A systematic review of circulating-free MYCN amplification status as a noninvasive and prominent prognostic and recurrence monitoring indicator for neuroblastoma

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Abstract

MYCN gene plays an important role in neuroblastoma cell growth, apoptosis, differentiation, tumor infiltration, metastasis, and angiogenesis. *MYCN* gene amplification status in tumor tissue is one of the important clinical indicators of neuroblastoma risk. However, there are several problems in detecting the *MYCN* gene in tumor tissues, such as complex procedures in detection approaches, high detection costs, and difficulty in obtaining tumor tissues. Studies have shown that circulating-free *MYCN* gene amplification in neuroblastoma is consistent with that in tumor tissue. Circulating-free *MYCN* gene amplification has the advantages of high sensitivity, high specificity, simple operation, and non-invasiveness as an indicator for prognosis and recurrence of neuroblastoma. Therefore, the application and future development of circulating-free *MYCN* gene amplification status in the prognosis and recurrence monitoring of neuroblastoma was examined in this article.

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Tables: 1

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Abbreviations

NB	neuroblastoma
M/N	the MYCN copy number and NAGK copy number ratio
MNA	MYCN gene amplification
INSS	international neuroblastoma staging system
INRG	International neuroblastoma risk group
FISH	in situ hybridization
OS	overall survival

Abstract: *MYCN* gene plays an important role in neuroblastoma cell growth, apoptosis, differentiation, tumor infiltration, metastasis, and angiogenesis.*MYCN* gene amplification status in tumor tissue is one of the important clinical indicators of neuroblastoma risk. However, there are several problems in detecting the *MYCN* gene in tumor tissues, such as complex procedures in detection approaches, high detection costs, and difficulty in obtaining tumor tissues. Studies have shown that circulating-free *MYCN* gene amplification in neuroblastoma is consistent with that in tumor tissue. Circulating-free *MYCN* gene amplification has the advantages of high sensitivity, high specificity, simple operation, and non-invasiveness as an indicator for prognosis and recurrence of neuroblastoma. Therefore, the application and future development of circulating-free *MYCN* gene amplification status in the prognosis and recurrence monitoring of neuroblastoma was examined in this article.

Introduction

Neuroblastoma (NB) is one of the most common solid tumors in young patients, accounting for 8-10% of pediatric malignancies and 15% of all tumor-related deaths in patients[1-3]. According to the demarcation of age at diagnosis, the International Neuroblastoma Staging System (INSS) stage, the tumor tissue *MYCN* status, the International Neuroblastoma Pathology Committee (INPC) classification, and ploidy, NB can be stratified into low-, intermediate- and high-risk groups[4]. Clinically, *MYCN*- amplified NB patients account for about 20% - 30% of NB cases, and most of them are high-risk patients[5-9]. Despite intensive multimodal therapy, patients with high-risk neuroblastoma have a 5-year overall survival (OS) rate of approximately 50%[10-15]. In addition, 50% to 60% of high-risk neuroblastoma patients relapse and metastasize, signifying a poor prognosis[16-18]. The primary sites of NB mainly reside in the adrenal gland, neck, chest and basin. The metastatic sites of NB mainly reside in the liver, bone, marrow, lymph node and pancreas (Figure 1). Therefore, NB has a wide range of primary and metastatic sites, which can lead to severe harm in patients.

The International Neuroblastoma Risk Group (INRG) database has shown a 5-year overall survival (OS) rate of 4% in patients with relapsed stage 4 *MYCN* amplification [16]. Therefore, *MYCN*- amplified NB should be accurately assessed for prognosis and monitored for recurrence, and corresponding comprehensive treatment should be administered to improve the survival rate of patients. *MYCN*status and the time of recurrence have all been shown to affect length of survival [16, 18-21]. At present, the amplification of*MYCN* in NB tissues is detected by fluorescence in situ hybridization (FISH) and Southern blotting. However, obtaining NB tissue is difficult and invasive, making the dynamic monitoring of NB recurrence implausible. In addition, the high heterogeneity of NB may lead to inaccurate test results [22-28]. Studies have shown that the circulating-free *MYCN* in serum and plasma (circulating-free*MYCN*) of NB patients is consistent with NB tissue. Therefore, NB circulating free *MYCN* is expected to be an indicator for*MYCN* prognostic assessment and recurrence diagnosis.

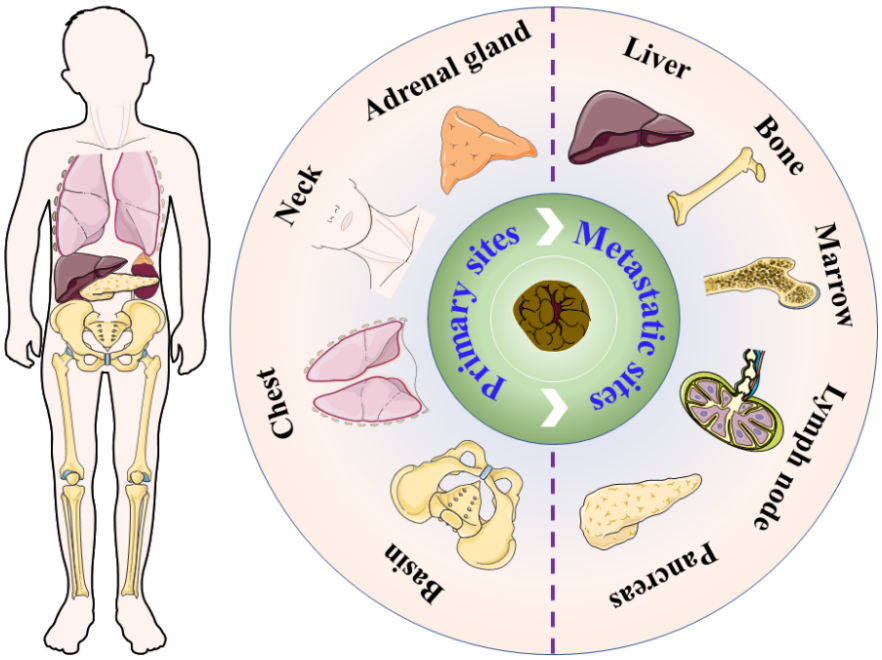

Figure 1 The primary and metastatic sites of NB

The role of *MYCN* gene in occurrence and development of NB

The *MYCN* gene is a member of the MYC oncogene family and is located on band 3 (2p24.3), region 24, on the short arm of chromosome 2. Structurally, the *MYCN* coding region is highly homologous to the MYC gene coding region, and its expressed protein is about 60-63 kDa[29, 30]. The *MYCN* protein plays an important role in cell signal transduction[31-39]. It is downstream of several important signal transduction pathways, such as the Wnt/β-catenin signaling pathway and PI3K/Akt/mTOR signaling pathway, etc.[40-43].*MYCN* is the main regulator of early embryonal development in pluripotent cells. Meanwhile, *MYCN* is highly expressed in the multipotent cells of the migratory and post-migratory neural crest[44-48]. During the differentiation of multipotent cells,*MYCN* expression is downregulated and the sympathoadrenal precursor cells or progenitor cells mature into different cell types of the autonomic neural cell lineage, such as sympathetic ganglion cells, chromaffin cells of the adrenal medulla or cells of the peripheral nervous system (Figure 2) [49, 50]. Therefore, *MYCN* plays an important role in coordinating neural crest cell proliferation and differentiation during embryonic development.

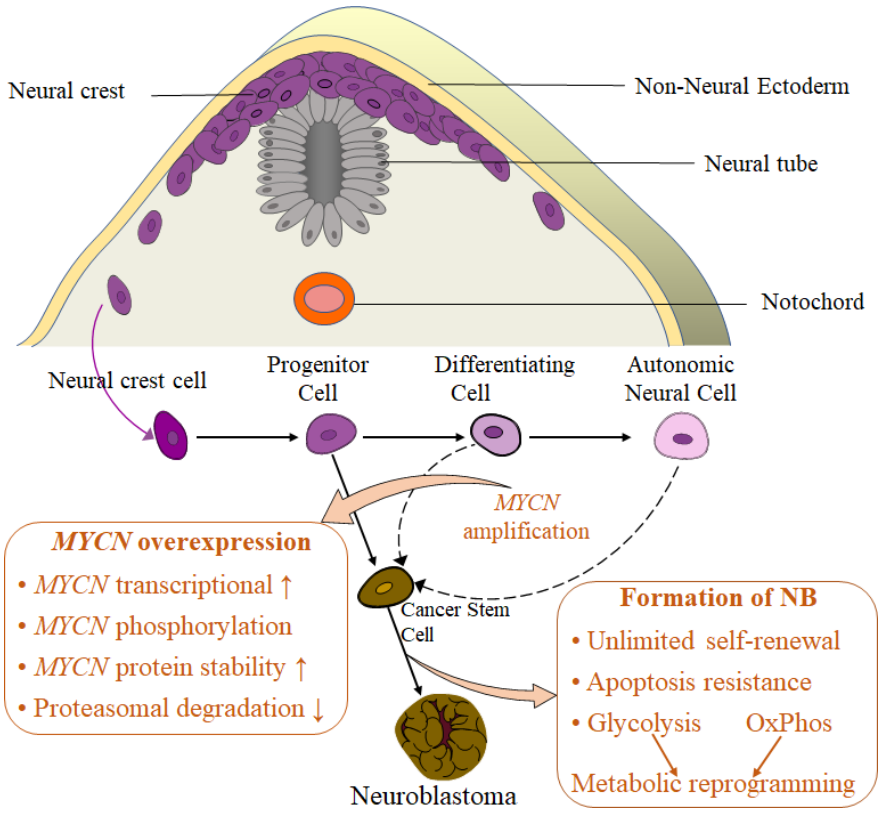

Figure 2 The role of *MYCN* gene in the occurrence and development of NB

Note: OxPhos: Oxidative Phosphorylation

Overexpression of the *MYCN* gene is common in tumors of the nervous system, such as NB, medulloblastoma, retinoblastoma, and glioblastoma multiforme. *MYCN* plays an important role in the proliferation, differentiation, metastasis, apoptosis and angiogenesis of NB cells[49, 51-61]. There are several mechanisms by which*MYCN* is overexpressed in tumors, ranging from induction of*MYCN* transcriptional activation, increased *MYCN* protein stability due to dysregulated *MYCN* phosphorylation, and reduced proteasomal degradation to *MYCN* gene amplification[62-67]. Additionally, the aberrant expression of *MYCN* induces a unique cancer stem cell-like phenotype by enabling infinite self-renewal, apoptotic resistance, and metabolic reprogramming characterized by increased glycolysis with active oxidative phosphorylation[67-71].*MYCN* is one of the earliest genetic markers discovered in neuroblastoma, and *MYCN* amplification status is one of the strongest predictors of poor prognosis[72-77]. In high-risk neuroblastoma patients, *MYCN* amplification, if present, will always be present at diagnosis. Patients with low-risk disease lack*MYCN* gene amplification and never progress to high-risk disease nor acquire additional copies of the *MYCN* gene[47, 63]. Studies by Schmidt et al., Park et al., Tomoko et al., and Meany et al. provide strong evidence that patients with *MYCN* amplification neuroblastoma, who are otherwise at low or intermediate risk, have an inferior prognosis compared to those with non-*MYCN* amplification NB [78-85]. This suggests that *MYCN* gene amplification is an early, perhaps initiating event, driving the development of high-risk neuroblastoma tumors. Therefore, *MYCN* amplification can be an effective indicator for prognostic assessment and recurrence detection in NB patients.

Problems facing *MYCN* copy number detection in NB tumor tissues

Currently, NB is clinically graded based on *MYCN* amplification in tumor tissues. However, most high-risk NB patients are already in advanced stages when they are diagnosed with large tumor sizes. At this

stage, direct surgical biopsy is very risky where accidents during anesthesia may occur in addition to serious complications such as intraoperative and postoperative massive bleeding, tumor rupture, and abdominal infection[22-25, 86]. Therefore, NB tissues, especially high-risk NB tissues, are difficult to obtain. In addition, in some high-risk NB patients who can be punctured, a tissue biopsy is unable to ensure a fully accurate assessment of *MYCN* amplification due to the high heterogeneity of NB[26-28]. Particularly, the detection of*MYCN* in primary tumors after toxic chemotherapy may be inaccurate, resulting in an insufficient therapeutic dose or duration of treatment[87]. In terms of detection methods, FISH and Southern blotting are usually used clinically to detect *MYCN* amplification in the tumor tissue cells of patients with NB[88-91]. However, these methods face disadvantages of high cost, complicated procedures and long detection time[92].

Consistent copy numbers of *MYCN* and circulating-free*MYCN* in NB tissues

Due to the aforementioned problems in the detection of NB, the study of non-invasive and dynamic monitoring of the *MYCN* amplification state is critical. Combaret et al. showed that circulating-free *MYCN* amplification status also existed in the tumor tissue of patients with *MYCN* -amplified NB and that there are 20-600 times more circulating-free *MYCN* -amplified NB patients than non-*MYCN* -amplified NB patients[22]. However, assessing the amplification of circulating-free *MYCN* based on PCR results in the absence of an internal reference gene may affect assay results due to changes in template DNA quality or chromosome 2 values. Therefore, most studies have selected the N-acetylglucosamine kinase gene (*NAGK*) as the internal reference gene. Tomoko et al. compared the ratio of circulating-free *MYCN* /*NAGK* (M/N) with the *MYCN* amplification status of tumor tissue in 43 NB patients and proved that the amplification status of circulating-free*MYCN* was consistent with the amplification status of *MYCN*in tumor tissue. Gotoh et al. examined circulating-free M/N ratios in 12*MYCN* -amplified patients and 33 *MYCN* -non-amplified patients. At the same time, these results were compared with the*MYCN* amplification status in tumor tissue, which indicated that the circulating-free *MYCN* amplification status of these patients were completely consistent with the results from FISH and Southern blotting. The study further proved that NB circulating-free was consistent with the *MYCN* amplification state in tumor tissue[93]. In addition, multiple studies based on real-time fluorescence quantitative PCR to detect circulating-free *MYCN*amplification in NB have demonstrated that the *MYCN* amplification status in NB tumor tissue is consistent with that of circulating-free*MYCN* [93, 94]. Studies have shown that because there is no*MYCN* amplification in most low- and intermediate-risk NB tumor tissues, there is no amplification of circulating free *MYCN* in most low- and intermediate-risk NB tumor tissues[23, 95, 96]. Therefore, the tumor tissue and circulating-free *MYCN*amplification status of patients with NB are consistent, and the detection of circulating-free *MYCN* amplification is expected to replace the detection of *MYCN* amplification in tumor tissue for the diagnosis, prognostic evaluation, and recurrence monitoring of NB.

Circulating-free *MYCN* copy number for prognostic assessment in NB patients

The probability of *MYCN* amplification in NB patients is 20 - 30%. In multi-lineage high-risk NB patients, *MYCN* amplification is an important indicator of NB risk grading and is significantly associated with poor prognosis in NB patients[97-99]. Tomoko et al. detected circulating-free *MYCN* amplification and tumor tissue*MYCN* amplification in 43 NB patients. The 4-year Event-free survival (EFS) rates of circulating-free *MYCN* amplification and*MYCN* non-amplified NB patients were 37.5% and 84.8%, respectively, and the 4-year OS rates were 56.3% and 96.8%. Yagyu et al. analyzed 82 patients with circulating-free *MYCN* amplification and found that they had a significantly lower overall survival rate than those without *MYCN* amplification (hazard ratio: 2.50; 95% confidence interval: 1.22 - 5.13, $P < 0.01$). Therefore, circulating-free *MYCN* amplification may serve as a prognostic indicator in NB patients [100]. Kojima et al. tracked the changes in circulating-free M/N ratio within a week post-surgery in five patients with *MYCN* -amplified tumors. Tumors in three patients were completely surgically removed and the M/N ratio dropped to 1.0 3-5 days after the surgery; the remaining two patients still had metastases after surgery and the M/N ratio remained above 1.0 after primary tumor resection. The authors also examined the circulating-free M/N ratios of 0.97, 1.23, 1.56, and 2.01 in four patients before the second surgery after neoadjuvant chemotherapy.

These M/N ratios were similar to the ratios of non-*MYCN* -amplified NB patients. Therefore, circulating-free M/N ratio can be used as an indicator for efficacy detection[94]. Su Yan et al. studied 116 cases MYCN non-amplified and MYCN amplified patients who had significantly difference 3-year OS rates of 94.6% and 26.7%, respectively[101]. In addition, multiple studies have demonstrated that the detection of circulating-free*MYCN* amplification can be used for the prognostic assessment of children with NB[22, 93, 95, 102, 103]. Therefore, circulating-free*MYCN* is expected to serve as a biomarker for the prognostic assessment of NB.

Circulating-free *MYCN* copy number for monitoring of recurrence in patients with NB

Relapse is one of the significant causes of death in NB. Nermine et al. showed that high-risk NB patients had a median time to relapse OS of 8.4 months (interquartile range = 3.0 – 17.4) and a median time to relapse EFS of 4.7 months (interquartile range = 2.1 – 7.1). For high-risk NB patients, *MYCN* -amplified cases were independently associated with a difference in OS time to relapse[104]. Combaret et al. followed eight patients whose circulating-free *MYCN* levels decreased after chemotherapy, but increased after relapse, including one patient with elevated circulating-free *MYCN* levels two months before relapse was clinically diagnosed[22]. Kojima et al. reported that the degree of reduction in circulating-free *MYCN* copy number at one week post-surgery in five patients with *MYCN* amplification was proportional to the degree of tumor resection[94]. Gotoh et al. followed up six patients with *MYCN* -amplified NB after surgery. Among them, the circulating-free M/N ratio of three NB patients with complete remission dropped to the normal range after surgery and remained low; the circulating-free M/N ratio of two patients with relapse after remission first dropped to the normal range and then increased at the time of diagnosis; and the circulating-free M/N value of one patient who failed to achieve remission did not decrease to the normal range and remained at a high level until death[93]. Ma et al. studied the circulating-free M/N value of 105 patients with NB and followed up 10 patients with *MYCN* -amplified NB. One of these patients had an increased circulating-free M/N ratio at recurrence (M/N ratio at diagnosis was 2.643 and M/N ratio at recurrence was 2.407). Therefore, circulating-free *MYCN* amplification is expected to be used for NB recurrence monitoring.

Circulating-free *MYCN* is a sensitive and specific NB biomarker

Sensitive and specific detection of circulating-free *MCYN*amplification in NB patients is crucial for the early and accurate diagnosis of NB patients. Valerie et al. analyzed *MYCN*amplification in 41 tumor tissues of 124 stage 4 patients, of which 35 had circulating-free *MYCN* amplification. Six of the 66 patients with stage 4S had *MYCN* amplification in tumor tissue and five of them had circulating-free *MYCN* amplification. The sensitivity of circulating-free *MYCN* amplification detection in NB patients was 85% and 83%, respectively, and the specificity was 100% (no false positive samples)[95]. Trigg et al. investigated the diagnostic accuracy of circulating free *MYCN* amplification analysis in 529 patients with advanced (stage 3 and 4) NB based on the mate analysis method. The results showed that the sensitivity and specificity of circulating-free *MYCN* amplification assay for the diagnosis of NB patients were 0.908 and 0.976, respectively[105]. Gotoh et al. studied the ratio of circulating-free M/N in 17 patients with*MYCN* -amplified NB and 70 patients with *MYCN* -non-amplified NB. The results showed that the mean circulating-free M/N of*MYCN* -amplified NB patients was 199.32, ranging from 17.1 to 901.6, and the mean circulating-free M/N of *MYCN* non-amplified patients was 0.87, ranging from 0.25 to 4.6. The sensitivity and specificity of NB diagnosis were both 100%. The detection of circulating-free *MYCN* amplification was completely consistent with Southern blotting results, and the positive and negative predictive values were both 100%[93]. In addition, most studies in literature have reported high sensitivity and specificity for the analysis of circulating-free *MYCN* amplification in patients with advanced disease, but M/N thresholds varied widely (Table 1)[94, 106]. Therefore, the detection of circulating free *MYCN* amplification can be used for high sensitivity and specificity diagnosis of NB.

Table 1 Circulating-free M/N value and cancer tissue M/N value and its clinical application

No.	M/N threshold	Group	Number of cases	circulating-free MNA status	MNA status of tumor tissue	Sensit
1	/	MNA	32	25 – 600[a]	MNA	/

No.	M/N threshold	Group	Number of cases	circulating-free MNA status	MNA status of tumor tissue	Sensit
		Non-MNA	70	/	Non-MNA	
2	10	MNA	17	17.1 - 901.6 [b]	MNA	100%
		Non-MNA	70	0.25 - 4.6 [b]	Non-MNA	
3	/	MNA	16	6.45 - 89.20 [a]	11.99 - 110.92 [a]	/
		Non-MNA	34	0.5 - 1.7 [a]	0.4 - 1.4 [a]	
4	5	MNA	73	53 cases	73 cases	75% -
		Non-MNA	194	0 cases	194 cases	
5	5	MNA	15	5.00 - 1204.32[b]	MNA	100%
		Non-MNA	65	0.00 - 8.00[b]	Non-MNA	
6	5	MNA	57	Median M/N ratio 118.27	MNA	86%
		Non-MNA	91	Median M/N ratio 2.45	Non-MNA	
7	1.6	Non-MNA	95	Non-MNA	Non-MNA	90.9%
		Non-MNA	91	Median M/N ratio 2.45	Non-MNA	
8	6.965	MNA	37	Median M/N ratio 69.07	MNA	86%
		Non-MNA	68	Median M/N ratio 1.27	Non-MNA	

Note: /: There is no relevant data in the text; [a]:*MYCN* copy number; [b]: M/N ratio;MNA:*MCYN* amplification.

Circulating-free *MYCN* detection method and problems in clinical application

At present, the main methods of circulating-free *MCYN*amplification detection are as follows: (1) Real-time quantitative PCR: Based on primers and probes specially designed for *MYCN* , the quantitative or qualitative detection of *MYCN* can be achieved by detecting the fluorescent signal of *MYCN* in real time. The convenience of this method and low cost makes it suitable for clinical application[95, 100]. (2) Digital PCR: Based on primers and probes specially designed for *MYCN* , a large amount of diluted*MYCN* solution is dispersed into microreactors or droplets of the chip, so that the number of *MYCN* in each reactor is less than or equal to one. After the PCR cycle, a reactor with a *MYCN* molecule will generate a fluorescent signal, and a reactor without a *MYCN*molecule will not generate a fluorescent signal, achieving an absolute quantitative detection of *MYCN* . However, digital PCR faces problems of a complicated procedure and high cost[107]. (3) Electrochemical method: The electrochemical signal generated by*MYCN* was detected by electrochemical analysis. Compared with traditional analytical methods, electrochemical methods have the advantages of a simple operation, short time consumption, good specificity, high sensitivity and low experimental cost[108]. Therefore, among these detection methods, real-time quantitative PCR is the most suitable method for clinical use.

The problems faced by the clinical application of circulating-free *MYCN* amplification detection are as follows: (1) Due to the lysis of leukocytes in the blood, the*NAGK* gene in leukocytes is released into the serum, which reduces the M/N ratio and affects the sensitivity of *MYCN* amplification detection. Therefore, high-speed centrifugation must be performed within a few hours of blood collection to remove the *NAGK* gene released by leukocytes[23, 93, 100, 103]. (2) At present, the evaluation criterion for the detection of circulating free *MYCN*amplification is the M/N ratio. However, the test results of different laboratories are quite different and there is no unified standard (see Table 1). (3) There is no kit for the detection of circulating-free *MYCN* amplification on the market, so it cannot be used clinically. Therefore, it is necessary to standardize serum collection procedures, blood circulating tumor DNA extraction methods, circulating-free *MYCN* amplification detection methods, and evaluation criteria to accelerate its clinical translation.

Conclusion

MYCN amplification is a poor prognostic biomarker in NB. Clinically, the detection of *MYCN* amplification in tumor tissue can be used for NB risk grading, but the detection of *MYCN*amplification in tumor tissue

faces the following problems: (1) For most high-risk NB patients who need preoperative chemotherapy, they are already in advanced stages and the tumor is large. At this time, a direct surgical biopsy involves of high risk and the biopsy tissue cannot be obtained, resulting in the inability to evaluate the *MYCN* amplification in time; (2) For some high-risk NB patients who can be punctured, due to the high heterogeneity of NB, a tumor biopsy cannot completely and accurately assess the *MYCN* amplification status of patients; (3) *MYCN* test results in primary tumors after chemotherapy may be inaccurate, resulting in insufficient doses or courses of drug therapy for NB patients. However, compared with tissue, blood is relatively simple to obtain and detection of circulating-free *MYCN* amplification is consistent with the detection of *MYCN* amplification in tissue. Circulating-free *MYCN* amplification can thus be used for NB diagnosis, prognosis assessment, and recurrence monitoring through early and dynamic. Moreover, it has the advantages of high sensitivity, good specificity, and simple detection method. However, there is heterogeneity in the detection standards of circulating-free *MYCN* amplification across various laboratories. Consequently, there is an urgent need to establish a standardized detection method for circulating-free *MYCN* amplification, thereby promoting the standardization of clinical detection and accelerating the clinical application of circulating-free *MYCN* detection.

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