MYCN Fish combined with immunohistochemistry is a more prognostic factor for neuroblastoma

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Research

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Abstract

Background: MYCN is an identified driver and important prognosticator of neuroblastoma and performs biological function at protein level. At present, most detection methodologies focus on MYCN DNA level and fail to detect MYCN protein expression for poor antibody, especially in China.

Results In the present study, through the analysis of neuroblastoma gene expression profile data, we found MYCN expression is closely related with prognosis of neuroblastoma, especially in stage I-III and IVs at RNA level in current medical conditions. FISH was almost accurate as WES to test MYCN DNA level based on our hospital data. In 56 neuroblastoma cases, IHC analysis by a newfound antibody (#84406s, Cell Signaling Technology) demonstrated a 75%-85% concordance with FISH. Further analyzing those cases with inconsistent results, we found patients with FISH⁺ but IHC score<9 all had no adverse events, while 63.6% patients with FISH⁺ and IHC score≥9 tended to have poor outcome. As to those MYCN FISH⁻ patients, once their tumor showed MYCN protein expression, all of them were poor prognosis, while 81% of the rest patients with neither MYCN gene nor protein expression had ideal prognosis.

Conclusions: The results suggest that this newfound antibody is reliable in IHC, and IHC combined with FISH performs better in evaluating prognosis and guiding treatment.

Highlights

1 MYCN as a biomarker is more correctly with prognosis of neuroblastoma in stage I-III and IVs than in stage IV at current medical condition.

2 FISH is almost accurate as WES to test MYCN DNA level. MYCN protein level is more relevant with prognosis of neuroblastoma than DNA level.

3 We found an MYCN antibody that can perform consistently and reliable at clinical outcome prediction and make up for FISH in DNA level at the same time.

Background

Neuroblastoma is the most common extra cranial solid tumor of children(Brodeur et al. 2003, Maris, Hogarty, Bagatell and Cohn et al. 2007), which is responsible for about 12% pediatric cancer mortality(Ladenstein et al. 2018). It arises from the developing neural crest, therefore tumor can develop anywhere of sympathetic nervous(Maris, Hogarty, Bagatell and Cohn et al. 2007, Westermarck, Wilhelm, Frenzel and Henriksson et al. 2011). For patients, both diverse clinical symptoms and definite diagnosis start manifesting from the first year of life(Pottoo et al. 2019). As a highly heterogeneous disease, some tumors have the ability of differentiation and spontaneous regression, while some are highly malignant and demonstrate local invasion as well as distant spread(Westermarck, Wilhelm, Frenzel and Henriksson et al. 2011, Pinto et al. 2015). It is important to find the prognostic factors and establish an accurate identification technology to guide the neuroblastoma treatment.

MYCN amplification, which occurs in about 22% neuroblastoma cases, is the first neuroblastoma genetic markers for its significant clinical and prognostic value. Current guidelines for the assignment of risk group used by the International Neuroblastoma Risk Group (INRG) and the Children' Oncology Group (COG) heavily weigh MYCN amplification. Till now, for no reliable MYCN antibody, a number of methodologies used in clinical and laboratory tests are confined to nucleic acid level, such as conventional polymerase chain reaction (PCR)(Ma et al. 2016) , quantitative real-time PCR(q RT-PCR)(Feng et al. 2007, Kojima et al. 2013), semi-quantitative differential PCR (SQ-PCR)(Souza, Souza, Sanabani, Giorgi and Bendit et al. 2009), droplet digital PCR (ddPCR)(Somasundaram et al. 2019), fluorescence in situ hybridization (FISH)(Somasundaram et al. 2019)chromogenic in situ hybridization(CISH)(Bhargava, Oppenheimer, Gerald, Jhanwar and Chen et al. 2005), multiplex ligation-dependent probe amplification (MLPA)(Vicha and Eckschlager et al. 2008)and so on. MYCN gene status tested by FISH is integrated into the risk classification presently(Pinto et al. 2015). However, several studies have identified that MYCN protein could be isolated from cases without gene amplification and patients with MYCN gene amplification could not express

protein(Chan et al. 1997, Niemas-Teshiba et al. 2018, Santiago et al. 2019). In fact, it is MYCN protein that exerts its biologic effects(Wang et al. 2015), so it is of great significance to find a rapid, reliable, and cost-effective strategy to detect MYCN protein expression.

In this study, we found a MYCN antibody. Then we performed comparative analysis and survival analysis to verify the feasibility, reliability, and accuracy of this MYCN commercial antibody with FISH. Our results found that this newfound antibody is reliable in IHC, and IHC combined with Fish performs better in evaluating prognosis and guiding treatment.

Results

High MYCN in neuroblastomas predicts poor prognosis by RNA sequence

To complement previous studies about the significance of MYCN in neuroblastoma, we analyzed the human neuroblastoma SEQC dataset including 498 patient samples with prognosis information, published previously and downloaded from the Gene Expression Omnibus website of the National Center for Biotechnology Information (Super Series GSE62564). Analysis of the dataset showed that the high levels of MYCN mRNA expression in neuroblastoma tissues were associated with both poor OS and EFS ($P<0.0001$, Kaplan-Meier survival analysis) (Fig. 1a). Importantly, high levels of MYCN mRNA expression in the 315 stage I-III and IVs neuroblastoma tissues ($n=315$) played an even more adverse role, especially on OS, compared with stage IV neuroblastoma tissues ($n=183$) (Fig. 1b, c). Furthermore, boxplot analysis showed the worse international neuroblastoma staging system (INSS) stage was, the higher MYCN mRNA expression showed (Fig, 1d). In addition, the mRNA expression of MYCN was also related with the risk of tumor metastasis and the rate of long-term survival (Fig 1e, f). All these analysis were consistent with previous reports that amplification of the MYCN oncogene was one of the most powerful predictors of outcome(Westermark, Wilhelm, Frenzel and Henriksson et al. 2011), and they also suggested that compared with stage IV patients, the expression of MYCN had a greater negative impact on other INSS stages in current clinical condition.

Identifying MYCN status is most valuable for INSS stage III to predict prognosis

As MYCN amplification directly correlates with the clinical course of neuroblastoma and poor patient survival, diagnosis of MYCN status in neuroblastoma is of great benefit for related patients. To date, FISH result is regarded as “the gold standard”(Somasundaram et al. 2019). In our hospital, we also used FISH to detect MYCN gene amplification. If the copy number was ≥ 5 per haploid genome, the gene was considered to be amplified (Fig.2c) and related patients would be classified as higher risk and given higher dose of chemotherapy (Table S2). We compared the ratio of MYCN amplification by FISH among SEQC dataset ($n=498$), TARGET dataset ($n=243$) and SCMC dataset ($n=108$). Results showed that the amplification of MYCN was 18.3% in SEQC dataset and 27.6% in TAGET dataset, which was about equal to 22%, a universally acknowledged positive rate(Cheung and Dyer et al. 2013) (Fig. 2a). While the positive rate, 8%, in SCMC dataset was far below than those data (Fig. 2a). To identify the FISH results in our hospital was reliable, we conducted the whole genome sequencing (WES). The results showed the detection rate was almost same between two methods (Fig. 2b), which meant the FISH result in our hospital could reflect reality. Then, we choose all SCMC neuroblastoma patients diagnosed during 2013-2014 to identify the role of MYCN gene status for EFS (Fig.2d). Results like before, the MYCN-amplified groups had a worse clinical outcome ($P<0.0001$, Kaplan-Meier survival analysis). Focusing on MYCN non amplified patients and further categorized them according to INSS stage, we found that the risk of poor outcome was greatly increased only when patients were progressed into stage IV (Fig 2e). However, for MYCN-amplified groups, once patients were progressed into high risk group (including stage III and stage IV), they would be expected to be associated with poor outcome (Fig 2f). The results suggested that stage I/II and IVs patients always showed a good outcomes after SCMC treatment, but for stage III Fish⁺ and stage all IV patients, if they were elder than 18 months at diagnosis, they tended to have poor prognosis even with the most aggressive treatment. Similar trends results were found in database analysis (Supplemental Fig. 1). All these results showed that verifying MYCN status is most valuable for stage III patients to predict clinical outcome, and MYCN status is the key factor to guide treatment at I, II and IVs stage.

Inconsistent of MYCN status among DNA, RNA, and protein level.

No matter FISH or WES, these two methods focus on MYCN gene status, while protein is the real one to perform biological functions and DNA transcribed into RNA which then translated into proteins is a quite long physiological process and undergoes various transcriptional regulation and post-translational modifications. We analysis the relation between MYCN mRNA expression and DNA copy number from TARGET dataset (n=54) (Fig.3a-c). Results showed that there were some cases with low DNA copy number but high RNA expression while some high DNA copy but low RNA expression (Fig. 3a,3c). The inconsistent ratio could be up to 5.9% (Fig.3b-c). To further verify this phenomenon, we chose 12 neuroblastoma samples (6 were MYCN amplification tested by FISH, 6 MYCN non-amplification) to test their MYCN expression at DNA and protein level, respectively (Fig 3d-f). Like results found by database analysis, the same patient's tissue could show totally different MYCN expression among different molecular biology level. For example, the #3 tissue showed high DNA copy but without protein expression and #4 tissue low DNA copy but high protein expression. We also used IHC to assay MYCN protein expression which got the consistent result with western blot in most cases (Fig 3g). Results showed that IHC assay could semi-quantitatively reflect MYCN protein status, and this method is more convenient and applicable to advocate clinically than western blot.

IHC combined with Fish provide more accurate prognosis of neuroblastoma

Between 2010 to2015, 127 patients were enrolled and detected MYCN status by FISH and IHC (MYCN antibody# 51705, Cell Signaling Technology) at same time in our hospital. Compared with FISH data, IHC results only had 43.1% concordance (50/116) for non-amplified samples and 36.4% concordance (4/11) for amplified samples (Table S1). As to the antibody we used in this experiment (#84406s, Cell Signaling Technology), it should be applied to more cases to test its detection rate. We obtained 56 pre-chemotherapy samples from SCMC and assessed their MYCN expression by IHC, half of which had been confirmed MYCN gene amplified status by FISH before (Table 1). Results showed their predominant staining intensity and positive proportion of malignant cells showed great difference (Fig. 4a). Based on these two points, we give them a composite IHC score (Fig. 4a). Concordance between FISH and IHC had been shown in Table 2. More than 75% cases with negative or positive FISH results could be identified by IHC (Table2, Fig.4b). As to those with inconsistent results, we took prognostic information into account to verify which method was more reliable (To ensure prognostic accuracy for individuals, we only involved patients diagnosed in 2016 or before 2016 when taking into account the follow-up time). Results showed that IHC score increased with INSS stages (Fig. 4c, 4d). In MYCN gene amplified group, 7 of the 11 patients with high MYCN protein expression (IHC score $\geq$ 9) had a poor prognosis, whereas 5 of the 5 patients with no or low protein expression recovered well. As to MYCN gene non-amplified patients, 17 of 17 patients with good prognosis all showed no expression of MYCN protein, while 6 of the 10 patients who eventually died of disease showed the different expression of MYCN protein (Table2-3). By further refining the grouping conditions, we found IHC results had 100% concordance (11/11) for non-amplified samples with 60-month EFS at least (Fig.4e), while 6 of 10 samples from those non-amplified but with event patients showed low MYCN protein expression (Table 3-4). As to some amplified patients with good prognosis, their tumor tissue expressed low or undetectable MYCN protein (Fig. 4c, 4e, Table 3-4). All these data suggested this MYCN antibody (#84406s) show a good specificity and affinity.

With a reliable antibody, IHC could provide correct clinical prognosis. To prove this, we conducted Kaplan-Meier survival analysis. We found that IHC results could further distinguish different clinical outcomes among MYCN-amplified or non-amplified patients (Fig. 4f, 4g), For example, if we only specified groups by FISH result, the event-free rates of MYCN gene amplified group and non-amplified group was 56.2% and 63.0%, respectively and the Kaplan-Meier survival analysis showed these two group has no statistic difference ($P>0.05$). Grouped according to IHC results did better in this respect and survival analysis of two group had statistical significance ($P<0.05$) (Fig.4h). Combing two method "Fish+ IHC score <0 and Fish- IHC=0 group vs Fish+ IHC $\geq$ 9 and Fish- IHC >0 group"could reach the best producing effect (Fig.4h). At present, though FISH result is regarded as an important standard to direct risk group and clinical treatment, once patients develop to stage III/IV, MYCN FISH result lost this indication role if their ages are elder than 18 months at diagnosis (Table S2). Its protein expression level, however, could be a good indicator of clinical outcome, especially with those MYCN FISH negative patients (Fig. 4i).In summary, our results suggested that this MYCN antibody is reliable in IHC, and IHC can make up for Fish in evaluating prognosis and guiding treatment.

Discussion

MYCN is an identified driver and important prognosticator of neuroblastoma(Khan et al. 2020, Weiss, Aldape, Mohapatra, Feuerstein and Bishop et al. 1997). Identifying the MYCN status has great clinical significance for treatment and prediction of outcome. In this article, we further clarify that MYCN plays a more adverse role in stage III based on SCMC data. We also justify the inconsistent of MYCN status among DNA, RNA, and protein level. IHC and FISH combining approach is a better choice to predict prognosis in neuroblastoma.

In clinical, most of NB patients would test MYCN status to guide risk group and determine chemotherapy regimen. FISH, the most widely used method in clinical diagnosis right now, focuses on DNA level to detect MYCN status(Mathew et al. 2001). But this method is technically demanding and expensive. Using RT-qPCR or conventional PCR to detect MYCN amplification in plasma/serum circulating-free DNA seems to be a simple and non-invasive approach, but it still focuses on DNA level and its accuracy of result is needed to be confirmed(Trigg, Turner, Shaw and Jahangiri et al. 2020, Gotoh et al. 2005). Now, an ultrasensitive quantitative RNA in situ hybridization technique, RNA scope, is applied(Chang et al. 2020). Compared with PCR, this method investigates the MYCN status at RNA level and establishes that high expression of MYCN RNA does better at predicting poor prognosis than by CISH at DNA level(Chang et al. 2020). All this method could be used to detect MYCN status, but all fail to identify MYCN protein expression, the real one to perform biological functions. IHC, as a simple and rapid way for detecting proteins expression, is worth of popularizing and applying if there is a reliable antibody, especially with the general use of automated IHC assay. At present, most hospital are forced to give up MYCN IHC for poor antibody, so that researches about correlation between MYCN gene status and MYCN protein expression is very few. MYCN antibody from Santa Cruz Biotechnology Inc(Somasundaram et al. 2019) or Abcam(Niemas-Teshiba et al. 2018, Santiago et al. 2019, Wang et al. 2015) are a few mentioned and used in research, but these two antibody both showed a quite high rate of false positive and false negative after a large amount of experiments in our hospital. By this study, we found a new antibody to detect MYCN protein expression by IHC. For neuroblastoma patients, especially for patients without MYCN gene amplification but protein expression, it has great values of clinical application. Significantly, we still need a large number of high quality randomized controlled trail research to provide more evidence.

During this study, we also observed that several cases with over expressed of MYCN both at DNA and protein level still show a good prognosis in good clinical condition. This means there were some else genes which could antagonize the function of MYCN and finally improve clinical outcome. For example, experiments have shown that TAp63, an isoform of p63 gene from p53 family, is highly correlated with inhibition of MYCN gene transcription and its high levels of expression indicate favorable outcome in neuroblastoma(Suenaga et al. 2019). Several genes, like RUNX3 (RUNX family transcription factor 3)(Yu et al. 2014), GRHL1 (Grainyhead-like 1)(Fabian et al. 2014) seems to overcome the aggressive behavior of MYCN. Focus on these patients might help us to find some newer and really core neuroblastoma suppressor genes and make the best therapeutic method in clinic.

Conclusions

In summary, MYCN is an important index for determining neuroblastoma prognosis. Using IHC combined Fish is a more prognostic factor for neuroblastoma.

Materials And Methods

The clinicals

From 2010 to 2019, there were about 346 pre-therapy neuroblastoma paraffin embedded tissue from 323 patients, including 41 MYCN amplified (FISH⁺) patients in our hospital (Shanghai Children's Medical Center, SCMC). Due to severe disruption of tumor tissue for a long time or too little tumor cells to detect protein expression, only 28 MYCN amplified patient's neuroblastoma tissue were involved in this trial. As a control, we also selected 28 MYCN non-amplified neuroblastoma tissue, including all patients (11/323) who died eventually and 17 patients with no event until follow-up time. All tumors were histologically

examined and classified. All patients were no elder than 12 years old at the time of diagnosis. 30 patients were boys and 26 were girls. The age, gender, clinical stage, risk group, primary sites, infiltrating sites, distant transference status, MYCN status tested by FISH, MYCN IHC score, therapy protocol, course of chemotherapy, prognosis, current status, and duration of follow-up from diagnosis are listed in Table 1.

The 108 samples tested by the whole exome sequencing (WES) were obtained from 2002 to 2016 at SCMC. 71 of them all tested by FISH at the same time. All these patients had a 3-year overall survival (OS) at least or had been dead within 3 years from diagnosis.

DNA Analysis of MYCN

In this part, we mainly selected 12 samples based on the MYCN gene status, protein expression and individual's outcome. 6 samples were with MYCN gene amplification but half of whom were with low protein expression (MYCN IHC score<9), 6 were with no MYCN gene amplification but half of whom were dead within 60 months from diagnosis. All tumor samples obtained at operation were stored at -80°C until use. Tumor tissues were lysed by 100-150µl solution A (0.05g NaOH, 0.0038g EDTA and ddH₂O to 500ml in total) at 100°C about 1 hour. When the solution cooled down to room temperature, the same volume of solution B (0.24228g Tris and ddH₂O to 50ml in total, pH 5.5) was added together and mixed sufficiently. Genomic DNA supernatant was collected after the solution were centrifuged at 4000 round per minute (rpm)*3min. Then, RT-PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on a 7500 Fast Real-Time PCR System (Applied Biosystems). The sequences of primers were: 5'-ACCCGGACGAAGATGACTTCT-3' (forward) and 5'-CAGCTCGTTCTCAAGCAGCAT-3'(reverse) for MYCN. The comparative threshold cycle ($\Delta\Delta Ct$) method was employed to quantify fold changes in the expression of target genes, relative to the housekeeping gene actin.

Western Blotting

Tumors mentioned above were lysed by RIPA lysis buffer (P0013B, Beyotime) on the ice and SDS lysis buffer (1.0 M Tris-HCL pH 7.5, glycerol, 25%SDS, 2-mecaptethanol, bromophenol blue) at 100°C, successively. Protein supernatant was collected after the samples were centrifuged. Then, standard Western Blotting was performed with the rabbit anti-MYCN antibody (1:1000) (#84406s, Cell Signaling Technology). A mouse anti-actin antibody (1:10000) (HF1024) was used as loading controls.

MYCN amplification detection by FISH

All 56 samples were evaluated MYCN status by FISH using 2-µm-FFPE sections. Laboratory-developed probes targeting the MYCN gene (2p24) and a control PAX3 probe (2q35) were used, which would provide two signals (Fig.2c). Tissue sections then underwent SSC washes and were mounted in 4', 6-diamidino-2-phenylindole for nuclei counterstaining. Results were analyzed and interpreted in accordance with probe manufacturer's instructions

IHC Analysis of MYCN

Tumors specimens were fixed in 10% formalin as soon as they were obtained from patients, and then embedded in paraffin (FFPE). 2-3 µm thick FFPE sections were selected by the pathologist. The expression of MYCN protein was analyzed by IHC with rabbit anti-MYCN antibody (#84406s, Cell Signaling Technology). Scores for MYCN staining intensity were graded on a scale of 0–3 (0 = negative, 1 = weak, 2= moderate, 3 = strong), while positive proportion were 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%). IHC grading were independently graded by two pathologists, who were blinded to FISH results, based on final scores =staining intensity * positive proportion. For this, grading scale of 0 was regarded as 'negative', 1-4 as 'low expression', 5-8 as "median expression", and 9-12 as "high expression".

Statistical Analysis

Data were analyzed with Graphpad Prim 8.0. Differences were examined for significance with two-sided unpaired t test for two groups or ANOVA among groups. Event-free survival (EFS) was calculated as the time from diagnosis till occurrence of an event like death, relapse or progression, if no event, the date of last follow-up. OS was calculated as the time from diagnosis to

death or until the last follow-up if the patient did not die. Kaplan-Meier EFS and OS analyses were performed using Graphpad Prim 8.0 and comparisons of survival curves were carried out using log-rank (Mantel-Cox) test. A P value < 0.05 was considered statistically significant.

Data and software availability

The accession number for all the RNA-sequencing data and clinical information reported in this paper are SEQC Dataset (GSE62564) and TARGET Dataset (Downloaded from the cancer genome atlas website).

Declarations

Ethics approval and consent to participate

The study was conducted according to the Ethical Principles of Measures for Ethical Review of Biomedical Research Involving Human Beings and the Declaration of Helsinki. Patient provided written informed consent prior to 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.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

No potential conflicts of interest were disclosed.

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Authors' contributions

This study was conceived by YL, JT. and MY designed the study; YY, JZ and YZ performed the experiments; JW, BY, HF, and YG 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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Tables

Table 1 Clinical data of patients

Case	Gender	Age	Stage	Risk	Primary Sites	Infiltrating Sites	FISH(MYCN)	IHC (MYCN)	Distant Transference	Therapy protocol	Course of Therapy	Event	EFS months	OS months	Follow up status
1	0	2.9	4	4	3	5	5	6	1	2016Super Risk	9	Relapse	15.2	16.3	Death
2	1	2.4	4	4	3	5+10	5	6	1	2016Super Risk	10		8.1	8.1	CR
3	1	1.9	3	3	3		5	2	0	2016Super Risk	8		10.5	10.5	CR
4	1	1.8	4	4	3	5+6+12	5	9	1	2010Super Risk	11	Process	16.5	18.9	CR
5	0	3.6	3	3	3		5	12	0	2016High Risk	7		27.1	27.1	CR
6	1	1.4	3	3	3		5	12	0	2010High Risk	6	Process	35.1	53.9	Death
7	1	4.2	4	4	3	5	1	4	1	2010Super Risk		Death	0.5	0.5	Death
8	1	4.1	4	4	3	5+6	1	0	1	2010Super Risk	10	Relapse	35.3	38.3	Death
9	1	7.1	4	4	3	5	1	0	1	2016Super Risk	8	Relapse	24.0	24.0	Death
10	1	0.3	1	1	2		1	0	0				62.1	62.1	CR
11	1	0.2	2	1	3	10	1	0	0				75.3	75.3	CR
12	1	0.1	5	2	3+4	9	1	0	1	2010Med Risk	7		71.5	71.5	CR
13	1	0.2	1	1	2		1	0	0				109.7	109.7	CR
14	1	12.0	1-3	2-3	3		1	0	0	2010High Risk	4		82.2	82.2	CR
15	0	3.0	3	3	1		1	0	0	2010High Risk	8		80.9	80.9	CR
16	0	0.3	1	1	3		1	0	0				65.9	65.9	CR
17	0	0.6	3	2	1		1	0	0	2010Med Risk	10	Death	10.3	10.3	Death
18	0	3.3	3	3	3		1	0	0	2010High Risk	6		96.6	96.6	CR
19	1	3.0	4	4	3	5+7+12	1	4	1	2010Super Risk	10	Relapse	19.8	20.6	Death
20	1	1.6	3	3	3		5	2	0	2010High Risk	6		55.0	55.0	CR
21	1	6.6	3	3	3		5	6	0	2008High Risk	3		2.6	2.6	CR
22	0	1.3	4	3	3	8	5	12	1	2010High Risk	9	Relapse	9.4	9.4	Death
23	1	3.8	4	4	3	5	1	2	1	2010Super Risk	10	Relapse	62.7	63.1	Death
24	1	1.2	4	2	3+4	7+10	1	0	1	2010Med Risk	8		61.6	61.6	CR
25	1	3.1	3	3	3		5	12	0	2010High Risk	7		42.7	42.7	CR
26	0	3.8	3	3	3	9+17	1	1	0	2010High Risk	10	Death	13.4	13.8	Death
27	0	1.0	1	2	3		5	6	0	2010Med Risk	4		74.2	74.2	CR
28	0	1.2	3	3	3+4		5	12	0	2010Med Risk	8		68.8	68.8	CR
29	0	2.9	3	3	3		5	12	0	2010High Risk	10	Relapse	11.8	11.8	Death
30	0	3.3	2	2	2		1	0	0	2010Med Risk	6		63.5	63.5	CR
31	1	4.0	4	4	3	10	5	9	1	2010Super Risk	10	Relapse	13.3	13.3	Death
32	0	1.7	3	3	3		5	12	0	2010High Risk	9	Relapse	6.2	14.1	Death
33	1	2.5	4	4	3	5+6	5	12	1	2010Super Risk	10		32.3	32.3	CR
34	0	2.5	4	4	2+3	5	1	6	1	2010Super Risk	10	Relapse	14.0	17.3	Death
35	1	0.6	1	1	2		1	0	1				58.4	58.4	CR
36	1	3.7	4	4	3	5+6	5	12	1	2010Super Risk	10		55.7	55.7	CR
37	0	0.2	3	2	1		1	0	0	2010Med Risk	6		54.4	54.4	CR
38	1	0.4	4	2	3	9+10	1	0	1	2010Med Risk	8		48.9	48.9	CR
39	0	3.3	4	4	3	5+6+7	1	0	1	2010Super Risk	10		53.7	53.7	CCR
40	1	0.4	5	2	3	9	1	0	1	2010Med Risk	8		58.8	58.8	CR
41	0	3.8	3	3	3		1	0	0	2016High	8	Relapse	21.1	30.5	Death

										Risk					
42	1	1.5	4	4	3	9	1	12	1	2016Super Risk	6	Relapse	4.7	5.9	Death
43	0	0.3	3	2	3		1	0	0	2016Med Risk	6		16.6	16.6	CR
44	0	2.1	3	3	3		5	12	0	2016High Risk	8	Relapse	13.8	20.2	CR
45	0	1.0	3	3	4		5	0	0	2016High Risk	8		45.0	45.0	CR
46	1	0.3	1	2	3		5	0	0	2016Med Risk	4		44.9	44.9	CR
47	0	0.2	1	1	2		1	0	0				11.2	11.2	CR
48	0	1.2	4	3	3	6	5	12	1	2016High Risk	10		28.0	28.0	PR
49	1	3.1	4	4	3	6	1	2	1	2016Super Risk	1	Death	1.0	1.0	Death
50	1	0.5	4	2	3		5	12	1	2016Med Risk			0.3	0.3	CR
51	1	3.9	4	4	3		5	12	1	2016Super Risk	10		12.8	12.8	CR
52	0	4.8	4	4	3	5+6	5	0	1	2016Super Risk	10		11.5	11.5	CR
53	0	3.4	4	4	3	5+6+7+9	5	0	1	2016Super Risk	10	Relapse	9.5	17.2	Death
54	0	1.5	4	4	3	5	5	12	1	2016Super Risk	10		7.2	7.2	CR
55	0	4.1	3	3	3		5	12	0	2016High Risk	10		6.5	6.5	CR
56	1	1.9	4	4	3	5+6	5	12	1	2016Super Risk	8		4.2	4.2	CR

Gender: 0 girl; 1 boy

Risk: 1 low risk; 2 med risk; 3 high risk; 4 super risk

Primary sites: 1 cervical part; 2 thorax; 3 abdomen; 4 pelvic cavity

Infiltrating sites: 5 distant bone; 6 bone marrow; 7 craniocerebrum; 8 soft tissue; 9 liver; 10 distant lymph nodes; 11 intraspinal canals; 12 hydrothorax; 13 breast; 14 testis; 15 lung; 16 spleen; 17 pancreas;

Table2 Inter-assay concordance analysis of human MYCN status determined by IHC (MYCN) and FISH in human neuroblastoma

Fish	IHC core=0	0<IHC score<9	9≤IHC score	Concordance by Fish	Discordance by Fish
Non-amplified(n=28)	21(75%)	6(21.4%)	1(3.6%)	75%	25%
Amplified(n=28)	4(14.3%)	6(21.4%)	18(64.3%)	85.7%	14.3%

Table3 Inter-assay concordance analysis of human MYCN protein expression with clinical consequences.

NB	Fish Amplified(n=16)	IHC core=0(n=2)	0<IHC score<9(n=3)	9≤IHC score(n=11)
Event-free	9(56.2%)	2(100%)	3(100%)	4(36.4%)
Event	7(43.8%)	0(0%)	0(0%)	7(63.6%)

NB	Fish Non-Amplified(n=27)	IHC core=0(n=21)	0<IHC score<9(n=5)	9≤IHC score(n=1)
Event-free	17(63.0%)	17(81.0%)	0(0%)	0(0%)
Event	10(37.0%)	4(19.0%)	5(100%)	1(100%)

Table4 Inter-assay concordance analysis of human MYCN DNA and protein expression with clinical consequences.

	Fish Non-Amplified/ IHC=0 (n=21)	Fish Non-Amplified/ 0<IHC (n=6)	Fish Amplified IHC<9 (n=5)	Fish Amplified 9≤IHC (n=11)
NB				
CR	17(81%)	0(0%)	5(100%)	6(54.5%)
Death	4(19%)	6(100%)	0(0%)	5(45.5%)

	Fish Non-Amplified/ IHC=0 (n=21)	Fish Non-Amplified/ 0<IHC (n=6)	Fish Amplified IHC<9 (n=5)	Fish Amplified 9≤IHC (n=11)
NB				
Event-Free	17(81%)	0(0%)	5(100%)	4(36.4%)
Event	4(19%)	6(100%)	0(0%)	7(63.6%)

Figures

Figure 1

The relationship of MYCN amplification with the Prognosis in NB

Figure 2

Fish of MYCN amplification with the Prognosis in NB

Figure 3

A caption was omitted by the authors in this version of the paper.

Figure 4

Fish and IHC of MYCN amplification with the Prognosis in NB

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