before initiating treatment in neuroblastoma. Therefore, amplified MYCN is established as a powerful clinical marker for high-risk neuroblastoma and is the sole genetic marker used for stratification of treatment in neuroblastoma clinical trials. Another genetic abnormality observed in neuroblastoma is the gain of the MYCN gene locus on the short arm of chromosome 2 (2p24). It is unclear whether MYCN gain represents a pre-stage of MYCN amplification.[9-11] Furthermore, the clinical significance of MYCN gain remains unclear.[12] Fluorescence in situ hybridization (FISH) is the method of choice for evaluating the MYCN gene copy number status. It can be performed within a relatively short turnaround time and offers higher sensitivity since the microscopist directly controls the quality of the hybridization process and counts the MYCN copy number in each individual cell. MYCN copy number is a discrete variable, but various groups have established cut-off points to define specific tumors as amplified or non-amplified. The Children's Oncology Group (COG) categorizes MYCN copy number into four groups: 1) wild-type (<2-fold increase in MYCN signal), 2) MYCN gain (=2-4-fold increase), 3) low-level MNA (=4-10-fold increase), and 4) high-level MNA (>10-fold increase).[4] In this study, we evaluate the MYCN copy number status using the FISH method and examine its relationship with clinical variables and tumor maturation after standard chemotherapy. We also compare disease outcomes in different MYCN copy number categories in childhood neuroblastic tumors.

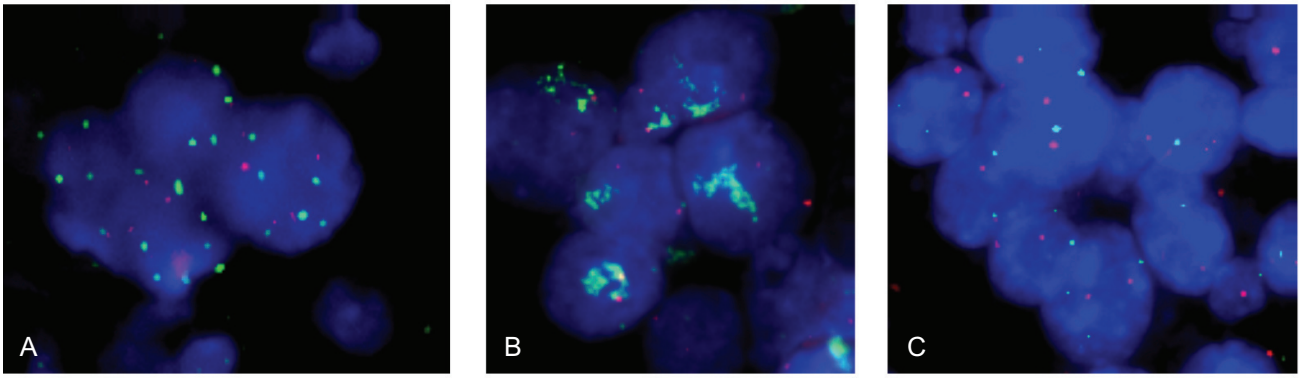

Figure 1. This figure shows examples of the copy number status of the MYCN gene in neuroblastoma tumors detected by interphase fluorescence in situ hybridization (FISH) examination. Probes used: green-MYCN locus (2p24) and red-control locus (2q11). (A) MYCN gain, (B) amplification, (C) normal.

Material and Methods

In this retrospective descriptive analysis of genetic alterations in neuroblastoma tumor samples before and after standard chemotherapy, we examined tumor samples from 20 patients diagnosed with neuroblastoma between 2014 and 2020. The patients were treated according to the current protocols of the European International Society of Pediatric Oncology Neuroblastoma Group. Clinical variables included age at diagnosis, gender, tumor stage, primary tumor site, metastatic involvement, and patient outcomes. This study was conducted in accordance with the ethics guidelines for clinical studies by the Research Ethics Committee of Shiraz University of Medical Sciences, with the ethical code of IR.SUMS.MED.REC.1399.477. Informed consent was not required for this descriptive retrospective analysis.

A total of 20 formalin-fixed paraffin-embedded pathologic samples taken before and after chemotherapy were enrolled in this study. The specimens were limited to the patients whose neuroblastoma diagnosis was based on histologic and immunohistochemical examinations, and tissue blocks were available both before and after chemotherapy treatment. The histologic sections of the tissue samples before chemotherapy were reviewed, and areas of interest (high tumor cell content and low stroma content) were manually dissected to reduce non-tumoral tissues and ensure consistency. The MYCN gene status at diagnosis was determined through interphase FISH examination, following international guidelines. Tissue sections, four micrometers thick, were deparaffinized and then digested in pepsin solution at 37 degrees Celsius for 15 minutes. After dehydration in an ethanol series, the appropriate probes were added. The dual-color set of fluorescence probes MYCN amplification (Zytolight SPEC MYCN/2q11) was hybridized to tumor tissue slides. The slides were incubated at 75 degrees Celsius for 10 minutes and at 37 degrees Celsius overnight. Subsequently, the slides were washed in post-hybridization buffers, stained with an anti-fade solution containing 4',6-diamidino-2-phenylindole (DAPI), and evaluated using a fluorescence microscope (Nikon ECLIPSE E600, Tokyo, Japan) by two pathologists independently. Fluorescence microscopy was performed with a microscope equipped with GenASIs software and filter sets for FITC, Texas red, and DAPI. The FISH signals were independently scored in 200 non-overlapping nuclei by two investigators, and the consensus result was recorded. The neuroblastoma patients were classified based on tumors with:

1- Wild type: <2-fold increase in MYCN signal

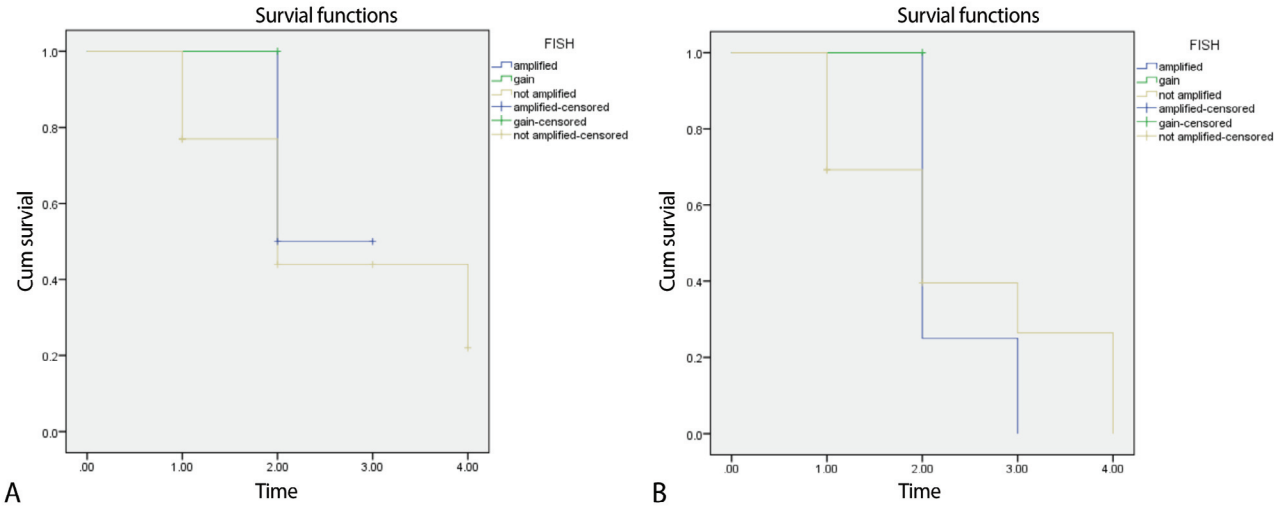

Figure 2. Kaplan-Meier estimates of (A) 5-year OS and (B) 5-year EFS without significant differences between specific subgroups in relation to the copy number status of the MYCN gene by FISH test.
OS: Overall survival; Cum: Cumulative; EFS: Event-free survival; FISH: Fluorescent in situ hybridization

Table 1. Clinicopathological features of patients with neuroblastoma

Age (months)	FISH	Metastasis	Stage	Outcome
24	Not amplified	Yes	4	Dead
12	Not amplified	No	3	Dead
120	Not amplified	No	3	Dead
84	Not amplified	Yes	4	Dead
12	High amplified	Yes	3	Dead
60	Not amplified	Yes	4	Alive
48	Not amplified	Yes	4	Alive
36	High amplified	Yes	4	Alive
24	Not amplified	-	-	Alive
19	Not amplified	Yes	3	Dead
60	Not amplified	Yes	2	Alive
36	Not amplified	No	2	Alive
24	Not amplified	Yes	4	Alive
108	Not amplified	No	3	Dead
16	Not amplified	Yes	3	Dead
42	MYCN gain	Yes	4	Alive
15	High amplified	Yes	3	Dead
30	Not amplified	No	2	Alive
12	High amplified	Yes	4	Alive
30	Not amplified	Yes	4	-

FISH: Fluorescence in situ hybridization

compared with the reference probe,

2-MYCN gain: 2-4-fold increase in MYCN signal compared with the reference probe,

3-Low-level MYCN amplification: 4-10-fold increase in MYCN signal compared with the reference probe,

4-High-level MYCN amplification: >10-fold increase in MYCN signal compared with the reference probe.

The best representative fields were imaged. The MYCN gene copy number status was compared with the maturation phenotype of tumors after chemotherapy. Finally, we compared the disease outcome of patients with different copy numbers of the MYCN gene.

The variables among the patients were analyzed using SPSS 22.0 software. The chi-square test or two-tailed Fisher's exact test was used to compare the predictive values of each analyzed variable. The results were considered significantly different when $P < 0.05$. Our primary predictor variable was the MYCN copy number category. Kaplan-Meier curves were plotted using SPSS 22.0 software. Clinical outcomes included event-free survival (EFS) and overall survival (OS). EFS was defined as the time from diagnosis to the first episode of disease recurrence, disease progression, death, or secondary malignancy. Patients without an event were considered at the date of the last contact. OS was defined as the time from diagnosis to death. Surviving patients were considered at the date of the last contact.

Results

We analyzed 20 pediatric neuroblastoma patient samples. Patient characteristics are summarized in table 1. There was a female predominance, with females accounting for 55% and males for 45%, resulting in a female-to-male ratio of 1.2:1. The median age at diagnosis was 40.6 months. Four (20%) out of the 20 cases analyzed using FISH showed MYCN amplification (Figure 1). One (5%) out of the 20 cases showed MYCN gain, while 15 (75%) out of 20 cases showed no MYCN amplification. The primary tumor site in all neuroblastoma samples was the adrenal gland. Three (15.8%) out of 20 cases analyzed were stage 2, 7 (36.8%) were stage 3, and 9 (47.4%) were stage 4. Of the 20 adrenalectomy samples, 11 (55%) showed ganglionic maturation after the initiation of a standard chemotherapy regimen compared with the primary trucat biopsy, while

9 (45%) did not show any maturation.

To compare the variables of interest, Fisher's exact test, chi-square, or Mann-Whitney U-test were used where appropriate. Copy numbers of MYCN were greater in males than in females, but the difference was not significant ($P = 0.51$). Although increased MYCN copy number had an intermediate relation to the absence of maturation phenotype, the correlation was not significant ($P = 0.10$). Patients with metastasis to lymph nodes showed increased MYCN copy number compared with patients without metastasis, but a statistically significant difference was not demonstrated ($P = 0.10$). Increased MYCN copy numbers in the FISH test corresponded to an intermediate relationship with metastasis in male patients ($P = 0.05$). A significant correlation was not detectable between different categories of MYCN gene copy numbers in the FISH test and various disease stages of patients ($P = 0.68$). Out of the 19 neuroblastoma cases with follow-up information available, 10 survived and 9 died at the time of writing up the study.

A significant relationship was found between advanced stages of neuroblastoma and decreased survival rate ($P = 0.034$, log rank = 6.74). Finally, we compared the disease outcome in specific subgroups of patients with different copy number statuses of the MYCN gene. Five years of EFS and OS were estimated using the Kaplan-Meier method, and survival curves were compared between the MYCN copy number categories using log-rank tests (Figure 2). There was no correlation between MYCN copy number statuses and poor prognosis in patients with or without maturation. The OS between different categories of MYCN copy number did not show statistical significance ($P = 0.53$, log-rank = 1.23). Although EFS was higher in the MYCN wild type compared to the MYCN amplified category, no significant correlation was demonstrated ($P = 0.51$, log rank = 1.33).

Discussion

In this study, we evaluated the MYCN copy number status, clinical characteristics, and histologic presentation of 20 pediatric neuroblastic tumors. We examined their association with the clinical outcome of the patients at diagnosis using interphase FISH examination. This examination enables the evaluation of MYCN status in single cells and is more sensitive in detecting low-level amplification. Our results showed that aberrant MYCN copy number was detected in 5 (25%) out of 20 neuroblastic samples. Among these, 4 (20%) exhibited amplification and 1 (5%) showed gain. Spitz et al. also reported 18% MYCN amplification and 6% MYCN gain, which is consistent with our study.[7] The results of this study demonstrate an extremely low incidence (0%) of low-level MYCN amplification, which agrees with the findings of Campbell et al.'s study (0.2%).[1] All four cases with MYCN amplification showed high-level amplification (>10-fold), suggesting different mechanisms leading to low-level amplification. However, the reason for this paucity of cases remains unclear. In line with previous reports, we found that the MYCN oncogene was most frequently amplified as DMs. We did not observe any HSRs in our amplified cases. The reason for this differential pattern is unclear, but it may be due to differences in the stage of tumor progression. DMs suggest an earlier process than intrachromosomal integration of HSRs. Although Santiago et al.,[8] demonstrated an association between undifferentiated or poorly differentiated morphology, high mitosis-karyorrhexis index (MKI), and MYCN gene amplification in neuroblastoma, we did not find a significant correlation in our study. Knowledge of the prognosis based on MYCN copy number may have therapeutic implications, as MYCN amplification is a well-established factor associated with unfavorable prognosis. Our study's results reveal ordered associations between MYCN copy number and several clinical and biological features of neuroblastoma. Patients with metastasis showed increased MYCN copy number compared with patients without metastasis. Survival rates decreased with advanced stages of neuroblastoma.[3,5]

For refractory and relapsed cases of neuroblastoma, alternative treatment modalities have been suggested, including the use of a histone deacetylase inhibitor (Panobinostat),[13] and 131I-

metaiodine benzyl guanidine (MIBG) therapy before and after autologous peripheral blood stem cell transplantation.[14] However, diagnosis at an age older than 30 months or high MYCN expression by the tumor significantly affects OS. According to a recent review article by Sait and Modak, effective immunotherapy holds promise for improving survival and quality of life by reducing exposure to cytotoxic agents. GD2, a surface glycolipid, is the most common target for immunotherapy, including monoclonal antibodies and anti-GD2 vaccine.[15]

Tang and colleagues showed that high-level MYCN expression in neuroblastoma lacking MYCN amplification results in a benign phenotype. Therefore, high MYCN expression confers the opposite biological inhibitor consequence in neuroblastoma, depending on whether or not MYCN is amplified.[16]

Our results, which demonstrate a relationship between MYCN amplification and EFS, are in agreement with the studies conducted by Campbell et al. and Tang et al.[1,16] However, we were unable to establish a statistically significant difference due to the limited sample size. We observed improved OS and EFS in the MYCN gain group; however, due to the small number of patients in this subgroup, it cannot be evaluated reliably. Therefore, further national studies in collaboration with other pediatric oncologists and molecular pathologists are required to better evaluate MYCN gain for future therapeutic immunotherapy and targeted therapy.

Furthermore, the novelty of our study lies in the comparison of the maturity status of tumor samples before and after chemotherapy. We observed that the absence of maturation after chemotherapy and the development of ganglioneuroblastic or ganglioneurotic differentiation in these patients are important indicators of poor prognosis, as previously described by Brodeur and Bagatel.[2] The major limitation of our study was the relatively small sample size.

Conclusion

The findings of the current study demonstrate the rarity of low-level MYCN amplification, decreased patient survival with increasing stages of the disease, the probable correlation of increased MYCN copy number with lymph node metastasis, and the absence of maturation histology after chemotherapy treatment. Further studies involving a larger population of patients are necessary to verify these results and incorporate them into clinical trials.

Conflict of Interest

None declared.

References

1. Campbell K, Gastier-Foster JM, Mann M, Naranjo AH, Van Ryn C, Bagatell R, et al. Association of MYCN copy number with clinical features, tumor biology, and outcomes in neuroblastoma: A report from the Children's Oncology Group. *Cancer.* 2017;123(21):4224-35. doi: 10.1002/cncr.30873.
2. Brodeur GM, Bagatell R. Mechanisms of neuroblastoma regression. *Nat Rev Clin Oncol.* 2014;11(12):704-13. doi: 10.1038/nrclinonc.2014.168.
3. Ambros PF, Ambros IM, Brodeur GM, Haber M, Khan J, Nakagawara A, et al. International consensus for neuroblastoma molecular diagnostics: report from the International Neuroblastoma Risk Group (INRG) Biology Committee. *Br J Cancer.* 2009;100(9):1471-82. doi: 10.1038/sj.bjc.6605014.
4. Mathew P, Valentine MB, Bowman LC, Rowe ST, Nash MB, Valentine VA, et al. Detection of MYCN gene amplification in neuroblastoma by fluorescence in situ hybridization: a pediatric oncology group study. *Neoplasia.* 2001;3(2):105-9. doi: 10.1038/sj.neo.7900146.
5. Szewczyk K, Wieczorek A, Młynarski W, Janczar S, Woszczyk M, Gamrot Z, et al. Unfavorable outcome of neuroblastoma in patients with 2p gain. Front Oncol. 2019;9:1018. doi: 10.3389/fonc.2019.01018.
6. Yoshimoto M, Caminada De Toledo SR, Monteiro Caran EM, de Seixas MT, de Martino Lee ML, de Campos Vieira Abib S, et al. MYCN gene amplification. Identification of cell populations containing double minutes and homogeneously staining regions in neuroblastoma tumors. *Am J Pathol.* 1999;155(5):1439-43. doi: 10.1016/S0002-9440(10)65457-0.
7. Spitz R, Hero B, Skowron M, Ernestus K, Berthold F. MYCN-status in neuroblastoma: characteristics of tumours showing amplification, gain, and non-amplification. *Eur J Cancer.* 2004;40(18):2753-9. doi: 10.1016/j.ejca.2004.05.002.
8. Santiago T, Tarek N, Boulos F, Hayes C, Jeha S, Raimondi S, et al. Correlation between MYCN gene

status and MYCN protein expression in neuroblastoma: a pilot study to propose the use of MYCN immunohistochemistry in limited-resource areas. *J Glob Oncol.* 2019;5:1-7. doi: 10.1200/JGO.19.00135.
9. Souzaki R, Tajiri T, Teshiba R, Higashi M, Kinoshita Y, Tanaka S, et al. The genetic and clinical significance of MYCN gain as detected by FISH in neuroblastoma. *Pediatr Surg Int.* 2011;27(3):231-6. doi: 10.1007/s00383-010-2781-4.
10. Wang M, Zhou C, Cai R, Li Y, Gong L. Copy number gain of MYCN gene is a recurrent genetic aberration and favorable prognostic factor in Chinese pediatric neuroblastoma patients. *Diagn Pathol.* 2013;8:5. doi: 10.1186/1746-1596-8-5.
11. Yue ZX, Huang C, Gao C, Xing TY, Liu SG, Li XJ, et al. MYCN amplification predicts poor prognosis based on interphase fluorescence in situ hybridization analysis of bone marrow cells in bone marrow metastases of neuroblastoma. *Cancer Cell Int.* 2017;17:43. doi: 10.1186/s12935-017-0412-z.
12. Taylor CP, Bown NP, McGuckin AG, Lunec J, Malcolm AJ, Pearson AD, et al. Fluorescence in situ hybridization techniques for the rapid detection of genetic prognostic factors in neuroblastoma. United Kingdom Children's Cancer Study Group. *Br J Cancer.* 2000;83(1):40-9. doi: 10.1054/bjoc.2000.1280.
13. Zareifar S, Shakibazad N, Zekavat OR, Bordbar M, Shahriari M. Successful treatment of refractory metastatic neuroblastoma with panobinostat in combination with chemotherapy agents and iodine-131-meta-iodobenzylguanidine therapy. *J Oncol Pharm Pract.* 2020;26(2):481-6. doi: 10.1177/1078155219852670.
14. Lee YJ, Hah JO. Palliative effect of 131I –MIBG in relapsed Neuroblastoma after autologous peripheral blood stem cell transplantation. *Korea J Ped.* 2008;51(2):214-8. doi: 10.3348/kjp.2008.512.2.214
15. Sait S, Modak S. Anti-GD2 immunotherapy for neuroblastoma. *Expert Rev Anticancer Ther.* 2017;17(10):889-904. doi: 10.1080/14737140.2017.1364995.
16. Tang XX, Zhao H, Kung B, Kim DY, Hicks SL, Cohn SL, et al. The MYCN enigma: significance of MYCN expression in neuroblastoma. *Cancer Res.* 2006;66(5):2826-33. doi: 10.1158/0008-5472.CAN-05-0854.