Implementation of the plasma MYCN/NAGK ratio to detect MYCN amplification in patients with neuroblastoma

Yan Su1, Lijun Wang2, Qian Zhao1, Zhixia Yue1, Wen Zhao1, Xisi Wang1, Chao Duan1, Mei Jin1, Dawei Zhang1, Shenglan Chen3, Jianfeng Yin3, Lihua Qiu2, Xianfeng Cheng2, Zhong Xu2 and Xiaoli Ma1

1 Beijing Key Laboratory of Pediatric Hematology Oncology, National Discipline of Pediatrics, Ministry of Education, MOE Key Laboratory of Major Diseases in Children, Hematology Oncology Center, Beijing Children’s Hospital, Capital Medical University, National Center for Children’s Health, Beijing, China
2 Beijing Keyin Technology Company Limited, Beijing Keyin Evergreen Institutes for Medical Research Company Limited, China
3 Taizhou Genewill Medical Laboratory Company Limited, China

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Correspondence
X. Ma, Hematology Oncology Center, Beijing Children’s Hospital, Capital Medical University, National Center for Children’s Health, Beijing 100045, China
Tel: +861059617612
E-mail: mxl1123@vip.sina.com
Z. Xu, Beijing Keyin Technology Company Limited, Beijing 100022, China
Tel: +861085179195
E-mail: xqirui@keyintt.com

Detection of amplification of the MYCN gene is essential for determining optimal treatment and estimating prognosis of patients with neuroblastoma (NB). DNA FISH with neuroblastoma tissues or patient-derived bone marrow cells is the standard clinical practice for the detection of MYCN amplification. As tumor cells may often be unavailable, we developed a method to detect MYCN amplification in the plasma of patients with neuroblastoma. Taking single-copy NAGK DNA as reference, we used real-time quantitative PCR (qPCR) to determine the MYCN/NAGK ratio in the plasma of 115 patients diagnosed with NB. An increased MYCN/NAGK ratio in the plasma was consistent with MYCN amplification as assessed by DNA FISH. The AUC for a MYCN/NAGK ratio equal to 6.965 was 0.943, with 86% sensitivity and 100% specificity. Beyond the threshold of 6.965, the MYCN/NAGK ratio correlated with a heavier tumor burden. Event-free and overall survival of two years were significantly shortened in stage 4 patients with a MYCN/NAGK ratio higher than 6.965. Plasma MYCN/NAGK ratios increased in patients with progressive disease and relapse. Thus, we conclude that the determination of the plasma MYCN/NAGK ratio by qPCR is a noninvasive and reproducible method to measure MYCN amplification in patients with NB.

1. Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor of pediatric malignancies and originates from sympathetic nervous system, accounting for approximately 10% of all pediatric tumors [1–3]. Although substantial progresses have been made in therapy of NB, including chemotherapy, radiotherapy, surgery, hematopoietic stem cell transfusion, and immunotherapy, the 5-year survival rate of patients with high-risk NB remains still less than 50% [4–9]. Besides age of onset, primary location, and metastasis, MYCN amplification is widely considered to correlate with risk stratification, relapse, progressive disease, and unfavorable survival rate in NB patients [5,10–17].

Abbreviations
AUC, area under the ROC curve; cfDNA, cell-free DNA; CI, confidence interval; EFS, event-free survival; FISH, fluorescence in situ hybridization; LDH, lactate dehydrogenase; NB, neuroblastoma; NSE, neuron-specific enolase; OS, overall survival; qPCR, quantitative PCR; ROC, receiver operating characteristic.
Therefore, the determination of MYCN amplification is necessary in patients with NB.

Histological and cytological investigation on tumor tissue, bone marrow, and circulating tumor cells provides significant clinical features, including diagnosis and risk stratification and genetic profile [10,18–20]. Presently, fluorescence in situ hybridization (FISH) is the most accurate way to evaluate status of MYCN amplification of tumor or metastatic bone marrow in NB [5,10,12,17,21,22]. However, checking status of MYCN amplification is impossible in case that tissue biopsy is unavailable or limited at the time of diagnosis. Bone marrow of metastasis could be selected as an alternative sample to examine MYCN amplification of bone marrow cells in patients with NB [12,17,21,23]. Unfortunately, negativity of MYCN amplification in cells from metastatic bone marrow could not prove MYCN nonamplification of tumor because of tumors' heterogeneity [21–23]. To depict tumor heterogeneity and minimal residual disease, liquid biopsy is recommended to estimate tumor dynamics [24–26]. Recently, plasma cell-free DNA (cfDNA) quantification is emerging as a promising and noninvasive method to predict tumor burden in NB [29–31]. More importantly, serum or plasma MYCN copy number quantification by real-time quantitative polymerase chain reaction (qPCR) was used to predict amplified MYCN of tumor in NB, at different cutoff value [32–37]. Remarkably, less work is done with plasma DNA and more clinical tests are needed. Hereby, the aim of our study was to predict MYCN amplification status of tumor using plasma cfDNA-based qPCR from patients with NB.

2. Materials and methods

2.1. Patients

A total of 115 patients with NB were recruited at the Hematology Oncology Center, Beijing Children’s Hospital between January 1, 2016, and December 31, 2019. The initial diagnosis of NB was made according to International Neuroblastoma Staging System (INSS) criteria. Unequivocal pathologic diagnosis was made from tumor tissue by light microscopy or bone marrow aspirate or trephine biopsy contained unequivocal tumor cells with increased urine/serum catecholamines/metabolites. In special cases of seriously ill patients without bone marrow metastasis, the initial clinical diagnosis was established by typical tumor localization with typical metastases (such as bone, liver, lymph node, and skin) detected by metaiodobenzylguanidine (MIBG) or fluorine-18-fluoroo-2-deoxy-D-glucose positron emission tomography/computed tomography (18F-FDG PET/CT) combined with abnormal tumor marker levels. Patients were staged according to the International Neuroblastoma Risk Group Staging System (INRGSS) and grouped by the INRG classification system. Tumors were classified in accordance with the International Neuroblastoma Pathology Classification System (INPC). All diagnosed patients were followed up by the end of December 2019. This study and the BCH-NB-2007 protocol were approved by the Beijing Children’s Hospital Institutional Ethics Committee (No. 2016-65). Informed consent was obtained from the patients’ parents or guardians according to the Declaration of Helsinki. The BCH-NB-2007 protocol is based on the Hong Kong Pediatric Hematology and Oncology Study Group guidelines [38] and the results of a study in Germany [39]. According to risk stratification of NB, multidisciplinary treatment was applied in HR-NB patients, including induction chemotherapy, surgery, consolidation therapy, radiotherapy, and autologous stem cell transplantation. The intermediate-risk patients received chemotherapy and surgery. The patients in low-risk group received surgery with or without chemotherapy. According to efficacy evaluation of HR-NB, after the second, fourth, and sixth course of chemotherapy, before stem cell transplantation, before maintenance treatment, every 3 months during maintenance treatment, tumor markers and imaging examination were performed to evaluate the size of tumor focus and metastasis site, and with bone marrow metastasis patients, bone marrow puncture of sternum and ilium were also performed.

2.2. Clinical test and evaluation

Upon initial diagnosis, bone marrow biopsies and/or aspirates were obtained for microscopic examination and identification of NB cells. Amplification of the MYCN gene was detected by FISH in both resected tumor tissues and bone marrow cells. MYCN amplification was defined as a > fourfold increase of MYCN signals in relation to the number of chromosome 2 in Fig. S1 as described [22]. Laboratory analysis was performed prior to treatment, and the interval between laboratory tests and biopsy was less than 15 days. Urinary vanillylmandelic acid (VMA) and homovanillic acid (HVA) were analyzed by gas chromatography-mass spectrometry (GC/MS), and their concentrations were expressed as a ratio to urinary creatinine concentration. Lactate dehydrogenase (LDH) and neuron-specific enolase (NSE) were measured in serum using...
routine clinical chemistry laboratory methods. During the period of follow-up, progressive diseases, relapse, and death were defined as events in patients with NB.

2.3. Sample collection

Venous blood samples were collected at the time of diagnosis. Serial blood samples were taken in several patients at 4th cycle of chemotherapy, postsurgery, event occurrence, and ending of follow-up. Blood samples were collected into ethylenediaminetetraacetic acid-coated tubes and centrifuged at 1600 g for 10 min. Supernatants were transferred to fresh tubes and centrifuged at 16 000 g for 10 min. Plasma was removed and stored at −80 °C until DNA extraction.

2.4. Plasma MYCN/NAGK ratio quantification

DNA was purified from 200 μL of plasma and eluted by 300 μL of elution buffer using QIAamp DNA Blood Mini Kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Taking NAGK (a single-copy gene) as a reference gene, plasma MYCN/NAGK ratio was quantified as previously described [33]. SYBR® Green qPCR was performed on a LightCycler LC480 PCR machine (Roche Molecular Systems, Inc., Pleasanton, CA, USA). The sequence of primers of MYCN and NAGK is as listed as follows:

MYCN forward, 5′-GCAGCACTTTGCTAAAGAA-3′;
MYCN reverse, 5′-CAGTGACTGTCCAGTGGTG-3′;
NAGK forward, 5′-TGAGCAGACACATCGTAGCAA-3′;
NAGK reverse, 5′-CACCTTCACCTCCCACCTCAAC-3′.

A serially diluted standardized solution of human genomic DNA (Thermo Fisher Scientific, Waltham, MA, USA) was used to create a reference standard curve. The MYCN/NAGK ratio was determined by the ratio of the MYCN dosage to the NAGK dosage according to the standard curve. The qPCRs were performed in triplicate, and mean values of the triplicates were used for further analysis. The qPCR mixture was 10 μL and contained 2 μL of the eluted DNA, 1 μL (final concentration 0.2 μmol) of each forward and reverse primer of MYCN or NAGK, 5 μL of UltraSYBR Mixture (Cwbiotech, Beijing, China), and 1 μL of double-distilled water. Cycling conditions were 1 min at 95 °C and 40 cycles of 95 °C for 8 s and 60 °C for 20 s. Each plate contained a plasma DNA sample, a negative control (water template), and seven serially diluted standard DNA solutions (10, 5, 1, 0.5, 0.25, 0.0625, and 0.015 ng·μL⁻¹).

2.5. Statistics analysis

Data are presented as the median and 95% confidence interval (CI), and were analyzed using the Mann–Whitney U-test or one-way ANOVA test and chi-square test in R statistical environment (version 3.4.0, R Foundation for Statistical Computing, Vienna, Austria). Event-free and overall survival curves were generated by Kaplan–Meier method, and curves were compared using a log-rank test. Receiver operating characteristic (ROC) curves were constructed and analyzed using the Bioconductor ROC package. A P-value of < 0.05 was considered significant.

3. Results

3.1. Clinical characters of patients with newly diagnosed NB

Clinical characters of 115 patients are analyzed in Table 1 and Table S1. The male and female patients diagnosed with NB were similar, 51.3% and 48.7%. The median age of diagnosed NB was 36 months, and most NB children ranged from 18 to 60 months. Ninety-six tumors (83.48%) are primarily found at abdomen. Determined by FISH in NB tumors, amplified MYCN was detected in 37 cases, 25 boys and 12 girls. The positive rate of MYCN amplification in boys was significantly higher than in girls, 42.37% vs 21.43%. More intriguingly, 37 MYCN amplification patients were all found in tumors originated at abdomen. This expression pattern provided an evidence that tumor of NB located in abdomen highly correlated to heavier tumor burden and high rate of MYCN amplification. In addition, 26 (55.32%) patients with MYCN amplification had NSE level more than 1, and 25 (80.65%) had LDH level more than 1500 IU·L⁻¹. For metastatic sites, including bone, bone marrow, lymph node, liver, spleen, and brain, 65 (56.52%) patients had less than three organs involved metastasis, 30 (26.09%) had 3, and 20 (17.39%) had more than 3 metastatic sites, respectively. However, there was no significant difference of MYCN amplification between metastatic organ sites.

3.2. High plasma MYCN/NAGK ratio predicting MYCN amplification status of tumor

According to the FISH results of tumor MYCN status (Table 2, Table S1), plasma MYCN/NAGK ratio was significantly higher in MYCN-positive patients with NB than in negative patients, 69.07 (median CI 95%,
vs 1.27 (median CI 95%, 1.00, 1.53). It represented plasma MYCN/NAGK ratio could discriminate MYCN amplification status significantly. To test the performance power of prediction by MYCN/NAGK ratio, receiver operating characteristic (ROC) analysis was applied in NB patients. The area under the ROC curve (AUC) was 0.943, with an optimal sensitivity and specificity of 86.5% and 100%, respectively, at a MYCN/NAGK ratio of 6.965 (Fig. 1).

Other than the powerful performance of MYCN/NAGK ratio prediction, higher plasma MYCN/NAGK ratio showed consistent with heavier tumor load. For instance, plasma MYCN/NAGK ratio was significantly different between primary sites of tumors, serum levels of NSE and LDH, and organ sites of metastasis (Table 2). In particular, plasma MYCN/NAGK ratio was significantly higher in NB patients with serum NSE more than 370 ng·mL⁻¹ and serum LDH more than 1500 IU·L⁻¹ than those with NSE less than 370 ng·mL⁻¹ and LDH less than 1500 IU·L⁻¹, 26.17 (median CI 95%, 1.71, 60.76) vs 1.32 (median CI 95%, 1.00, 1.57), 81.43 (median CI 95%, 40.74, 149.6) vs 1.54 (median CI 95%, 1.30, 1.73), or vs 1.13 (median CI 95%, 0.89, 1.54), respectively. Patients with metastatic sites more than three had higher MYCN/NAGK ratio than those with metastatic sites less than 3, 5.29 (median CI 95%, 1.25, 40.74) vs 1.53 (median CI 95%, 1.12, 1.71). Thus, the higher plasma MYCN/NAGK ratio indicated heavier tumor burden in NB patients.

### 3.3. High plasma MYCN/NAGK ratio predicting poor outcome of patients with NB in INSS stage 4

Set the threshold of 6.965 according to AUC analysis, event-free survival time (EFS) and overall survival time (OS) of patients with NB in INSS stage 4 were

### Table 1. Demographic and clinical features of patients with newly diagnosed NB. Amp, amplification; Nonamp, nonamplification; NSE, neuron-specific enolase; LDH, lactate dehydrogenase; P: chi-square test.

| Characters         | Total N = 115 | N  | %   | MYCN amp by FISH | Amp     | Nonamp | % of Amp | P       |
|--------------------|---------------|----|-----|------------------|---------|--------|----------|---------|
| Gender             |               |    |     |                  |         |        |          |         |
| Male               | 59            | 51.30 | 25 | 34 | 42.37  | < 0.05 |
| Female             | 56            | 48.70 | 12 | 44 | 21.43  |         |
| Age (months)       |               |    |     |                  |         |        |          |         |
| < 18               | 17            | 14.78 | 6  | 11 | 35.29  | > 0.05 |
| ≥ 18 and ≤ 60      | 74            | 64.35 | 24 | 50 | 32.43  |         |
| > 60               | 24            | 20.87 | 7  | 17 | 29.17  |         |
| Primary site       |               |    |     |                  |         |        |          |         |
| Abdomen            | 96            | 83.48 | 37 | 59 | 38.54  | < 0.05 |
| Thorax and other   | 19            | 16.52 | 0  | 19 | 0.00   |         |
| NSE (ng·mL⁻¹)      |               |    |     |                  |         |        |          |         |
| < 370              | 68            | 59.13 | 11 | 57 | 16.18  | < 0.01 |
| ≥ 370              | 47            | 40.87 | 26 | 21 | 55.32  | < 0.01 |
| LDH (IU·L⁻¹)       |               |    |     |                  |         |        |          |         |
| < 500              | 42            | 36.52 | 4  | 38 | 9.52   |         |
| ≥ 500 and < 1500   | 42            | 36.52 | 8  | 34 | 19.05  | < 0.01 |
| ≥ 1500             | 31            | 26.96 | 25 | 6  | 80.65  |         |
| Metastasis sites   |               |    |     |                  |         |        |          |         |
| < 3                | 65            | 56.26 | 15 | 50 | 23.08  | > 0.05 |
| 3                  | 30            | 26.09 | 12 | 18 | 40.00  |         |
| > 3                | 20            | 17.39 | 10 | 10 | 50.00  |         |

Values in bold highlight the statistical significance with P value less than 0.05 or 0.01.

### Table 2. High plasma MYCN/NAGK ratio predicting MYCN amplification status of tumor.

| Character    | Subgroup | N    | Plasma (median, CI 95%) | MYCN | P-value |
|--------------|----------|------|------------------------|------|---------|
| FISH-        | Amp      | 37   | 69.07 (53.39, 142.50)   | < 0.01<sup>a</sup> |
| MYCN         | Nonamp   | 68   | 1.27 (1.00, 1.53)       |      |         |
| Gender       | Male     | 59   | 1.73 (1.30, 5.53)       | > 0.05<sup>a</sup> |
|              | Female   | 56   | 1.55 (1.25, 1.78)       |      |         |
| Age (months) | < 18     | 17   | 1.71 (0.9, 53.39)       | > 0.05<sup>b</sup> |
|              | ≥ 18 and ≤ 60 | 74 | 1.57 (1.37, 2.21)       |      |         |
| Primary site | Abdomen  | 96   | 1.60 (1.37, 2.63)       | > 0.05<sup>a</sup> |
|              | Thorax and other | 19 | 1.56 (0.89, 1.83)       |      |         |
| NSE (ng·mL⁻¹) | < 370   | 68   | 1.32 (1.00, 1.57)       | < 0.01<sup>a</sup> |
|              | ≥ 370   | 47   | 26.17 (1.71, 60.76)     |      |         |
| LDH (IU·L⁻¹) | < 500   | 42   | 1.13 (0.89, 1.54)       | < 0.01<sup>b</sup> |
|              | ≥ 500 and < 1500 | 42 | 1.54 (1.30, 1.73)       |      |         |
|              | ≥ 1500  | 31   | 81.43 (40.74, 149.60)   |      |         |
| Metastasis sites | < 3    | 65   | 1.53 (1.12, 1.71)       | < 0.05<sup>b</sup> |
|              | > 3     | 20   | 5.29 (1.25, 40.74)      |      |         |

<sup>a</sup>Mann–Whitney U-test.  
<sup>b</sup>Kruskal–Wallis test.

53.39, 142.50) vs 1.27 (median CI 95%, 1.00, 1.53). It represented plasma MYCN/NAGK ratio could discriminate MYCN amplification status significantly. To test the performance power of prediction by MYCN/NAGK ratio, receiver operating characteristic (ROC)
investigated by Kaplan–Meier curve during 2 years. Events are referred as relapse, progressive disease, and death during follow-up period. Thirteen in 30 patients (43.33%) appeared to have events in patients with high plasma MYCN/NAGK ratio, which was significantly higher than those with low MYCN/NAGK ratio 15 in 63 (23.81%) (Table 3). Consistently, EFS was statistically significantly higher for patients with low plasma MYCN/NAGK ratio than with high plasma MYCN/NAGK ratio (86.61% vs 35.10%; Fig. 2). In similarity, the mortality rate was significantly higher in patients with high plasma MYCN/NAGK ratio than those with low plasma MYCN/NAGK ratio, 33.33% (10 in 30) vs 6.35% (4 in 63) (Table 4). Patients with low plasma MYCN/NAGK ratio had significantly higher OS than those with high plasma MYCN/NAGK ratio (88.41% vs 37.59%; Fig. 3).

3.4. Increased plasma MYCN/NAGK ratio indicating therapeutic efficiency and events in patients with NB

Monitoring therapeutic response and events in patients is necessary and important. Changes of plasma MYCN/NAGK ratio were hypothesized to indicate therapeutic response and events happening. In Fig. 4, plasma MYCN/NAGK ratio was measured in four patients with MYCN amplification at three time points, including diagnosis, adjuvant chemotherapy, event occurrence, and ending of follow-up. All four patients had high level of plasma MYCN/NAGK ratio at diagnosis, ranged from 26.17 to 177.29, far beyond the threshold value 6.965. After four cycles of chemotherapy, plasma MYCN/NAGK ratio declined obviously, less than 10. However, patients’ situations were different after multidiscipline treatment. In patient 15 with event-free by the end of 2-year follow-up period, plasma MYCN/NAGK ratio was...
consistently low. In contrast, among patients 3, 14, and 19 with event occurrence, plasma MYCN/NAGK ratios raised and were beyond the threshold value 6.965 (in newly diagnosed patients). Remarkably, plasma MYCN/NAGK ratio in patient 19 was dramatically higher at the time of event confirmation than at the time of newly diagnosis, 317.37 vs 177.29.

4. Discussion

Patients with high-risk NB frequently suffer from low survival rate, less than 50% [7,9,19]. Generally, minimal residual disease results in insufficient treatment and recurrence in NB [40–42]. In clinic, amplification of the MYCN is a reliable and powerful indicator both of high-risk stratification and poor prognosis in NB [11,12,17,43]. Unfortunately, serial assessment of MYCN amplification of tumor is not possible due to the lack of primary tumor tissue and heterogeneity of tumor in NB. Nowadays, liquid biopsy test is emerging as a promising and repeatable method to examine tumor load in clinic [24,26,44–46]. Using blood cfDNA from patients with NB sheds light on predicting MYCN amplification repeatedly [34–36]. In our study, the most concern is whether plasma MYCN/NAGK ratio could evaluate amplified MYCN of NB tumor accurately.

Decade ago, a method-based real-time quantitative polymerase chain reaction was developed to measure serum MYCN/NAGK ratio in NB [33]. When cutoff of serum MYCN/NAGK ratio was set at 10, the sensitivity and specificity to distinguish MYCN amplification were both 100%. Furthermore, patients with MYCN/NAGK ratio beyond 5 had worse overall survival, particularly in those less than 18 months of age [37]. After surgery or neoadjuvant chemotherapy in NB, plasma copy numbers of MYCN were significantly decreased [35]. In patients with recurrence or unsuccessful treatment, serum MYCN/NAGK ratio was in the higher level than the cutoff value at the time of diagnosis [33]. However, less sensitivity of serum MYCN/NAGK ratio in stage 1 or 2 of NB remains improved further [11].

Previous studies demonstrated that plasma cfDNA could be a promising and reproducible method to examine tumor burden of NB [29–31]. In the present study, plasma MYCN/NAGK ratio was tested to predict MYCN amplification by qPCR in patients with NB. When threshold was set at 6.965, the performance of plasma MYCN/NAGK ratio was 0.943, with 86.5%
sensitivity and 100% specificity to discriminate amplified MYCN in NB (Fig. 1). The optimal AUC and cutoff value of MYCN/NAGK ratios were similar to other investigations by serum [32,37]. During 2-year follow-up, poor prognosis appeared in patients in INSS stage 4 with plasma MYCN/NAGK ratio higher than 6.965 (Figs 2 and 3). The powerful prediction of prognosis with plasma MYCN/NAGK ratio is consistent to that with FISH examination of MYCN amplification in histology [11,17]. Another advantage of plasma MYCN/NAGK ratio to predicting MYCN amplification is monitoring therapeutic effect and recurrence disease in NB. Patients with sufficient remission would keep dramatically lower level of plasma MYCN/NAGK ratio, while those with progression or recurrence maintain higher level or ascend significantly in NB (Fig. 4). These data are matched with previous finding [33]. It is known that NB tumor originated from abdomen and nonabdomen sites has different outcome and genomic profiles [14,16]. Consistently, our data show that all 37 tumors with MYCN amplification are detected at abdomen exclusively (Table 1).

5. Conclusions

In conclusion, plasma MYCN/NAGK ratio may be a promising indicator of MYCN amplification of tumor in NB. Combined with other clinical features, plasma MYCN/NAGK ratio could successfully distinguish heavier tumor burden, insufficient treatment, and poor prognosis. More importantly, plasma MYCN/NAGK ratio is a promising, noninvasive, less time-consuming, and repeatable method to check MYCN amplification of tumors in NB when tumor tissues are limited and MYCN nonamplification is detected in bone marrow cells by FISH test.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

XM and ZX designed the study. XM, YS, and LW conceptualized the study. XM, YS, LW, and QZ revised the manuscript. SC, JY, LQ, XC, and ZX analyzed and interpreted data. ZY, WZ, XW, QZ, CD, MJ, and DZ collected patients’ samples. SC, JY, ZY, and LW performed qPCR. WZ, XW, QZ, CD, MJ, and DZ wrote the manuscript. All authors read and approved the final manuscript.

Data accessibility

The raw data are available upon reasonable request from the corresponding authors.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Typical fluorescence in situ hybridization (FISH) images of bone marrow cells from patients with bone marrow metastatic neuroblastoma. (A) The status of normal *MYCN* was detected using a dual-
color probe. Green signals represent the specific probe for MYCN (n=2), and red signals stand for centromeric chromosome 2 probes. (B) The status of MYCN amplification. MYCN signals show more than 50 copies (n=66) within the nuclei and red signals show that there are 3 chromosome 2. (C) The status of MYCN amplification. MYCN signals show 20-30 copies (n=26) within the nuclei. Bars=5μm.

Table S1. All patients’ data (supplement). All data of patients are listed, including demographic, diagnosis, staging, pathology, clinical examinations, plasma MYCN/NAGK ratios, events and treatment.