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MYCN protein stability is a better prognostic indicator in neuroblastoma

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Abstract

Objective: *MYCN* oncogene amplification is associated with treatment failure and poor prognosis in neuroblastoma. To date, most detection methods of MYCN focus on DNA copy numbers instead of protein expression, which is the real one performing biological function, for poor antibodies. The current investigation was to explore a fast and reliable way to detect MYCN protein expression and evaluate its performance in predicting prognosis.

Methods: Several MYCN antibodies were used to detect MYCN protein expression by immunohistochemistry (IHC), and one was chosen for further study. We correlated the IHC results of MYCN from 53 patients with *MYCN* fluorescence in situ hybridization (FISH) and identified the sensitivity and specificity of IHC. The relationship between patient prognosis and MYCN protein expression was detected from this foundation.

Results: *MYCN* amplification status detected by FISH was most valuable for INSS stage 3 patients. In the cohort of 53 samples, IHC test demonstrated 80.0–85.7% concordance with FISH results. Further analyzing those cases with inconsistent results, we found that patients with *MYCN* amplification but low protein expression tumors always had a favorable prognosis. In contrast, if patients with *MYCN* non-amplified tumors were positive for MYCN protein, they had a poor prognosis.

Conclusion: MYCN protein level is better than *MYCN* amplification status in predicting the prognosis of neuroblastoma patients. Joint of FISH and IHC could confirm MYCN protein stability and achieve better prediction effect than the singular method.

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Highlights

The *MYCN* gene test is most valuable for INSS stage 3 patients to predict prognosis.

Compared to gene status, MYCN protein expression is more relevant to prognosis.

Combining FISH with IHC, MYCN protein stability could be identified.

Keywords: Neuroblastoma, MYCN, IHC, FISH, Protein stability

Background

Neuroblastoma is the most common extracranial solid tumor in children [1, 2], and causes up to approximately 12% of pediatric cancer-related mortality [3]. *MYCN* oncogene amplification is a genetic marker detected in about 20–30% of neuroblastoma patients [4]. As a member of the *MYC* oncogene family, the overexpression of MYCN is closely correlated with high-grade malignancy, early distant metastasis, and poor clinical prognosis [5]. Even with increased intensity treatment, the five-year overall survival (OS) rate of patients with *MYCN* amplified tumors, independent of the risk stratification, is still less than that of patients with *MYCN* non-amplified tumors [6].

Since no reliable MYCN antibody is used in IHC, clinicians and researchers usually detect *MYCN* amplification status at the nucleic acid level. Conventional polymerase chain reaction (PCR) [7], quantitative real-time PCR (qPCR) [8, 9], semi-quantitative differential PCR (SQ-PCR) [10], droplet digital PCR (ddPCR) [11], FISH [12], chromogenic in situ hybridization (CISH) [13], and multiplex ligation-dependent probe amplification (MLPA) [14] are some common methods. The FISH result is an important index of risk stratification [15]. However, several studies have found that MYCN protein could be isolated from tumors without gene amplification, and tumors with *MYCN* amplification could not express protein [16–18]. For protein exerts the biological function [19], finding a rapid, reliable, and cost-effective strategy to detect MYCN protein expression is significant.

We compared the performance of several antibodies in IHC and finally chose one for further study in this research. Comparative analysis and survival analysis were performed to verify its feasibility in IHC. The correlation of MYCN protein expression with patient prognosis was another focus. Our results demonstrated that the antibody is reliable in IHC. Compared to gene status, MYCN protein expression and stability better predict outcomes.

Methods

Study population

A cohort of 53 neuroblastoma patients was selected as the main study object. They received curative surgery at Shanghai Children's Medical Center (SCMC), Shanghai, China, between January 2010 and September 2019. 28 tumor samples of this cohort were *MYCN* amplification tested by FISH (*MYCN* FISH$^+$), which was the maximum count of *MYCN* FISH$^+$ prechemotherapy samples suitable for the IHC test during this time. As a control, 25 patients with *MYCN* FISH$^-$ tumors were chosen according to their clinical consequences: 1) 8 patients died from tumors, 2) 17 patients had a favorable long-term prognosis. Follow-up within this cohort was completed on December 31, 2019. To ensure prognostic accuracy for individuals, only 41 patients (including 16 with FISH$^+$ tumors and 25 with FISH$^-$ tumors) of this cohort diagnosed in or before 2016 were included when referred to the follow-up time. More detailed clinical information was listed in Table 1 and Table S1.

Another two cohorts of 71 and 127 patients were identified as the validation cohorts for FISH and IHC results, respectively. Diagnostic tumor samples from the cohort of 71 patients were tested by whole exome sequencing (WES) and FISH at the same time, and those from the cohort of 127 patients were tested by MYCN IHC (MYCN antibody: # 51705, Cell Signaling Technology) and FISH at the same time. Because their other clinical information was not involved in this study, we would not further enumerate them.

MYCN gene status tested by FISH

All 53 samples were evaluated *MYCN* amplification status by FISH using 2 μm formalin-fixed, paraffin-embedded (FFPE) sections. Laboratory-developed probes targeting *MYCN* gene (2p24) were used. Tissue sections were washed with SSC buffer and mounted in 4′, 6-diamidino-2-phenylindole for nuclear counterstaining. The results were analyzed and interpreted following the probe manufacturer's instructions. *MYCN* FISH$^+$ at region 2p24 showed red signals (Fig. 1a). If the copy numbers of *MYCN* were ≥ 5 per haploid genome, related patients were classified into the "*MYCN* FISH$^+$" group.

MYCN protein expression tested by IHC

Tumor specimens were fixed in 10% formalin and embedded in paraffin as soon as they were obtained from

Table 1 Key characteristics of the patient cohort

Characteristics	FISH^{-}($n=25$)	FISH^{+}($n=28$)	Total($n=53$)
IHC (MYCN)			
0	20(37.74%)	4(7.55%)	24(45.28%)
1 ~ 8	4(7.55%)	6(11.32%)	10(18.87%)
≥9	1(1.89%)	18(33.96%)	19(35.85%)
Age			
> 18 m	13(24.53%)	19(35.85%)	32(60.38%)
≤18 m	12(22.64%)	9(16.98%)	21(39.62%)
Stage			
Stage 1	5(9.43%)	2(3.77%)	7(13.21%)
Stage 2	2(3.77%)	0(0.0e+ 0%)	2(3.77%)
Stage 3	8(15.10%)	12(22.64%)	20(37.74%)
Stage 4	7(13.21%)	14(26.42%)	21(39.62%)
Stage 4S	3(5.66%)	0(0.0e+ 0%)	3(5.66%)
Risk			
Low	6(11.32%)	0(0.0e+ 0%)	6(11.32%)
Med	7(13.21%)	3(5.66%)	10(18.87%)
High	5(9.43%)	14(26.42%)	19(35.85%)
Very High	7(13.21%)	11(20.75%)	18(33.96%)
Complete primary tumor resection			
No	5(9.43%)	6(11.32%)	11(20.75%)
Yes	20(37.74%)	22(41.51%)	42(79.25%)
Autologous stem cell transplantation			
No	21(39.62%)	26(49.06%)	47(88.68%)
Yes	4(7.55%)	2(3.77%)	6(11.32%)
External Radiotherapy			
No	18(33.96%)	13(24.53%)	31(58.49%)
Yes	7(13.21%)	15(28.30%)	22(41.51%)
Event			
No	17(32.08%)	19(35.85%)	36(67.92%)
Yes	8(15.09%)	9(16.98%)	17(32.08%)
EFS months			
Median [min-max]	58.43[4.70,109.73]	13.52[0.33,74.17]	28.00[0.33,109.73]
Follow up status			
CR	17(32.08%)	21(39.62%)	38(71.70%)
Death	8(15.09%)	7(13.21%)	15(28.30%)
OS months			
Median [min-max]	58.43[5.93,109.73]	16.73[0.33,74.17]	30.47[0.33,109.73]

patients. Pathologists chose specimens with the highest tumor content by H&E staining to navigate tumor pathological heterogenicity. MYCN IHC was performed on the same specimen in which *MYCN* FISH was performed or on a different specimen of the same tumor if that was unavailable. The performance of two anti-MYCN antibodies (#84406 s and # 51705, Cell Signaling Technology) in IHC was compared. Subcutaneous tumors of *MYCN* amplified SK-N-BE(2) cell line were used as positive controls, and subcutaneous tumors of *MYCN* non-amplified SY-5Y cell line as negative controls. Scores for staining intensity were graded on a scale of 0–3 (0 = negative, 1 = weak, 2 = moderate, 3 = strong), while the positive proportion was graded on a scale of 0–4 (0 for 0%, 1 for < 25%, 2 for 25–50%, 3 for 50–75%, 4 for 75–100%). The IHC score was calculated independently by two pathologists blinded to the FISH results, based on the formula that final score = staining intensity * positive proportion.

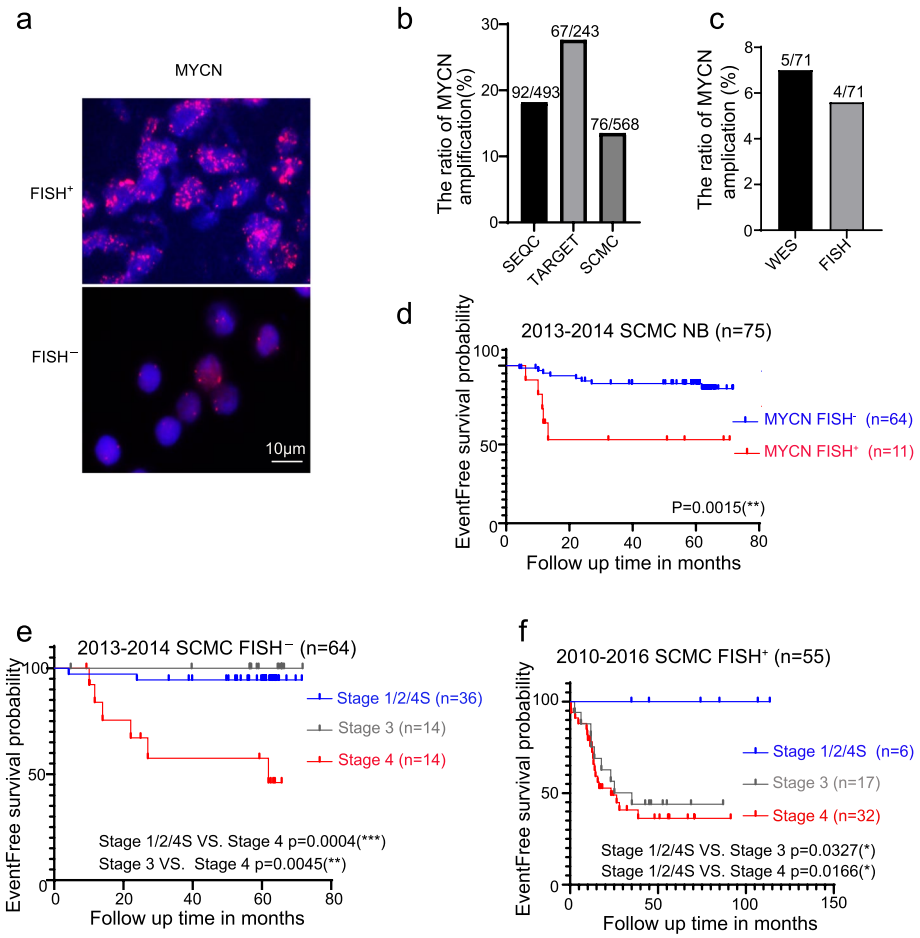

Fig. 1 Identifying *MYCN* amplification status is most valuable for INSS stage 3 patients. **a** Representative *MYCN* FISH images of tumors from *MYCN* FISH$^+$ and FISH$^-$ samples (Red: *MYCN* region probe). **b** The ratio of *MYCN* amplification among SEQC, TARGET, and SCMC Dataset. **c** Comparing *MYCN* amplified rates detected by WES and FISH at SCMC. **d** Survival curve analysis of EFS cut off by *MYCN* amplification status. Log-rank (Mantel-Cox) test was used to generate the *p*-value. **e-f** Survival curve analysis of EFS cut off by INSS stages in *MYCN* FISH$^-$ group (**e**) and *MYCN* FISH$^+$ group (**f**). Log-rank (Mantel-Cox) test was used to generate the p-value

Samples with scores of 0 were defined as "IHC = 0". Samples with scores of 1–9 were classified into the "low expression" group. Beyond that, samples belonged to the "high expression" group.

RNA sequence

Total RNA was extracted and purified using the Qiagen RNeasy Mini kit (Valencia, CA, USA) according to the manufacturer's instructions. The quality of RNA was assessed by a bioanalyzer before sequencing [20]. RNA libraries for RNA-seq were based on TruSeq Stranded Total RNA Gold library by Novaseq S4 PE150 (Illumina). Regrettably, only 14/53 samples detected by MYCN IHC had frozen tumor tissues and had been fully sequenced.

Western blotting (WB)

WB was performed as previously described [21] against the following antibodies: rabbit anti-MYCN antibody (1:1000) (#84406 s, Cell Signaling Technology), mouse anti-β-actin antibody (1:10000) (HF1024, HuaAn).

Statistical analysis

Data were processed with GraphPad Prism 8.0. An unpaired T-test was used to determine the statistical difference between groups. The time of event-free survival (EFS) was calculated from diagnosis until an event such as death, relapse, or progression; if there was no event, the date of last follow-up. The OS time was from diagnosis to death or the date of last follow-up. Kaplan-Meier EFS and OS analyses were performed using GraphPad

Prism 8.0, and comparisons of survival curves were carried out using the log-rank (Mantel-Cox) test. A p-value < 0.05 was considered statistically significant.

Data availability

The accession numbers of RNA-seq data and clinical information reported in this paper were SEQC Dataset (GSE62564) from the GEO website and TARGET Dataset deposited at the cancer genome atlas website.

Results

Identifying *MYCN* amplification status is most valuable for INSS stage 3 patients

As *MYCN* amplification closely correlates with the neoplastic prognosis of neuroblastoma [4], identifying *MYCN* amplification status is greatly important for related patients. The ratios of *MYCN* amplification among the SEQC dataset ($n = 493$), TARGET dataset ($n = 243$) and SCMC dataset ($n = 568$) were compared. Among the results, ratios in the SEQC dataset ($n = 18.7\%$) and TAGET dataset ($n = 27.6\%$) were within the universally acknowledged positive rate [22], while the ratio in the SCMC dataset ($n = 13.4\%$) was far below (Fig. 1b). WES was performed to test the accuracy of SCMC FISH results. The results showed that the positive rates were almost equal between two methods (Fig. 1c), which meant that the FISH results in our hospital were reliable.

Then, patients diagnosed at SCMC during 2013–2014 were further analyzed. The prognosis of patients with *MYCN* FISH$^+$ tumors was poor ($p < 0.05$, Kaplan-Meier survival analysis) (Fig. 1d). By categorizing patients with *MYCN* FISH$^-$ tumors according to INSS stage, we found that the adverse event rate significantly increased when patients progressed to stage 4 (Fig. 1e). However, for patients with *MYCN* FISH$^+$ tumors, their prognosis was poor once they developed to stage 3 (Fig. 1f). These results suggested that stage 1/2 and 4S patients usually had a promising future after rational treatment. If stage 3 patients with *MYCN* FISH$^+$ tumors or stage 4 patients were older than 18 months at first diagnosis, their prognosis

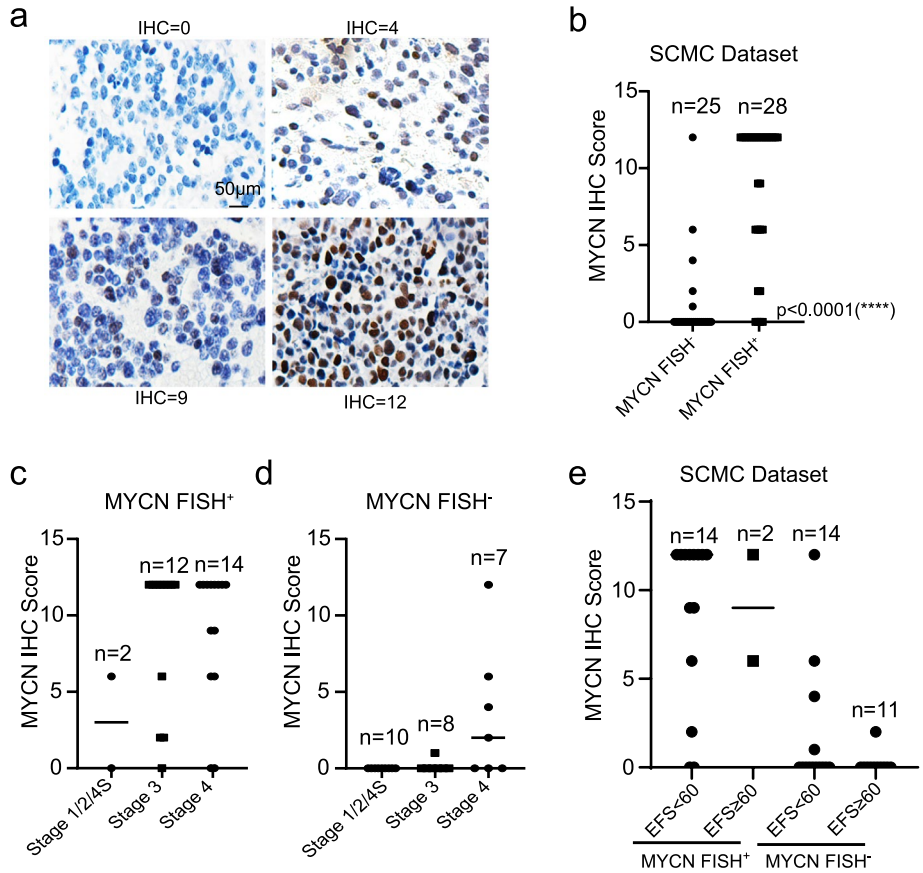

Fig. 2 MYCN IHC showed good specificity and sensitivity. **a** Representative IHC images of tumors with different levels of MYCN protein expression (Immunostaining with hematoxylin counterstaining, Original*40). **b** The concordance of IHC and FISH about MYCN amplification status. An unpaired T-test was used to generate the p-value. **c-e** The IHC score of individuals distributed at different INSS stages and clinical outcomes

Table 2 Inter-assay concordance analysis of MYCN amplification status determined by IHC and FISH in neuroblastoma

FISH	IHC = 0	0 < IHC < 9	IHC ≥ 9	Concordance by FISH	Discordance by FISH
FISH$^-$(n = 25)	20(80.0%)	4(16.0%)	1(4.0%)	80.0%	20.0%
FISH$^+$(n = 28)	4(14.3%)	6(21.4%)	18(64.3%)	85.7%	14.3%

could hardly be sanguine even under the most aggressive treatment. Similar trends were found in database analysis (Supplemental Fig. 1). Overall, the *MYCN* gene test is most valuable for INSS stage 3 patients in predicting prognosis.

MYCN IHC showed good specificity and sensitivity

Whether FISH or WES, both focus on *MYCN* gene status, while protein is the real one performing biological functions. However, there is no reliable antibody that could be clinically useful in IHC. Pathologists at SCMC tried various MYCN antibodies for a long time and finally chose one (MYCN antibody: #51705, Cell Signaling Technology) used in IHC. Between 2010 and 2015, 127 tumor samples were detected MYCN amplification status simultaneously by FISH and IHC (MYCN antibody: #51705, Cell Signaling Technology) at SCMC. Compared with FISH data, IHC results only had 43.1% concordance (50/116) in the *MYCN* FISH$^-$ group and 36.4% concordance (4/11) in the *MYCN* FISH$^+$ group (Table S2), which meant its specificity and sensitivity were substandard. Finding a reliable antibody that could rapidly and accurately detect MYCN protein expression was urgent. An antibody (#84406 s, Cell Signaling Technology), never used in the IHC test before, was chosen.

53 prechemotherapy samples were obtained, and 28 were *MYCN* FISH$^+$ (Table 1). IHC detection revealed that their staining intensity and positive proportion of malignant cells showed a remarkable difference (Fig. 2a). With FISH results as standard, MYCN IHC could accurately detect more than 80% of cases regardless of whether the *MYCN* gene was amplified (Table 2, Fig. 2b). For those cases with inconsistent results, prognostic information was involved to verify which method was more reliable. The results showed that IHC scores increased with INSS stage (Fig. 2c, d). In the *MYCN* FISH$^+$ group, 7/11 patients with high MYCN protein expression (IHC score ≥ 9) had a poor prognosis, whereas 5/5 with no or low (IHC score < 9) recovered well. In the *MYCN* FISH$^-$ group, MYCN protein was not detected in 17/17 event-free patients' tumors, but it was positive for 5/8 patients who died of neuroblastoma (Table 3-4, Fig. 2e). These data suggest that this MYCN antibody (#84406 s) has reasonable specificity and sensitivity.

Table 3 Inter-assay concordance analysis of MYCN protein expression with clinical consequences

NB	FISH$^+$(n = 16)	IHC = 0(n = 2)	0 < IHC < 9(n = 3)	IHC ≥ 9(n = 11)
Event-free	9(56.3%)	2(100.0%)	3(100.0%)	4(36.4%)
Event	7(43.8%)	0(0.0%)	0(0.0%)	7(63.6%)
NB	FISH$^-$(n = 25)	IHC = 0(n = 20)	0 < IHC < 9(n = 4)	IHC ≥ 9(n = 1)
Event-free	17(68.0%)	17(85.0%)	0(0.0%)	0(0.0%)
Event	8(32.0%)	3(15.0%)	4(100.0%)	1(100.0%)

Table 4 Inter-assay concordance analysis of MYCN stability with clinical consequences

NB	FISH$^-$&IHC = 0 (n = 20)	FISH$^-$&IHC > 0 (n = 5)	FISH$^+$&IHC < 9 (n = 5)	FISH$^+$&IHC ≥ 9 (n = 11)
CR	17(85.0%)	0(0.0%)	5(100.0%)	**6(54.5%)**
Death	3(15.0%)	5(100.0%)	0(0.0%)	5(45.5%)
Event-Free	17(85.0%)	0(0%)	5(100%)	**4(36.4%)**
Event	3(15.0%)	5(100%)	0(0%)	7(63.6%)

Conjoint analysis of IHC and FISH provides better prognostic prediction

More analyses were performed to detect the value of MYCN IHC in clinical prediction. The results showed that IHC could further distinguish patients with different clinical outcomes in the *MYCN* FISH$^+$ and FISH$^-$ groups (Fig. 3a-b). If specifying patients only by FISH results, the *MYCN* FISH$^+$ and FISH$^-$ groups' EFS rates were 56.3 and 68.0%, respectively. There was no significant difference ($p > 0.05$, Kaplan-Meier survival analysis). Grouped according to IHC results did better, and their difference was statistically significant ($p < 0.05$, Kaplan-Meier survival analysis). Combining IHC and FISH reached the best predicting effect among the three methods (Fig. 3c). As shown in Fig. 3d and Table 4, patients with *MYCN* FISH$^-$ but protein-expressing tumors always had a poor prognosis. In contrast, patients with *MYCN* FISH$^+$ but low protein expression tumors always had a favorable prognosis (Fig. 3d, Table 4). In summary, our results suggest that IHC could make up for FISH in predicting prognosis.

MYCN protein stability is crucial for the prognosis of neuroblastoma

FISH focused on DNA copy numbers, while IHC centered on protein expression. We wondered what exactly affected MYCN protein expression. Western blotting was performed to test MYCN protein expression (Fig. 4a). As predicted, their protein level varied from DNA copy numbers. For instance, the #3 tissue detected no protein expression but high DNA copy numbers. Based on RNA-seq data, we found that their *MYCN* mRNA expression

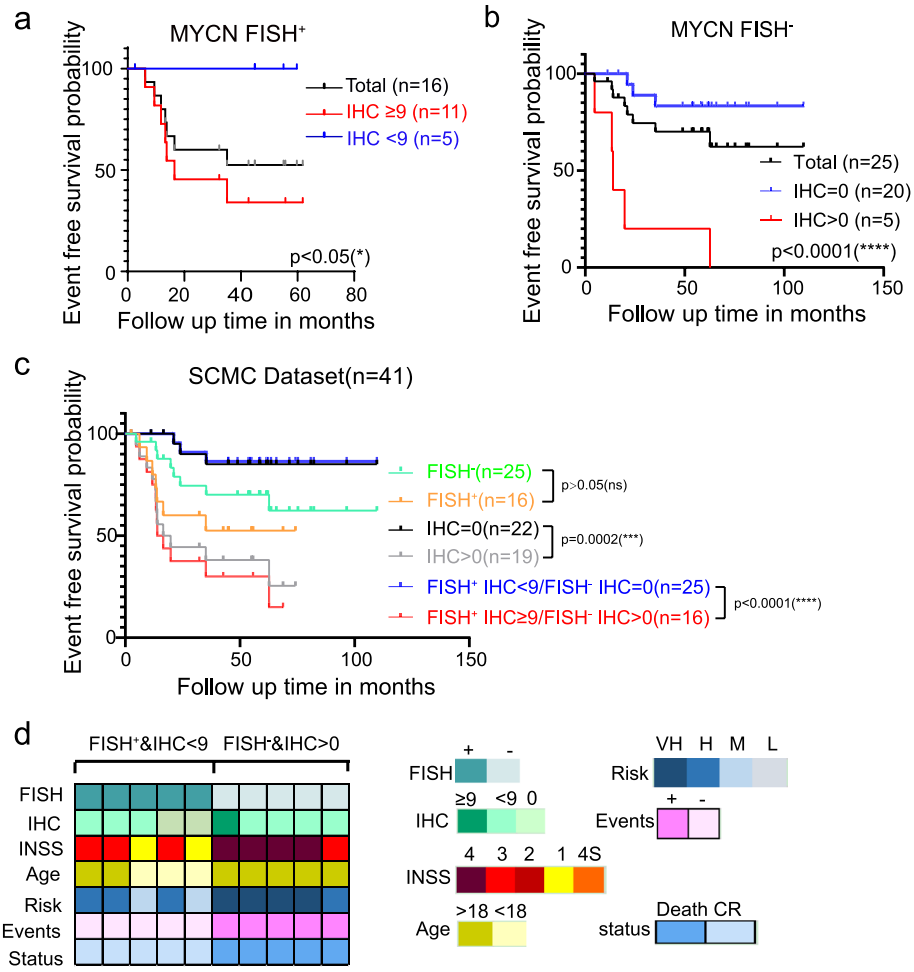

Fig. 3 Conjoint analysis of IHC and FISH provides better prognostic prediction. **a-b** Survival curve analysis of EFS cut off by IHC score in the *MYCN* FISH$^+$ group ($n = 16$) (**a**) and *MYCN* FISH$^-$ group ($n = 25$) (**b**). Log-rank (Mantel-Cox) test was used to generate the p-value. To ensure prognostic accuracy for individuals, only 41 patients (including 16 with *MYCN* FISH$^+$ tumors and 25 with *MYCN* FISH$^-$ tumors) diagnosed in or before 2016 were included from the cohort of 53 patients. **c** Survival curve analysis of EFS cut off by IHC and FISH results ($n = 41$). Log-rank (Mantel-Cox) test was used to generate the p-value. Sample selection was the same with 3a-b. **d** The chromaticity diagram of several significant clinical factors

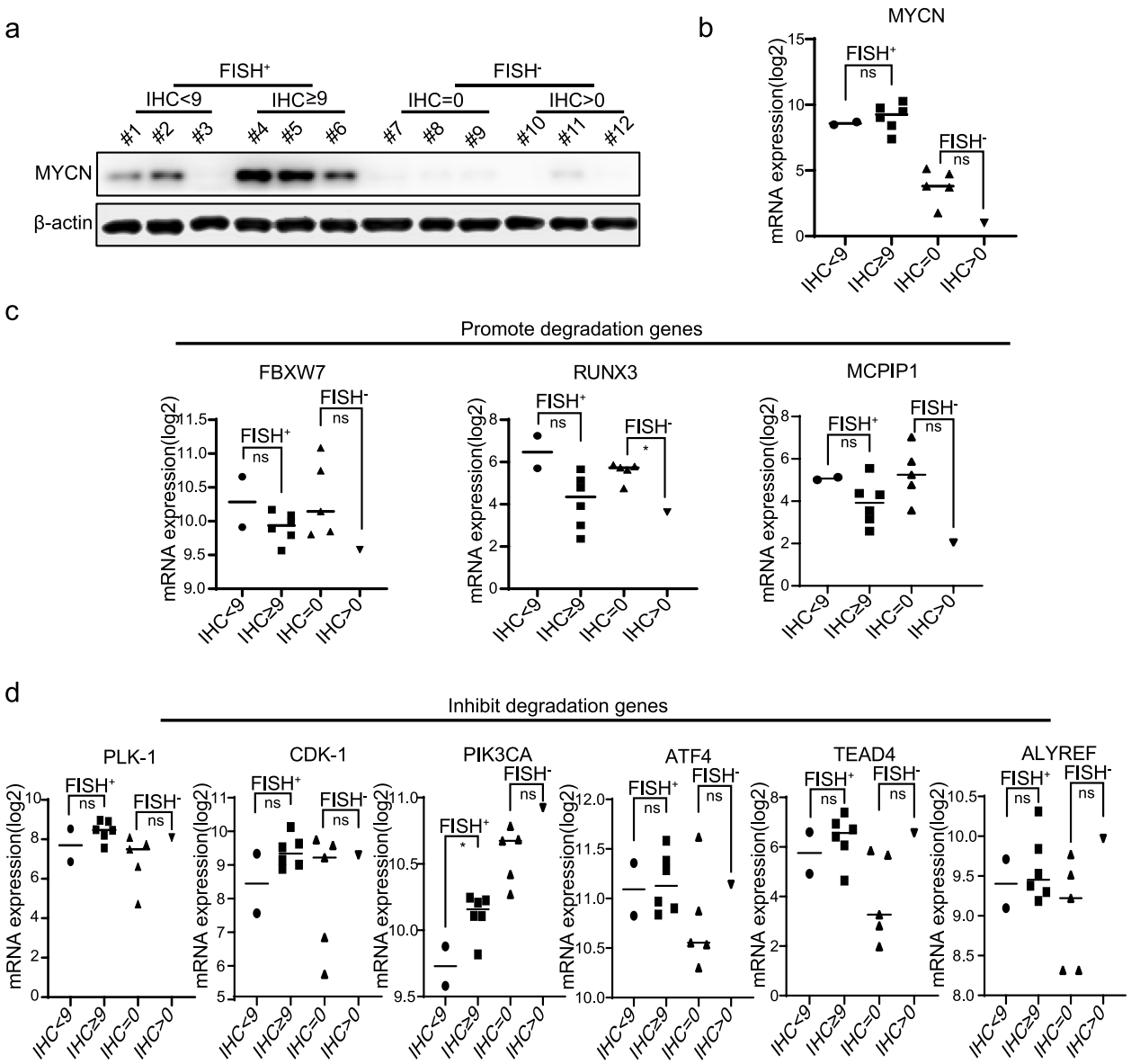

Fig. 4 MYCN protein stability is crucial for the prognosis of neuroblastoma. **a** The MYCN protein expression tested by western blotting among 12 samples at SCMC. **b-d** The mRNA expression of *MYCN* and genes related to MYCN protein expression grouped by MYCN FISH and IHC results. An unpaired T-test was used to generate the p-value (* $p < 0.05$, ns: no significant)

was more consistent with the FISH results (Fig. 4b). These data demonstrated that aberrant protein expression might not stem from DNA copy numbers but be related to RNA translation or protein stability. Previous studies have shown that FBW7 [23], RUNX3 [24], and MCPIP1 [25] play a fundamental role in MYCN ubiquitination and degradation, and all these genes showed declining trends in the high MYCN protein expression group (Fig. 4c) (ns: no significant, unpaired T-test). PLK-1 [26], CDK-1 [27], PIK3CA [28], ATF4 [23], TEAD4 [29] and ALYREF [30] could enhance MYCN protein stability and sustain MYCN expression in neuroblastoma. The expression of these genes showed a rising trend in the high MYCN protein expression group (Fig. 4d) (* $p < 0.05$, ns: no significant, unpaired T-test). More factors that could affect MYCN protein expression were analyzed. The results showed that their expression was more consistent with the MYCN protein level detected by IHC (Fig. S2). Overall, some genes could influence MYCN protein expression through post-transcriptional or post-translational

modification. RNA-seq revealed that their expression changed among different samples, which might be the reason why the results of MYCN IHC could be different from those of FISH. Conjoint analysis of IHC and FISH could test MYCN protein stability, a key prognostic factor.

Discussion

MYCN is an identified driver and reliable genomic hallmark of aggressive tumor behavior [16]. Detecting *MYCN* amplification status has great significance for clinical treatment and prognostic prediction. This article clarified that identifying *MYCN* amplification status is most valuable for INSS stage 3 patients. MYCN stability detected by the joint of IHC and FISH is a good choice to predict the prognosis of neuroblastoma.

In clinical management, *MYCN* amplification status is a metric used to identify risk groups and determine chemotherapy regimens. FISH focuses on the *MYCN* DNA level; its result is regarded as the "gold standard" in clinical practice [11, 31]. Patients with *MYCN* $FISH^+$ tumors would be classified into a higher risk group and given a higher dose of chemotherapy (Table S3). An ultrasensitive quantitative RNA in situ hybridization technique, RNA scope, is emerging [32]. This method investigates *MYCN* amplification status at the RNA level. Compared with CISH at the DNA level, it better predicts prognosis [32]. It is reasonable to suspect that identifying MYCN protein levels is the best choice to assess prognosis among the above.

However, most hospitals have to give up MYCN IHC for poor antibodies, so there is little research about the correlation between *MYCN* amplification status and protein expression. MYCN antibodies from Santa Cruz Biotechnology Inc. [11] or Abcam [17–19] are several antibodies mentioned in research, but high false-positive and false-negative rates were observed in IHC using the antibodies mentioned above in our hospital. In this study, we extended the application of a commercial MYCN antibody in IHC. Both specificity and sensitivity of this antibody showed bright futures. Compared with before, the accuracy of IHC was vastly improved. In addition, experimental results would be less affected by human factors with the application of IHC autostainer. For neuroblastoma patients, especially those without *MYCN* amplification but with abnormal protein expression, MYCN IHC has great clinical value. A joint of IHC and FISH could obtain a more complete understanding of MYCN expression level. It could reduce the shortcoming of any single method and obtain a better predictive effect. However, our research is limited by relatively small samples; we need more high-quality randomized controlled trials to provide more evidence.

In addition to *MYCN* gene status, age of diagnosis is an important indicator of risk stratification and is closely related to prognosis. Children younger than 12 months at first diagnosis may be classified to stage 4S, and they might resolve themselves whether the *MYCN* gene is amplified or not. The elucidation of the intrinsic mechanisms of stage 4S patients with a good prognosis could offer new ideas to cure neuroblastoma.

In summary, MYCN is a vital index influencing neuroblastoma prognosis. The combination of IHC and FISH to determine MYCN stability could potentially be of greater importance as prognostic indicators for patients diagnosed with neuroblastoma compared to singular factors.

Abbreviations

FISH: Fluorescence in situ hybridization; OS: Overall survival; WES: Whole exome sequencing; PCR: Polymerase chain reaction; qPCR: Quantitative real-time PCR; SQ-PCR: Semi-quantitative differential PCR; ddPCR: Droplet digital PCR; CISH: Chromogenic in situ hybridization; MLPA: Multiplex ligation-dependent probe amplification; IHC: Immunohistochemistry; FFPE: Formalin-fixed, paraffin-embedded; VCR: Vincristine; CTX: Cyclophosphamide; CBP: Carboplatin; VP-16: Etoposide; Adr: Adriamycin; CDDP: Cisplatin; IFOS: Ifosfamide; THP: Pirarubicin; Topo: Topotecan; DOXO: Doxorubicin.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12887-022-03449-1.

Additional file 1: Supplemental Fig. 1. a-d Survival curve analysis of EFS (a-b) and OS(c-d) when *MYCN* amplification (b,d) or not (a,c). Log-rank (Mantel-Cox) test was used to generate the *p*-value.

Additional file 2: Supplemental Fig. 2. a Heat map grouped by MYCN FISH and IHC results. The mRNA expression of *MYCN* and genes related to MYCN protein stability were shown.

Additional file 3.

Additional file 4.

Additional file 5.

Acknowledgments

Not applicable.

Authors' contributions

This study was conceived by YL, JT, MY and YG; YY, JZ, YZ and TF performed the experiments; JW and BY analyzed and interpreted the data; YY wrote the paper with comments from all authors. All authors read and approved the final manuscript.

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Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Declarations

Ethics approval and consent to participate
The study was approved and supervised by the SCMC Ethics Committee (SCMCIRB-K2014050), according to the Declaration of Helsinki. All subjects provided written consent for banking of tissue and future research use of the samples, following the regulations of the institutional review board of SCMC. The patient provided written informed consent before participating in any study-specific procedures.

Consent for publication
Individuals may consent to participate in a study, but object to having their data published in a journal article. Authors should make sure to also seek consent from individuals to publish their data prior to submitting their paper to a journal. This is in particular applicable to case studies. A consent to publish form can be found.

Competing interests
The authors declare no conflicts of interest

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References

1. Fetahu IS, Taschner-Mandl S. Neuroblastoma and the epigenome. Cancer Metastasis Rev. 2021;40(1):173.
2. Zafar A, Wang W, Liu G, Wang X, Xian W, McKeon F, et al. Molecular targeting therapies for neuroblastoma: Progress and challenges. Med Res Rev. 2021;41(2):961.
3. Ladenstein R, Pötschger U, Valteau-Couanet D, Luksch R, Castel V, Yaniv I, et al. Interleukin 2 with anti-GD2 antibody ch14.18/CHO (dinutuximab beta) in patients with high-risk neuroblastoma (HR-NBL1/SIOPEN): a multicentre, randomised, phase 3 trial. Lancet Oncol. 2018;19(12):1617.
4. Otte J, Dyberg C, Pepich A, Johnsen JI. MYCN Function in Neuroblastoma Development. Front Oncol. 2020;10:624079.
5. Liu R, Shi P, Wang Z, Yuan C, Cui H. Molecular Mechanisms of MYCN Dysregulation in Cancers. Front Oncol. 2020;10:625332.
6. Nakagawara A, Li Y, Izumi H, Muramori K, Inada H, Nishi M. Neuroblastoma. Japan J Clin Oncol. 2018;48(3):214.
7. Ma Y, Lee JW, Park SJ, Yi ES, Choi YB, Yoo KH, et al. Detection of MYCN Amplification in Serum DNA Using Conventional Polymerase Chain Reaction. J Korean Med Sci. 2016;31(9):1392.
8. Feng C, Tang S-Q, Wang J-W, Liu L-Z, Gao X-N, Long H. Detection of MYCN mRNA in neuroblastoma cell lines by quantitative RT-PCR. Zhongguo dang dai er ke za zhi = Chinese journal of contemporary pediatrics. 2007;9(1):47.
9. Kojima M, Hiyama E, Fukuba I, Yamaoka E, Ueda Y, Onitake Y, et al. Detection of MYCN amplification using blood plasma: noninvasive therapy evaluation and prediction of prognosis in neuroblastoma. Pediatr Surg Int. 2013;29(11):1139.
10. Souza AC, Souza DR, Sanabani SS, Giorgi RR, Bendit I. The performance of semi-quantitative differential PCR is similar to that of real-time PCR for the detection of the MYCN gene in neuroblastomas. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas. 2009;42(9):791.
11. Somasundaram DB, Aravindan S, Yu Z, Jayaraman M, Tran NTB, Li S, et al. Droplet digital PCR as an alternative to FISH for MYCN amplification detection in human neuroblastoma FFPE samples. BMC Cancer. 2019;19(1):106.
12. 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.
13. Bhargava R, Oppenheimer O, Gerald W, Jhanwar SC, Chen B. Identification of MYCN gene amplification in neuroblastoma using chromogenic in situ hybridization (CISH): an alternative and practical method. Diagn Mol Pathol. 2005;14(2):72.
14. Costa RA, Seuánez HN. Investigation of major genetic alterations in neuroblastoma. Mol Biol Rep. 2018;45(3):287.
15. Pinto NR, Applebaum MA, Volchenboum SL, Matthay KK, London WB, Ambros PF, et al. Advances in Risk Classification and Treatment Strategies for Neuroblastoma. J Clin Oncol. 2015;33(27):3008.
16. Chan HS, Gallie BL, DeBoer G, Haddad G, Ikegaki N, Dimitroulakos J, et al. MYCN protein expression as a predictor of neuroblastoma prognosis. Clin Cancer Res. 1997;3(10):1699.
17. Niemas-Teshiba R, Matsuno R, Wang LL, Tang XX, Chiu B, Zeki J, et al. MYC-family protein overexpression and prominent nucleolar formation represent prognostic indicators and potential therapeutic targets for aggressive high-MKI neuroblastomas: a report from the children's oncology group. Oncotarget. 2018;9(5):6416.
18. 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.
19. Wang LL, Teshiba R, Ikegaki N, Tang XX, Naranjo A, London WB, et al. Augmented expression of MYC and/or MYCN protein defines highly aggressive MYC-driven neuroblastoma: a Children's Oncology Group study. Br J Cancer. 2015;113(1):57.
20. Lv D, Li Y, Zhang W, Alvarez AA, Song L, Tang J, et al. TRIM24 is an oncogenic transcriptional co-activator of STAT3 in glioblastoma. Nat Commun. 2017;8(1):1454.
21. Zhang L, Zhang W, Li Y, Alvarez A, Li Z, Wang Y, et al. SHP-2-upregulated ZEB1 is important for PDGFRα-driven glioma epithelial-mesenchymal transition and invasion in mice and humans. Oncogene. 2016;35(43):5641.
22. Cheung N-KV, Dyer MA. Neuroblastoma: developmental biology, cancer genomics and immunotherapy. Nat Rev Cancer. 2013;13(6):397.
23. Xia Y, Ye B, Ding J, Yu Y, Alptekin A, Thangaraju M, et al. Metabolic Reprogramming by MYCN Confers Dependence on the Serine-Glycine-One-Carbon Biosynthetic Pathway. Cancer Res. 2019;79(15):3837.
24. Yu F, Gao W, Yokochi T, Suenaga Y, Ando K, Ohira M, et al. RUNX3 interacts with MYCN and facilitates protein degradation in neuroblastoma. Oncogene. 2014;33(20):2601.
25. Boratyn E, Nowak I, Durbas M, Horwacik I, Sawicka A, Rokita H. MCPIP1 Exogenous Overexpression Inhibits Pathways Regulating MYCN Oncoprotein Stability in Neuroblastoma. J Cellu Biochem. 2017;118(7):1741.
26. Xiao D, Yue M, Su H, Ren P, Jiang J, Li F, et al. Polo-like Kinase-1 Regulates Myc Stabilization and Activates a Feedforward Circuit Promoting Tumor Cell Survival. Mol Cell. 2016;64(3):493.
27. Chen Y, Tsai YH, Tseng SH. Inhibition of cyclin-dependent kinase 1-induced cell death in neuroblastoma cells through the microRNA-34a-MYCN-survivin pathway. Surgery. 2013;153(1):4.
28. Mohlin S, Hansson K, Radke K, Martinez S, Blanco-Apiricio C, Garcia-Ruiz C, et al. Anti-tumor effects of PIM/PI3K/mTOR triple kinase inhibitor IBL-302 in neuroblastoma. EMBO Mol Med. 2019;11(8):e10058.
29. Rajbhandari P, Lopez G, Capdevila C, Salvatori B, Yu J, Rodriguez-Barrueco R, et al. Cross-Cohort Analysis Identifies a TEAD4-MYCN Positive Feedback Loop as the Core Regulatory Element of High-Risk Neuroblastoma. Cancer Discov. 2018;8(5):582.
30. Nagy Z, Seneviratne JA, Kanikevich M, Chang W, Mayoh C, Venkat P, et al. An ALYREF-MYCN coactivator complex drives neuroblastoma

tumorigenesis through effects on USP3 and MYCN stability. Nat Commun. 2021;12(1):1881.
31. 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 (New York, NY). 2001;3(2):105.
32. Chang H-H, Tseng Y-F, Lu M-Y, Yang Y-L, Chou S-W, Lin D-T, et al. MYCN RNA levels determined by quantitative in situ hybridization is better than MYCN gene dosages in predicting the prognosis of neuroblastoma patients. Modern Pathol. 2020;33(4):531.
33. Monclair T, Brodeur GM, Ambros PF, Brisse HJ, Cecchetto G, Holmes K, et al. The International Neuroblastoma Risk Group (INRG) staging system: an INRG Task Force report. J Clin Oncol. 2009;27(2):298.
34. Matthay KK, Maris JM, Schleiermacher G, Nakagawara A, Mackall CL, Diller L, et al. Neuroblastoma. Nat Rev Dis Prim. 2016;2:16078.

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