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ARTICLE

MYCN RNA levels determined by quantitative in situ hybridization is better than *MYCN* gene dosages in predicting the prognosis of neuroblastoma patients

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Abstract

The aim of this study was to investigate the prognostic role of *MYCN* RNA expression by quantitative RNA in situ hybridization and its association with *MYCN* amplification in neuroblastoma. *MYCN* RNA expression in 69 neuroblastoma tumors was evaluated by an ultrasensitive quantitative RNA in situ hybridization technique, RNAscope. The correlations between *MYCN* RNA expression, *MYCN* amplification, and other clinicopathologic variables of neuroblastoma were analyzed. High expression levels of *MYCN* RNA were detected 30 of 69 (43%) of neuroblastomas, mainly in those with undifferentiated or poorly differentiated histology. High expression of *MYCN* RNA was significantly associated with *MYCN* amplification ($P < 0.001$) and other adversely prognostic factors, including older age at diagnosis (>18 months, $P = 0.017$), advanced clinical stage (International Neuroblastoma Staging System stage 3, 4, $P = 0.002$), unfavorable International Neuroblastoma Pathology Classification tumor histology ($P < 0.001$), and high-risk Children's Oncology Group risk group ($P = 0.001$). In Kaplan–Meier analysis, *MYCN* RNA levels determined by quantitative in situ hybridization were better than *MYCN* gene dosages determined by chromogenic in situ hybridization in discriminating good and poor prognostic groups of neuroblastoma patients. In multivariate analysis, we further confirmed that high expression of *MYCN* RNA was an independent adverse prognostic factor for event-free and overall survival. Furthermore, high expression of *MYCN* RNA predicted unfavorable survival outcomes for neuroblastoma patients with *MYCN* non-amplification or high-risk Children's Oncology Group risk group. In conclusion, our study is the first report to show the application of *MYCN* RNA in situ hybridization in neuroblastoma and established that high expression of *MYCN* RNA could be a better biomarker than *MYCN* amplification for predicting poor prognosis of neuroblastoma patients.

Introduction

Neuroblastoma is a cancer of the sympathetic nervous system that is thought to arise during neural crest progenitor cell differentiation [1]. Neuroblastoma represents the most common extracranial solid tumor in children as well as the most frequently diagnosed malignancy during infancy [2]. It is exceedingly rare in adults, with >96% of neuroblastoma patients diagnosed <10 years of age [3]. Although neuroblastoma accounts for around 5% of childhood cancers, it disproportionately causes up to 10–15% of cancer deaths in children [4].

Neuroblastoma is a heterogeneous disease [1]. The disease is remarkable for its broad spectrum of clinical behavior and displays a variety of morphologic and functional characteristics that result in a histologic spectrum of tumors ranging from highly undifferentiated tumors with very poor

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outcomes to the most differentiated benign ganglioneuroma or neuroblastoma with high probability of spontaneous regression and hence favorable prognosis [2]. Patients with neuroblastoma can be classified into different risk groups based on relevant biological or prognostic factors [5]. Among these factors, *MYCN* proto-oncogene amplification, which is detected in about 20–25% of neuroblastoma, is considered as the most reliable genomic hallmark of aggressive tumor behavior and treatment failure [6–8]. However, neuroblastoma patients with *MYCN* non-amplification or in high-risk group still showed heterogeneity in survival outcomes [9]. The inconsistency between *MYCN* gene dosage, mRNA and protein expression, and clinical outcome [10] has led to a question of whether *MYCN* gene copy number should be replaced by other molecular markers more indicative of *MYCN* function [11]. In addition, RNA biomarkers or gene expression signature have emerged as a major class of cancer biomarker to guide treatment, such as the widespread use of genome-wide gene expression profiling technologies [12–15]. The *MYCN* gene copy number was shown to be correlated with RNA expression in neuroblastoma in one study [16], but two other studies showed the absence of correlation between DNA amplification and RNA expression [10, 17]. Most of these studies used quantitative real-time polymerase chain reaction as the platform which has disadvantages of being unable to map the observed signals to individual cells and the observed signals are prone to be interfered by unintended cell types, such as non-cancer cells [12]. In contrast, RNA in situ hybridization allows the integration of molecular information with histopathology for optimal clinical interpretation, but use of conventional RNA in situ hybridization has been limited to highly expressed genes due to lacking the sensitivity and specificity required to measure low-abundance RNA biomarkers reliably [12, 18].

A new ultrasensitive quantitative RNA in situ hybridization method, RNAscope, was developed recently which has advantages of single-molecule visualization in individual cells and a hybridization-based signal amplification system to simultaneously amplify signals and suppress background [12]. This approach can be used with archival formalin-fixed, paraffin-embedded tissue samples on glass slides, and the stained slides can be visualized under either a standard bright-field microscope or an epifluorescent microscope [12]. We therefore hypothesized that *MYCN* RNA expression evaluated by RNAscope could be a novel biomarker for predicting the clinical outcome of neuroblastoma patients. The aim of this study is to investigate the prognostic role of *MYCN* RNA expression evaluated by RNAscope and compare the results of *MYCN* amplification in primary neuroblastoma tumors.

Materials and methods

Patients and tissue samples

From January 2002 to December 2018, histologically proved neuroblastoma patients at diagnosis with sufficient tumor tissues and complete follow-up were enrolled in this study. All diagnoses of tumors were confirmed by histologic assessment of a specimen obtained from the primary or metastatic tumor at surgery or biopsy before starting chemotherapy. Based on the criteria of the International Neuroblastoma Pathology Classification [19], the differentiating status of the tumor histology was categorized into undifferentiated neuroblastoma (Schwannian stroma poor), poorly differentiated neuroblastoma (Schwannian stroma poor), differentiating neuroblastoma (Schwannian stroma poor), and ganglioneuroblastoma, intermixed (Schwannian stroma-rich) according to the percentage and degree of differentiation of the neuroblastoma cells. The nodular type ganglioneuroblastoma was classified into either undifferentiated or differentiating neuroblastoma according to the morphology of their neuroblastoma nodules, since the prognosis of this type of tumors depends mainly on their neuroblastoma nodules [20]. The tumor staging was based on the International Neuroblastoma Staging System [21]. *MYCN* copy number status of the tumor tissue was evaluated by chromogenic in situ hybridization analysis of formalin-fixed paraffin-embedded tissues or single cells from fresh tumor samples. *MYCN* amplification was defined as ≧10 copies/nuclei [22]. Based on risk classifications of the Children's Oncology Group, patients were classified into low-, intermediate-, and high-risk groups and were treated with surgery only or a combination of multiple modalities including chemotherapy, radiotherapy, and/or autologous bone marrow transplantation according to the patient's risk grouping [23, 24]. The clinical parameters, including age at diagnosis, sex, tumor stages, primary tumor sites, differentiating status of tumor histology, and treatment of neuroblastoma patients were collected for prognostic and survival analysis. This study was approved by the Institutional Review Board of the National Taiwan University Hospital.

In situ hybridization for *MYCN* oncogene mRNA detection

The reagents and probes were purchase from Advanced Cell Diagnostics (Hayward, CA, USA). Tissue sections (5 μm thick) were deparaffinized in xylene, followed by rehydration in an ethanol series. Tissue sections were then incubated in a citrate buffer (10 nmol/L, pH 6.0) maintained at a boiling temperature (100–103 °C) using a hot plate for 15 min, rinsed in deionized water, and treated with 10 μg/mL

protease (Sigma-Aldrich, St. Louis, MO) at 40 °C for 30 min in a bacterial incubator. The cells were then incubated in order at 40 °C with the following solutions: target probes in hybridization buffer A [6× SSC (1× SSC is 0.15 mol/L NaCl, 0.015 mol/L Nacitrate), 25% formamide, 0.2% lithium dodecyl sulfate, blocking reagents] for 3 h; pre-amplifier (2 nmol/L) in hybridization buffer B (20% formamide, five SSC, 0.3% lithium dodecyl sulfate, 10% dextran sulfate, blocking reagents) for 30 min; amplifier (2 nmol/L) in hybridization buffer B at 40 °C for 15 min; and label probe (2 nmol/L) in hybridization buffer C (five SSC, 0.3% lithium dodecylsulfate, blocking reagents) for 15 min. After each hybridization step, slides were washed with wash buffer (0.1 SSC, 0.03% lithium dodecyl sulfate) three times at room temperature. The endogenous housekeeping gene UBC was used as positive control to assess both tissue RNA integrity and assay procedure. The tissue sections were counterstained with hematoxylin. The slides were observed under a bright field microscope. Any intracellular brown staining was considered to be positive signal and the signals were graded semiquantitatively as negative, 1+, 2+, and 3+ according to the amount of RNA signal per cell.

Real-time reverse transcription polymerase chain reaction

The SYBR green-based real-time polymerase chain reaction was performed to determine the levels of *MYCN* mRNA using the QuantStudio™ 7 Flex Real-time RCR System (Thermo Fisher Scientific, Waltham, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, served as a control for RNA quantity. The primers for *MYCN* were *MYCN*-F (5′-ACCACAAGGCCCTCAGTACCTC-3′) and *MYCN*-R(5′-TGACAGCCTTGGTGTTGGAGGA-3′). The primers used for GAPDH were GAPDH-F (5′-AGCCTCAAGATCATCAGCAATGCC-3′) and GAPDH-R (5′-TGTGGTCATGAGTCCTTCCACGAT-3′). In a volume of 20 µl polymerase chain reaction, 1 µl of complementary DNA template was mixed with 10 µl of 2× Power SYBR® polymerase chain reaction master mix (Applied Biosystems, Waltham, MA, USA), 200 nM of paired primers, and distilled water. Polymerase chain reaction amplification included initial incubation at 50 °C for 2 min, denaturing at 95 °C for 10 min, and 40 cycles of denaturing at 95 °C for 15 s and annealing at 60 °C for 1 min. Melting curves were analyzed after each run to verify the size of polymerase chain reaction product. The relative expression of *MYCN* in each sample was calculated with the following formula: relative mRNA expression $= 2^{-\Delta Ct} \times 256$. $\Delta Ct = Ct_{MYCN} - Ct_{GAPDH}$.

Statistical analysis

Pearson's chi-square test was used to assess associations between pairs of categorical variables. Event-free survival (defined as the time interval from the date of diagnosis to the date of the first event [tumor progression, relapse, or death] or last follow-up) and overall survival (defined as the time interval from the date of diagnosis to the date of death or last follow-up) estimates were calculated using the Kaplan–Meier method and compared using the log-rank test. The Cox proportional hazards model was used to identify independent prognostic factors with respect to event-free survival and overall survival. All statistical analyses were conducted using Statistical Package for the Social Sciences for Windows, software version 16.0 (SPSS Inc., Chicago, IL). All statistical tests were two-sided, and those with a P value < 0.05 were considered to be statistically significant.

Results

Patient characteristics

There were 69 neuroblastoma patients diagnosed at our hospital enrolled for analysis in this study. The mean age of the 69 patients was 29 months (range, 0.2–184 months). Male patients were slightly predominant, with a male/female ratio of 41:28. The median follow-up after diagnosis was 37 months (range of 1–139 months). The 5-year event-free and overall survival rates for this cohort were 46 ± 6.7% and 56.8 ± 7%, respectively. A summary of the demographic information and clinical characteristics of these patients is provided in Table 1.

MYCN RNA expression and clinicopathologic factors of neuroblastoma

To investigate the clinical significance of *MYCN* RNA expression as well as its relationship with *MYCN* amplification and other clinicopathologic factors of neuroblastoma, we examined *MYCN* RNA expression by in situ hybridization staining with RNAscope in our 69 neuroblastoma tumors. The signals were graded as negative, 1+, 2+, and 3 + according to the expression of dot-like signals in neuroblastoma tumors. The representative pictures of *MYCN* RNA in situ hybridization staining were shown in Fig. 1. The expression signals of "2+" to "3+" were pooled as high expression in contrast to those of "negative" to "1+" as low expression for statistical analysis. Our in situ hybridization staining result revealed that high expression of *MYCN* RNA could be detected in 30 (43%) of the 69 neuroblastoma tumors, majorly in tumors with

Table 1 Clinicopathological characteristics of patients ($n = 69$) with neuroblastoma

Variables	Case number (%)
Age at diagnosis (months)	29 (0.2–184)
≦18 months	32 (46%)
>18 months	37 (54%)
Gender	
Male	41 (59%)
Female	28 (41%)
International Neuroblastoma Staging System stage	
1	15 (22%)
2	2 (3%)
3	10 (14%)
4	36 (52%)
4S	6 (9%)
Primary tumor site	
Adrenal	53 (77%)
Extraadrenal	16 (23%)
Pathologic histology	
Undifferentiated neuroblastoma	22 (32%)
Poorly differentiated neuroblastoma	30 (44%)
Differentiating neuroblastoma	7 (10%)
Ganglioneuroblastoma	10 (14%)
MYCN	
Amplified	17 (25%)
Non-amplified	52 (75%)
Children's Oncology Group risk group	
Low	19 (27%)
Intermediate	6 (9%)
High	44 (64%)

undifferentiated or poorly differentiated histology (Table 2). High expression of *MYCN* RNA by in situ hybridization staining was significantly associated with *MYCN* amplification ($P < 0.001$) and other adversely prognostic factors, including older age at diagnosis (>18 months, $P = 0.017$), advanced clinical stage (International Neuroblastoma Staging System stage 3, 4, $P = 0.002$), unfavorable International Neuroblastoma Pathology Classification tumor histology ($P < 0.001$), and high-risk Children's Oncology Group risk group ($P = 0.001$) (Table 2).

MYCN RNA expression by in situ hybridization in neuroblastoma predicts an unfavorable survival outcome

Kaplan–Meier analysis showed that patients with high expression of *MYCN* RNA by in situ hybridization staining have a predicted 5-year event-free and overall survival rates of 9.2 ± 6% and 16.1 ± 8.2%, respectively, compared with the 5-year event-free and overall survival rates of 76.3 ± 7.9 and 79.1 ± 8.6% for those with low expression (Fig. 2a, b and Table 3, $P < 0.001$ by log-rank test), suggesting that high expression of *MYCN* RNA by in situ hybridization staining could predict an unfavorable outcome. In univariate analysis, in addition to high expression of *MYCN* RNA, older age at diagnosis (>18 months), advanced clinical stage (International Neuroblastoma Staging System stage 3/4), unfavorable International Neuroblastoma Pathology

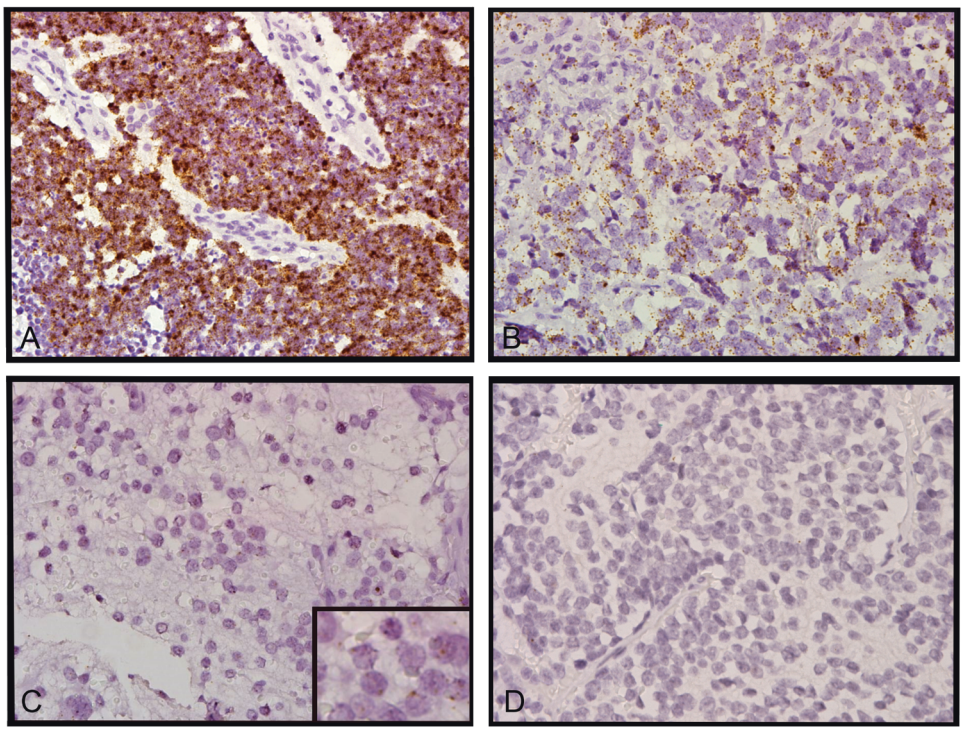

Fig. 1 *MYCN* RNA expression by in situ hybridization with RNAscope in neuroblastoma. Staining images of neuroblastoma tumors represent four categories of *MYCN* RNA expression according to the intensity of expression. **a** 3+, **b** 2+, **c** 1+, and **d** negative. Hematoxylin was used as counterstaining

Table 2 *MYCN* RNA in situ hybridization expression and clinicopathologic factors in patients ($n = 69$) with neuroblastoma

Variables	Case number	*MYCN* RNA in situ hybridization low expression	*MYCN* RNA in situ hybridization high expression	*P* value
Age at diagnosis (months)				
≦18 months	32	23 (72%)	9 (28%)	0.017*
>18 months	37	16 (43%)	21 (57%)	
Gender				
Male	41	24 (59%)	17 (41%)	0.683
Female	28	15 (54%)	13 (46%)	
International Neuroblastoma Staging System stage				
1, 2, 4s	23	19 (83%)	4 (17%)	0.002*
3, 4	46	20 (43%)	26 (57%)	
Primary tumor site				
Adrenal	53	27 (51%)	26 (49%)	0.089
Extraadrenal	16	12 (75%)	4 (25%)	
International Neuroblastoma Pathology Classification histology				
Favorable	33	27 (82%)	6 (18%)	<0.001*
Unfavorable	36	12 (33%)	24 (67%)	
MYCN				
Amplified	17	3 (18%)	14 (82%)	<0.001*
Non-amplified	52	36 (69%)	16 (31%)	
Children's Oncology Group risk group				
Nonhigh risk	25	21 (84%)	4 (16%)	0.001*
High risk	44	18 (41%)	26 (59%)	

*$P < 0.05$ by Pearson's chi-squared test

Classification histology, *MYCN* amplification, and high-risk Children's Oncology Group risk group predicted a significant worse 5-year event-free survival or overall survival outcome (Table 3). To be noteworthy, although *MYCN* amplification was a significant factor for predicting worse 5-year event-free survival or overall survival outcomes, *MYCN* RNA levels discriminated good and poor prognostic groups far better than *MYCN* gene dosage (Fig. 2). In multivariate analysis through the Cox proportional hazard model, it was further confirmed that high expression of *MYCN* RNA, in addition to advanced clinical stage, is an independent adverse prognostic factor for event-free survival and overall survival outcomes in our cohort of neuroblastoma patients (Table 4).

The significance of *MYCN* RNA expression by in situ hybridization staining in prognostic discrimination was further stratified by *MYCN* amplification status and Children's Oncology Group risk groups. The prognosis of neuroblastoma patients with *MYCN* amplification could not be distinguished by *MYCN* RNA expression, and this may be due to the high frequency of *MYCN* RNA high expression and very poor survival outcome for these patients. Nevertheless, among patients without *MYCN* amplification, high expression of *MYCN* RNA clearly predicted an unfavorable outcome (Fig. 3a, $P < 0.001$ by log-rank test). Although *MYCN* RNA expression might not be suitable for the prognosis discrimination of neuroblastoma patients with low-risk Children's Oncology Group risk group due to a low rate of *MYCN* RNA high expression and favorable outcome for these patients, high expression of *MYCN* RNA predicted the unfavorable prognosis for patients with high-risk Children's Oncology Group risk group (Fig. 3b; $P < 0.001$ by log-rank test).

Correlation of *MYCN* RNA expression by in situ hybridization and real-time reverse transcription polymerase chain reaction

Fresh frozen tissue samples of eleven neuroblastoma tumors were available from our archive. Four were scored as 3+, three were scored as 2+, one was scored as 1+, and three were scored as negative in the *MYCN* RNA in situ hybridization analysis by RNAscope. RNA samples were extracted from tumor tissues. Real-time reverse transcription polymerase chain reaction assays showed the average expression levels of 3+, 2+, and 1+ tumors were 55.5, 5.9, and 3.7-fold of that of the negative tumors, respectively. This result showed the authenticity of the *MYCN* RNA in situ hybridization assay by RNAscope in our neuroblastoma tumors.

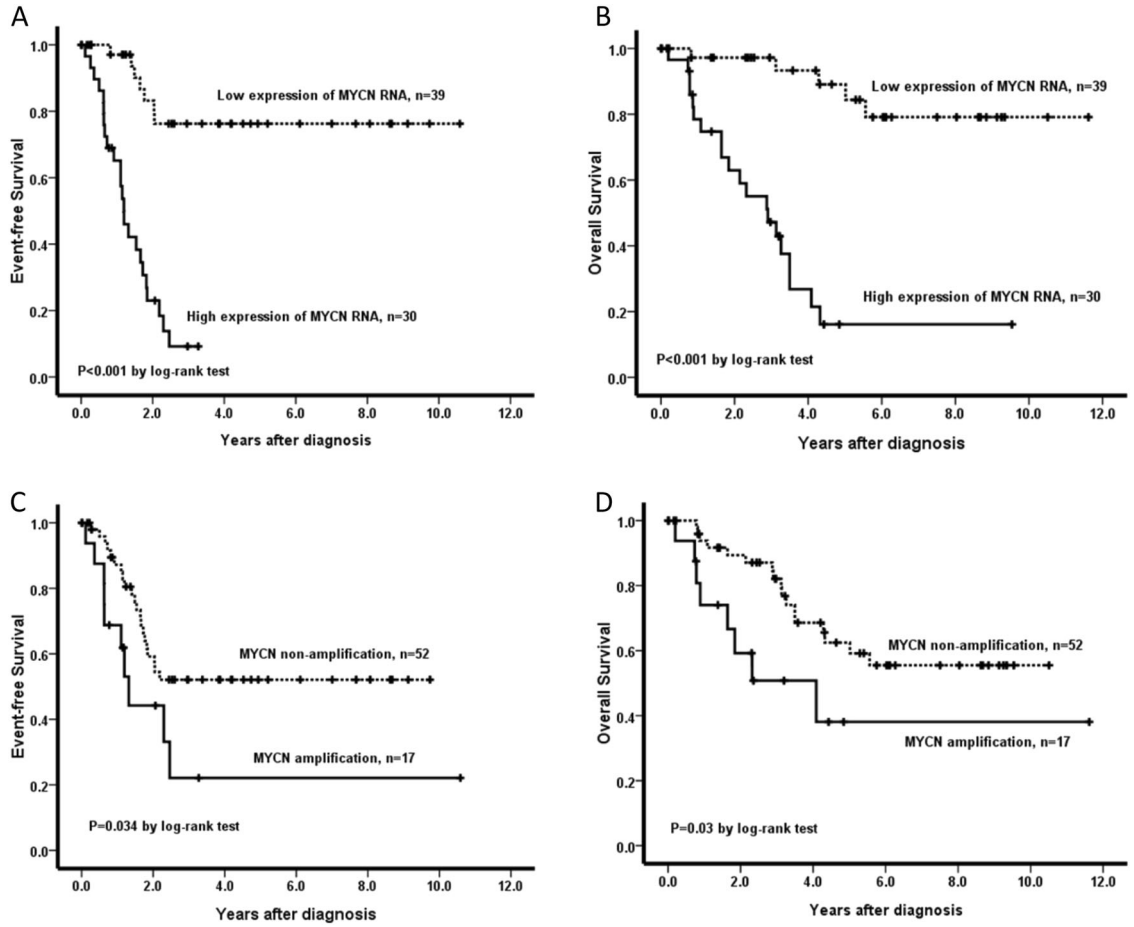

Fig. 2 Kaplan–Meier survival analysis according to the *MYCN* RNA expression levels and *MYCN* amplification status in our neuroblastoma patients ($n = 69$). **a** Probability of event-free survival and **b** overall survival in patients with low or high *MYCN* RNA expression by in situ hybridization. **c** Probability of event-free survival and **d** overall survival in patients with *MYCN* amplification or non-amplification. *P* value was calculated using log-rank test

Discussion

Our present study showed that, within a cohort of 69 neuroblastoma patients, high expression levels of *MYCN* RNA determined by in situ hybridization with RNAscope could be detected in a significant number (30/69, 43%) of neuroblastoma tumors. The high expression of *MYCN* RNA was mainly found in neuroblastoma tumors with undifferentiated or poorly differentiated histology and significantly associated with *MYCN* amplification as well as other adversely clinical prognostic factors. In Kaplan–Meier analysis, *MYCN* RNA levels discriminated good and poor prognostic groups far better than *MYCN* gene dosage in our neuroblastoma patients. Thus *MYCN* RNA levels determined by quantitative in situ hybridization are better than *MYCN* gene dosages determined by chromogenic in situ hybridization in predicting the prognosis of neuroblastoma patients. In spite of the currently used method for *MYCN* copy-number determination is fluorescence in situ hybridization in Children's Oncology Group clinical trials [25], chromogenic in situ hybridization used in this study is also a reliable method to detect *MYCN* amplification in neuroblastoma [22]. In univariate and multivariate analysis, high expression of *MYCN* RNA by in situ hybridization with RNAscope predicted an unfavorable survival outcome and was an independent poor prognostic factor in neuroblastoma patients. In further analysis of prognostic discrimination, high expression of *MYCN* RNA predicted the unfavorable survival outcomes for neuroblastoma patients with *MYCN* non-amplification or high-risk Children's Oncology Group risk group. The current study is the first report to show the application of *MYCN* RNA in situ hybridization in neuroblastoma tumor and established that high expression of *MYCN* RNA by in situ hybridization could be a novel biomarker for predicting poor prognosis of neuroblastoma patients and provide complimentary prognostic information in addition to *MYCN* amplification status and Children's Oncology Group risk grouping.

Although *MYCN* amplification remains the one of the most important genomic abnormalities associated with

Table 3 Univariate analysis of prognostic factors in patients ($n = 69$) with neuroblastoma

Variables	Case number	5-year event-free survival, % (SE)	*P* value	5-year overall survival, % (SE)	*P* value
Age at diagnosis (months)			0.001*		0.001*
≦18 months	32	75.8 (8)		81.4 (7.7)	
>18 months	37	20.1 (7.5)		21.1 (9.6)	
International Neuroblastoma Staging System stage			<0.001*		<0.001*
1, 2, 4s	23	90.2 (6.6)		95.2 (4.6)	
3, 4	46	22 (7.1)		38.2 (8.5)	
Primary tumor site			0.57		0.412
Adrenal	53	45.3 (7.4)		52.3 (8)	
Extraadrenal	16	46.7 (15.1)		76 (12.3)	
International Neuroblastoma Pathology Classification histology			<0.001*		<0.001*
Favorable	33	77.1 (8.3)		86.5 (7.4)	
Unfavorable	36	19.9 (7.2)		33 (9)	
MYCN			0.034*		0.03*
Amplified	17	22.1 (13)		38.1 (15)	
Non-amplified	52	52.1 (7.6)		62.5 (7.9)	
Children's Oncology Group risk group			<0.001*		<0.001*
Nonhigh risk	25	90.4 (6.5)		93.3 (6.4)	
High risk	44	21.8 (7)		38.9 (8.5)	
MYCN RNA in situ hybridization expression			<0.001*		<0.001*
Low	39	76.3 (7.9)		79.1 (8.6)	
High	30	9.2 (6)		16.1 (8.2)	

*$P < 0.05$

highly malignant clinical behaviors, the role of *MYCN* in pathogenesis of neuroblastoma tumor is still under investigation. An early study confirmed that target expression of *MYCN* was sufficient to initiate genesis of neuroblastoma in mice [26]. Several studies identified signaling pathway influenced by *MYCN* expression and showed that high *MYCN* expression increases the expression of numerous genes involved in cell proliferation, and inhibits expression of differentiation or apoptosis-related genes [27–29]. These observations suggested that high expression of *MYCN* may enhance malignant phenotypes of neuroblastoma and could be the explanation for our study results that high expression of *MYCN* RNA was associated with poor survival outcomes in neuroblastoma patients.

Microarray, quantitative real-time polymerase chain reaction, and now even next-generation sequencing are common tools for disease profiling of gene expression signature, but clinically relevant information regarding cellular and tissue context, as well as spatial variation of the expression patterns, is lost in the process. In situ hybridization is a technique involves using a nucleotide sequence that is tagged or labeled with a detection molecule and is used to localize nucleic acid sequences in tissue sections or cell culture samples [30]. It allows detection of specific DNA or RNA sequences with cellular and subcellular resolution within complex tissue architecture and could provide detailed spatial as well as contextual information about gene expression [31]. Conventional non-radioisotopic RNA in situ hybridization methods lack the sensitivity and specificity required to measure low-abundance RNAs [31]. Some strategies have been used to increase the sensitivity of detection for RNA in situ hybridization, such as amplifying the mRNA targets before hybridization [32], or amplifying the signals after target hybridization [33], and tyramide signal amplification [34]. However, these strategies have the disadvantage of increased non-specific noise accompanying increased signals [31]. RNAscope is a new RNA in situ hybridization method [12] and used a novel target probe design strategy (a double-Z design) to enhance its specificity and sensitivity.

Table 4 Multivariate analysis of prognostic factors in patients ($n = 69$) with neuroblastoma

Variables	Case number	Event-free survival HR (95% CI)	*P* value	Overall survival HR (95% CI)	*P* value
Age at diagnosis (months)			0.147		0.331
≦18 months	32	1		1	
>18 months	37	2.525 (0.721–8.845)		2.182 (0.453–10.519)	
International Neuroblastoma Staging System stage			0.006*		0.013*
1, 2, 4s	23	1		1	
3, 4	46	10.577 (1.981–56.47)		22.833 (1.95–267.362)	
Primary tumor site			0.211		0.357
Adrenal	53	1.811 (0.714–4.595)		1.752 (0.532–5.774)	
Extraadrenal	16	1		1	
International Neuroblastoma Pathology Classification histology			0.1		0.092
Favorable	33	0.338 (0.093–1.231)		0.24 (0.046–1.261)	
Unfavorable	36	1		1	
MYCN			0.156		0.465
Amplified	17	1		1	
Non-amplified	52	0.545 (0.235–1.26)		0.712 (0.286–1.772)	
MYCN RNA in situ hybridization expression			0.009*		0.011*
Low	39	1		1	
High	30	4.403 (1.457–13.306)		5.368 (1.464–19.68)	

*$P < 0.05$

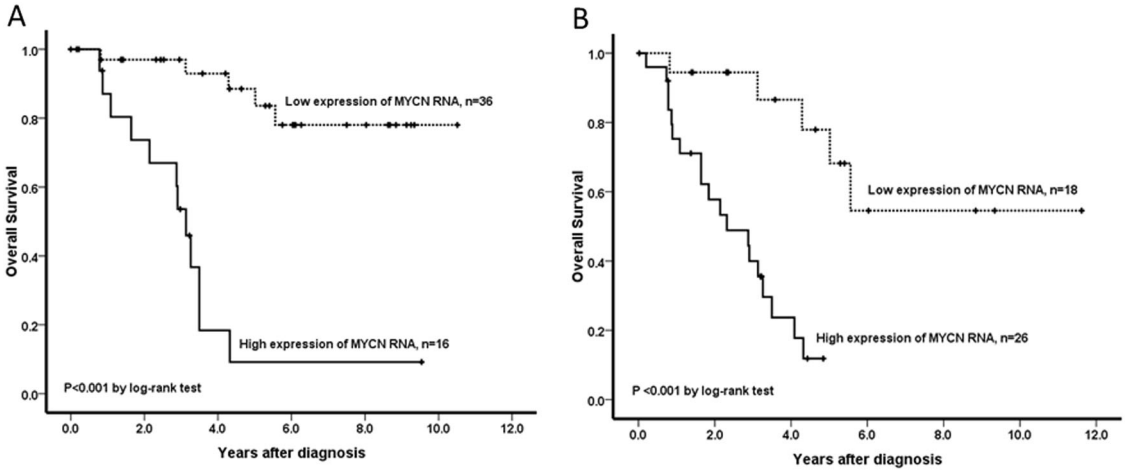

Fig. 3 Impact of *MYCN* RNA expression by in situ hybridization with RNAscope on survival outcomes for subgroups of neuroblastoma patients. **a** Overall survival analysis according to the *MYCN* RNA levels in neuroblastoma patients with *MYCN* non-amplification ($n = 52$). **b** Overall survival analysis according to the *MYCN* RNA levels in neuroblastoma patients with high-risk grouping ($n = 44$). *P* value was calculated using log-rank test

In this assay, a series of target probes were designed to hybridize to the specific target RNA molecule. Each target probe contains an 18- to 25-base region complementary to the target RNA, a spacer sequence, and a 14-base tail sequence. The two tail sequences (double Z) together form a 28-base hybridization site for signal amplification. This double Z design ensures superior background control by reducing the chance of a non-specific hybridization even

being amplified [31]. Although RNAscope has above advantages, it still has some shortcomings. If the tissue samples are of poor quality of fixation, they will not be well stained. In addition, one study showed RNA in situ hybridization signals detected by RNAscope may be reduced or lost as age of tissue block increased [35]. These disadvantages should be taken into consideration when applying RNAscope for clinical practices. Our study included paraffin specimens of different age, but we did not lower positive rate in older specimens, indicating at least for the *MYCN* probe we used, specimen age was not a confounding factor for this analysis.

Our study revealed RNA expression of *MYCN* evaluated by RNAscope was well correlated with *MYCN* amplification. Another study explored the correlation between HER2 RNA expression evaluated by RNAscope and HER2 gene amplification in gastric carcinoma showed similar results [36]. However, *MYCN* amplification is unable to predict all cases of poor clinical outcomes in neuroblastoma, and around 80–75% of neuroblastomas do not have *MYCN* amplification [6–8]. It has been noted previously that enhanced *MYCN* RNA expression was not confined to tumors with *MYCN* oncogene amplification in neuroblastoma [10, 17]. Other factors, such as transcription factor binding, epigenetic modification, and transcriptional regulation may affect the expression of *MYCN*. In our cohort, 31% of tumors without *MYCN* amplification showed high expression of *MYCN* RNA. Furthermore, *MYCN* RNA expression level was better than *MYCN* gene dosage in predicting patient outcome. This is not unexpected because the protein level usually correlates better with mRNA level than gene dosage. Therefore, assessing *MYCN* RNA expression by in situ hybridization with RNAscope could provide complement prognostic information in addition to known prognostic factors, which in turn to help clinicians to determine the most appropriate therapy for the neuroblastoma patients

There were several limitations in this study which should be considered when interpreting the results. First, it was a retrospective analysis, not a prospective study, and it is possible to be associated with observation and selection biases. Second, the sample size in this study was small. Although we found a novel prognostic role of *MYCN* RNA expression by in situ hybridization with RNAscope in neuroblastoma tumor, the results of our study may need a prospective study with larger number of neuroblastoma patients to confirm our observations.

In summary, our study showed that a significant percentage of neuroblastoma tumors have high expression of *MYCN* RNA by in situ hybridization which was significantly correlated with *MYCN* amplification and other adverse prognostic factors. *MYCN* RNA levels discriminated good and poor prognostic groups better than *MYCN* gene dosage in our neuroblastoma patients. High expression of *MYCN* RNA by in situ hybridization is an independent adverse prognostic factor for survival outcomes in our neuroblastoma patients and also predicted worse prognosis for patients with *MYCN* non-amplification status or high-risk Children's Oncology Group grouping. Our study is the first report to show the application of *MYCN* RNA in situ hybridization in neuroblastoma tumor and established that high expression of *MYCN* RNA by in situ hybridization could be a novel biomarker for predicting poor prognosis of neuroblastoma patients, providing complimentary prognostic information to *MYCN* amplification status and Children's Oncology Group risk grouping. Our findings suggest *MYCN* RNA levels determined by quantitative in situ hybridization are better than *MYCN* gene dosages determined by chromogenic in situ hybridization in predicting the prognosis of neuroblastoma patients and could provide invaluable insights to determine the most appropriate risk classification as well as therapy for neuroblastoma patients.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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