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Original Article

TOP2A expression predicts an unfavourable prognosis in neuroblastic tumors

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Abstract: Neuroblastic tumors (NTs) are the most common extracranial solid tumors in children. Topoisomerase (DNA) II alpha (TOP2A) is a critical nuclear enzyme involved in DNA replication and mitosis, which is targeted by several chemotherapeutic agents. To date, little is known about the expression of TOP2A in neuroblastic tumors. This study was conducted to evaluate TOP2A protein expression and *TOP2A* gene alteration, as well as its prognostic value in neuroblastic tumors. We analyzed 88 cases of NTs with immunohistochemistry for protein expression, and 80 cases using fluorescence in situ hybridisation (FISH) for gene status of *TOP2A*. Statistical analyses were performed to evaluate the correlations between TOP2A expression and the clinicopathological characteristics of the patients as well as the predictive value of TOP2A. The results showed that TOP2A protein was expressed in 59.09% of NTs, and 23.86% of NTs showed high TOP2A expression in >50% of tumor cells. The high expression of TOP2A was significantly linked to aggressive NT type (P=0.006), and also strongly correlated with high Ki-67 index (P=0.000) and mitosis-karyorrhexis index (MKI) (P=0.001). *TOP2A* amplification was found in 35.00% of NTs and was more frequently detected in the advanced clinical stage neuroblastomas (stage III and IV) (P=0.028), older patients (>18 months) (P=0.000) and high risk cases (P=0.001). It was discovered that the *TOP2A* gene amplification did not correlate with protein overexpression. Survival analyses showed that high TOP2A protein expression was significantly associated with worse survival (P=0.005). Multivariate analyses demonstrated that TOP2A expression was an independent predictive factor for poor prognosis (P=0.010). In summary, high TOP2A protein expression was more frequently found in aggressive type of NTs, and in cases with high Ki-67 index or high MKI. *TOP2A* amplification was more frequently presented in late stage neuroblastomas, older patients (>18 months) and high risk group. Furthermore, TOP2A protein expression was significantly associated with shorter survival. It is therefore concluded that TOP2A protein expression is an independent unfavorable predictive factor in NTs.

Keywords: Neuroblastic tumors, TOP2A, prognosis, FISH, immunohistochemistry

Introduction

Neuroblastic tumors (NTs) are the most common extracranial solid tumors in children, arising from primordial neuroepithelial cells of the neural crest [1]. Neuroblastic tumors are commonly categorized into neuroblastoma (NB), ganglioneuroblastoma (GNB) and ganglioneuroma (GN) based on the differentiation of neoplastic cells and Schwannian stroma amount [2]. Among them, neuroblastoma is the most immature, undifferentiated and malignant tumor, accounting for 13% of pediatric cancer morbidity with current cure rates less than 50% [3, 4]. Ganglioneuroblastoma is composed of both mature gangliocytes and immature neuroblasts and has intermediate malignant potential. The most benign tumor is the ganglioneuroma, which is composed of gangliocytes and mature Schwannian stroma [2]. The behavior of NB is markedly heterogeneous, ranging from spontaneous differentiation or regression into GNB or GB with a favorable prognosis to highly undifferentiated tumors with a rapid progression and very poor outcomes [1]. Most of NB patients have fatal outcomes despite the imple-

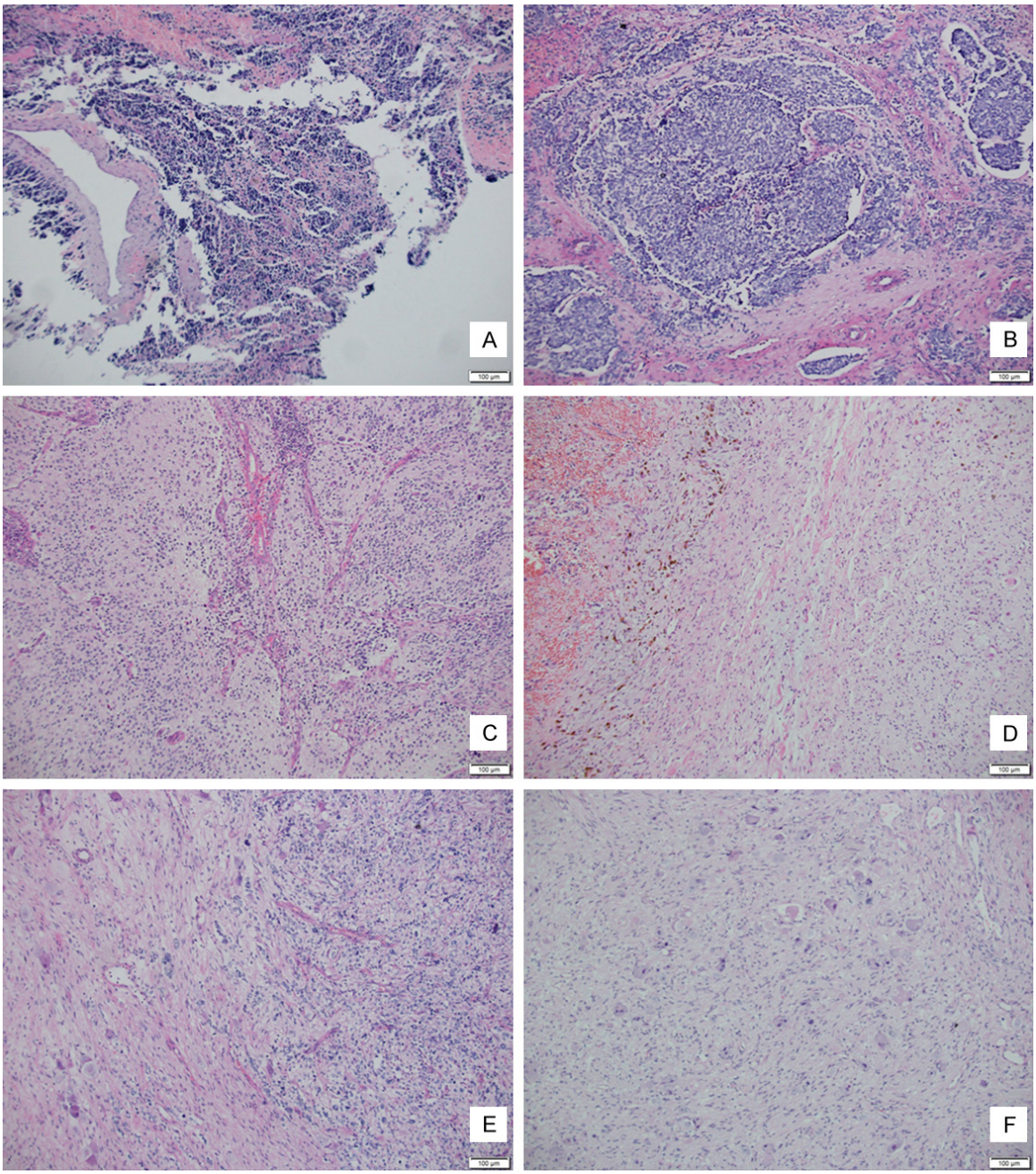

Figure 1. Representative cases showing typical morphology of NTs. A. NB (undifferentiated) showing small round malignant cells. B. NB (poorly differentiated): less than 5% of tumor cells presenting differentiation. C. NB (differentiated): more than 5% of tumor cells displaying gangliocyte differentiation. D. GNB (nodular) being composed of both gangliocyte nodules and immature neuroblastoma nodules. E. GNB (intermixed) showing nested neuroblastoma cells distributed in the gangliocyte stroma (more than 50%). F. GN being composed of gangliocytes and mature Schwannian stroma.

mentation of maximal modern therapy. Neuroblastoma frequently shows complex patterns of genetic aberrations including *MYCN* amplification, deletion of chromosome 1p or 11q, and gain of chromosome 17q [5]. Chromosomal 17q21-ter gain is the most common chromosomal alteration in 50% to 95% of NB cases [6]. Gene products encoded on 17q21-qter that have been proposed to contribute to the development or progression of NB include survivin, ncRNA, the insulin-like growth factor-2 mRNA-binding protein 1 (IGF2BP1) and

Table 1. Clinical and pathological characteristics of the 89 patients with neuroblastic tumors

Variable	Neuroblastoma	Ganglioneuroblastoma	Ganglioneuroma
Cases (n.)	68	18	3
Male/Female	40/28	10/8	1/2
Median Age (months)	24	44.5	32
Site			
Adrenal/retroperitoneal	43/68 (63.24%)	10/18 (55.56%)	1/3 (33.33%)
Mediastinum	17/68 (25%)	7/18 (38.89%)	1/3 (33.33%)
Lymph nodes in the neck	2/68 (2.94%)		
Intraspinal	3/68 (4.41%)	1/18 (5.56%)	1/3 (33.33%)
Bone Marrow	3/68 (4.41%)		
Age			
≤18 months	28/68 (41.18%)		
>18 months	40/68 (58.82%)	18/18 (100%)	3/3 (100%)
Clinical Stage*			
1, 2, 4 s	3/68 (4.41%)	2/18 (11.11%)	
3, 4	37/68 (54.41%)	9/18 (50%)	1/3 (33.33%)
Undetermined	28/68 (41.18%)	7/18 (38.89%)	2/3 (66.67%)
Risk stratification**			
Low	10/68 (14.71%)		
Middle	5/68 (7.35%)	2/18 (11.11%)	
High	29/68 (42.65%)	9/18 (50%)	1/3 (33.33%)
Undetermined	24/68 (35.29%)	7/18 (38.89%)	2/3 (66.67%)
Ki-67 Index			
<20%	23/68 (33.82%)	14/18 (77.78%)	3/3 (100%)
20-50%	24/68 (35.29%)	3/18 (16.67%)	
>50%	12/68 (17.65%)		
Undetermined	9/68 (13.24%)	1/18 (5.56)	
MKI***			
<2%	12/68 (17.65%)	4/18 (22.22%)	
2%-4%	9/68 (13.24%)	1/18 (5.56%)	
>4%	3/68 (4.41%)		
Undetermined	44/68 (64.71)	13/18 (72.22%)	3/3 (100%)
MYCN			
Normal	9/68 (13.24%)	2/18 (11.11%)	2/3 (66.67%)
Gain	49/68 (70.56%)	16/18 (88.89%)	1/3 (33.33%)
Amplification	10/68 (14.71%)		

Note: *Clinical stage: defined by International Neuroblastoma Staging System (INSS) [13]. **Risk stratification: defined by International Neuroblastoma Risk Group Staging System (INRGSS) [13]. ***MKI: mitosis-karyorrhexis index (Counting the number of mitosis and nuclear fragmentation in 5000 cells. Low MKI (<100/5000), intermediate MKI (100-200/5000), high MKI (>200/5000) [14].

others [7-9]. Identifying more efficient biomarkers of NTs is essential in designing more effective individualized therapy regimes.

Topoisomerase (DNA) II alpha (TOP2A) gene is located at chromosome 17q21-q22. It encodes TOP2A, a critical nuclear enzyme in DNA replication, chromosome condensation, chromatid separation, maintenance of genomic stability, *etc* [10]. The expression and genetic alteration of TOP2A have been investigated in various tumors, such as malignancies from breast, ovary, liver and lung *etc* [10]. TOP2A protein is targeted by several chemotherapeutic agents such as anthracyclines and epipodophyllotoxins, which are frequently utilized to treat NT patients [11]. TOP2A alterations have been reported to influence response to anthracycline-

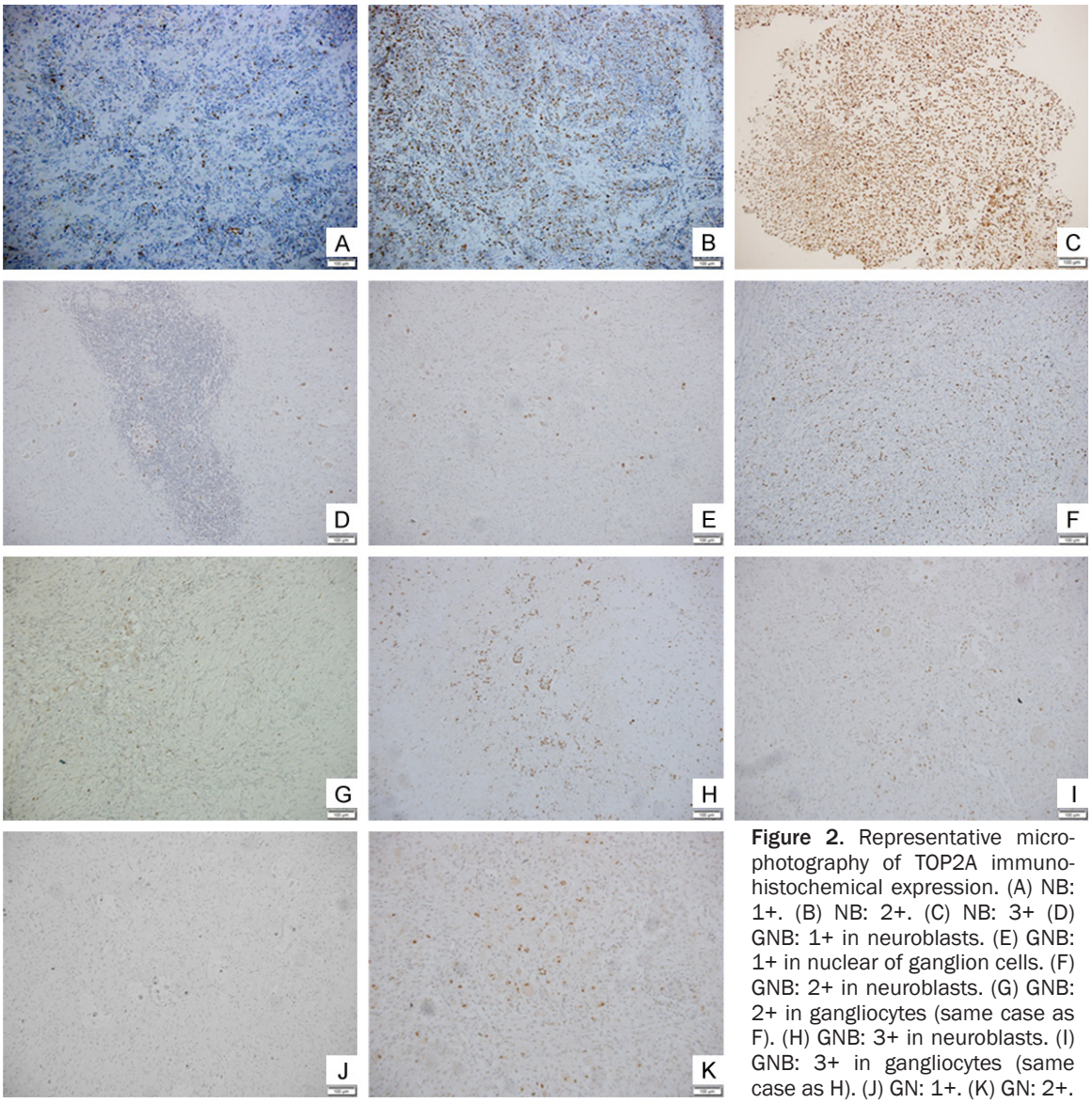

Figure 2. Representative microphotography of TOP2A immunohistochemical expression. (A) NB: 1+. (B) NB: 2+. (C) NB: 3+ (D) GNB: 1+ in neuroblasts. (E) GNB: 1+ in nuclear of ganglion cells. (F) GNB: 2+ in neuroblasts. (G) GNB: 2+ in gangliocytes (same case as F). (H) GNB: 3+ in neuroblasts. (I) GNB: 3+ in gangliocytes (same case as H). (J) GN: 1+. (K) GN: 2+.

based chemotherapy for breast cancer patients [12]. However, to our knowledge TOP2A protein expression and its potential clinicopathological role in NT are not fully understood yet.

The current study was to evaluate the copy number and protein status of TOP2A in a series of neuroblastic tumors, and to explore the prognostic value of TOP2A in these lesions.

Methods and materials

Ethics statement

The study was approved by the Ethics Committee of Capital Medical University, China (registration number 2015SY71). Written informed consent was obtained from all of the participants involved. This study was performed at Capital Medical University, Beijing, China.

Patients and tumor samples

This retrospective analysis included a total of 89 primary tumor specimens (68 NB, 18 GNB and 3 GN), which were obtained at diagnosis from Beijing Children's Hospital (Beijing, China), Peking University Third Hospital, Children's Hospital of Hunan Province, 301 and 304 Hospitals between January 2011 and December 2014. Specimen was randomly selected from the patients whose diagnoses of NTs were based on histological and immunohistochemical examination. Selected clinical and

laboratory data (e.g., age at diagnosis, gender, tumor site) were retrieved from the Beijing Children's Hospital. Representative histological morphology of every type of NTs is shown in **Figure 1** and the clinical and pathological characteristics of patients are described in **Table 1**. NB risk assessment was defined by the International Neuroblastoma Staging System (INSS) [13]. Individual patients' data were provided in Supplementary Table 1. The study was approved by Capital Medical University Ethics Committee.

Immunohistochemistry (IHC)

TOP2A protein expression was detected using immunohistochemical Elivision method, according to the manufacturer's introduction with the following modifications. Briefly, sections were deparaffinized in xylene and rehydrated. Antigens were retrieved by heating the slides in citrate buffer (10 mmol/L, pH 6.0). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide. The primary monoclonal antibody was mouse anti-human TOP2A (monoclonal, clone 1E2; Gene Tex, Irvine, CA; diluted at 1:800). The second-generation biotin-free polymer detection system (Advance; Dako) was used to visualize antigen-antibody reaction. Stains were developed with 3,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO), and sections were counterstaining with Mayer hematoxylin. All reactions were performed with appropriate positive and negative controls. The reactive hyperplasia of amygdale was used as positive control and tissues with omission of the primary antibody were used as negative control. Slides were examined by two pathologists (Gong LP, Chen JM), who were blinded to the clinical data using an Olympus BX41 light microscopy (**Figure 2**). The primary antibody for TOP2A generated nuclear immunostaining. The TOP2A immunostaining was assessed semi-quantitatively, based on the percentage of positive cells in the tissues. The section was scored 0 when there was no stain; 1 for nuclear staining in <20% of tumor cells; 2 if 20-50% of tumor cells were stained; and 3 if >50% of tumor cells were positive. Samples were considered to be negative if they received a score of <2, weakly positive if =2, and strongly positive if =3. Accordingly, the definition of positive rate was the percentage of tumor cells with more than 20% TOP2A expressed in each NT group, and high expression rate was defined as the percentage of tumor cells with more than 50% TOP2A expressed in each NT group.

Fluorescence in situ hybridization (FISH)

TOP2A gene amplification was assessed by fluorescence in situ hybridization using TOP2A DNA probe kit (Vysis Inc., Downers Grove, Illinois, USA), according to the procedure specified by the manufacturer with the following modifications. Briefly, the 4 μm-thick sections were deparaffinized, hydrated, and pressure-cooked in 1 mM ethylene diamine tetraacetic acid (EDTA) buffer for 3 min. Next, the slides were subjected to protease digestion in 0.1% pepsin solution at 37°C for 20 min, dehydration and incubation with the probes in StatSpin® Abbott TermoBrite A. The probe and target DNA were codenatured at 75°C for 20 min and hybridized at 37°C for 24 h. The next day, the slides were rinsed in post-hybridization buffers, stained with anti-fade solution containing 4,6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA) and examined on a fluorescence microscope (BX63; Olympus, Tokyo, Japan) by two investigators independently. Signals were counted under a fluorescence microscope (Olympus BX61) equipped with a 100 W mercury lamp. To view signals, single bandpass filters for spectrum green, spectrum orange, and DAPI were used. At least a total number of 100 interphase nuclei were scored from most of specimens. TOP2A status was determined according to previous studies [15]. TOP2A gene amplification was described as a TOP2A/centromere of chromosome 17 (TOP2A/CEP17) ratio ≥2.0, while TOP2A deletion was described as a TOP2A/CEP17 ratio ≤0.8. The case was regarded as no alteration or normal when the ratio of TOP2A/CEP17 was between 0.8 and 2.0. Images of nuclei were captured by Olympus cellSense Microscope Imaging Software (Olympus, UK).

Statistical analysis

Statistical analyses were performed with SPSS 17.0 package (SPSS, Chicago, Illinois, USA). Spearman's correlation coefficient analysis was used for correlation analysis between TOP2A expression and clinico-pathological characteristics, while Kappa consistency test was used to compare the measurements by IHC and FISH methods. The Kaplan-Meier (Log-rank test) was used for univariate survival anal-

Table 2. Association between TOP2A expression and clinico-pathological features in a cohort of neuroblastic tumors

Variable	TOP2A protein expression					*P* value
	<20%	20-50%	>50%	Positive Rate**	High Expression Rate***	
Type						
NB#	21/67 (31.34%)	28/67 (41.79%)	18/67 (26.87%)	46/67 (68.66%)	26.87% (18/67)	P=0.006*
GNB##	14/18 (77.78%)	1/18 (5.56%)	3/18 (16.67%)	4/18 (22.22%)	3/18 (16.67%)	
GN###	1/3 (33.33%)	2/3 (66.67%)	0/3 (0)	2/3 (66.67%)	0/3 (0)	
Ki-67 Index						
<20%	27/40 (67.5%)	9/40 (22.5%)	4/40 (10%)	32.5% (13/40)	10% (4/40)	P=0.000*
20%-50%	7/26 (26.92%)	12/26 (46.15%)	7/26 (26.92%)	73.08% (19/26)	26.92% (7/26)	
>50%	0/12 (0)	6//12 (50%)	6/12 (50%)	100% (12/12)	50% (6/12)	
MKI						
<2%	10/14 (71.43%)	4/14 (28.57%)	0/14 (0)	28.57% (4/14)	0 (0/14)	P=0.001*
2%-4%	2/10 (20%)	5/10 (50%)	3/10 (30%)	80% (8/10)	30% (3/10)	
>4%	0/3 (0)	2/3 (66.67%)	1/3 (33.33%)	100% (3/3)	33.33% (1/3)	

Note: #NB: Neuroblastoma; ##GNB: Ganglioneuroblastoma; ###GN: Ganglioneuroma; *P<0.05; **positive rate: percentage of tumor cells with more than 20% TOP2A expressed in each NT group; ***high expression rate: percentage of tumor cells with more than 50% TOP2A expressed in each NT group.

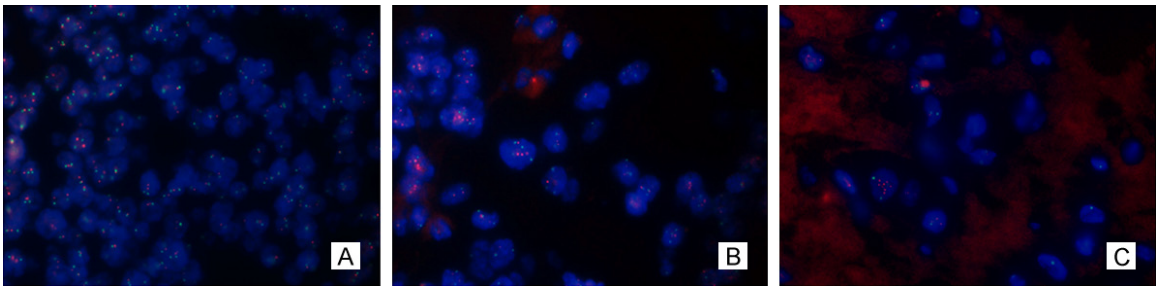

Figure 3. Representative FISH image of NT cells showing TOP2A gene status. A. Normal: Cells with 2 TOP2A signals (orange) and 2 CEP17 signals (green). B. No alteration (no amplification and deletion): The Ratios of TOP2A signals (orange)/CEP17 signals (green) in the tumor cell nuclei were between 0.8 to 2.0, and chromosome 17 polysomia existed in the nuclei of tumor cells. C. Amplification: The numbers of TOP2A signals (orange) were more than 2.0 copies of the CEP17 probe signals (green).

ysis and Cox proportional hazard model for multivariate survival analysis. All reported *P*-values are two-sided. *P*-values <0.05 were considered statistically significant.

Results

TOP2A protein expression in NTs

Of all the 88 NTs with sufficient material, fifty-two cases (52/88, 59.09%) were positive for TOP2A immunostaining, and 21 cases (21/88, 23.86%) showing high expression of this protein, scored as "3". The association between TOP2A protein expression and clinico-pathological features in a cohort of NTs, such as NT types, Ki-67 index and MKI (mitosis-karyorrhexis index), was summarized in **Table 2**. Interestingly, TOP2A expression was significantly associated with tumor types, Ki-67 index and MKI (*P*<0.001). The high expression rate for TOP2A strongly was significantly associated with aggressive tumor type (*P*=0.006), with 26.87% in neuroblastoma (NBs), as compared to 16.67% in GNB and 0% in GB. The positive rate of TOP2A expression was significantly correlated with Ki-67 index (*P*=0.000), which was 32.5% in the cases of Ki-67 index <20%, as compared with 100% in those >50%. In addition, TOP2A expression was also significantly associated with MKI (*P*=0.001).

TOP2A protein expression did not associate with other clinico-pathological features such as gender (*P*=0.700), age at diagnosis (*P*=0.132), site (*P*=0.657), clinical stage (*P*=0.725), risk classification (*P*=0.208) and *MYCN* amplification status (*P*=0.300).

Table 3. Relationship between *TOP2A* gene status and clinico-pathological features in a cohort of neuroblastic tumors

Variable	*TOP2A* gene status			*P* value
	Amplification	No alteration	Amplification Rate	
Category				
NB	16/59 (27.12%)	43/59 (72.88%)	27.12% (16/59)	*P*=0.014*
GNB	10/18 (55.56%)	8/18 (44.44%)	55.56% (10/18)	
GN	2/3 (66.67%)	1/3 (33.33%)	66.67% (2/3)	
Age				
≤18 m	2/28 (7.14%)	26/28 (92.86%)	7.14% (2/28)	*P*=0.000*
>18 m	25/52 (48.08%)	27/52 (51.92%)	48.08% (25/52)	
Clinical stage				
1, 2, 4 s	0/7 (0)	7/7 (100%)	0 (0/7)	*P*=0.028*
3, 4	17/40 (42.5%)	23/40 (57.5%)	42.5% (17/40)	
Risk				
Low	0/8 (0)	8/8 (100%)	0 (0/8)	P=0.001*
Intermediate	0/7 (0)	7/7 (100%)	0 (0/7)	
High	17/35 (48.57%)	18/35 (51.43%)	48.57% (17/35)	
Ki-67 Index				
<20%	16/39 (41.03%)	23/39 (58.97%)	41.03% (16/39)	*P*=0.040*
20%-50%	3/22 (13.64%)	19/22 (86.36%)	13.64% (3/22)	
>50%	2/10 (20%)	8/10 (80%)	20% (2/10)	

Note: *P<0.05.

TOP2A copy number alteration in NTs

Of the 80 NT cases with satisfactory fluorescence signals, twenty-eight cases (28/80, 35.00%) presented TOP2A gene amplification. TOP2A amplification rates in NB, GNB and GN groups were 27.12% (16/59), 55.56% (10/18) and 66.67% (2/3), respectively, and there were significant differences between the three groups (P=0.014), indicating that TOP2A amplification status was positively correlated with increasing tumor differentiation. Representative TOP2A gene status is shown in **Figure 3**. TOP2A amplification rate was found significantly higher in the older patients (>18 months) (25/52, 48.08%), as compared with the patients <18 months (2/28, 7.14%) (P=0.000). Furthermore, higher amplification rate of TOP2A was more frequently disclosed in tumors in advanced INSS stages (stage III and IV) (17/40, 42.5%), while the lower TOP2A gene status was frequently discovered in tumors in early and intermediate stages (I, II and IVs) (0/7, 0). The difference was statistically significant between the two groups (*P*=0.028). *TOP2A* amplification rate in low-risk, intermediate-risk and high-risk group were 0 (0/8), 0 (0/7) and 48.57% (17/35), respectively. And *TOP2A* amplification was significantly associated with high risk cases (*P*=0.001). Cases with Ki-67 index <20% had significantly higher *TOP2A* amplification rate (16/39, 41.03%), suggesting that *TOP2A* amplification was inversely interrelated with Ki-67 expression in NTs. The associations between *TOP2A* gene status and clinico-pathological features, including histological category, age, clinical stage, risk and Ki-67, were provided in **Table 3**.

Spearman rank correlation analysis showed that TOP2A amplification had no correlation with gender (*P*=0.850), onset location (*P*= 0.062), MKI (*P*=0.810), or *MYCN* gene status (*P*=0.532). Notably, no significant correlation was indicated between TOP2A protein expression and *TOP2A* gene amplification (*P*=0.223).

Follow-up study and survival analysis

A total of 54 cases were included in the follow-up study, comprising of 12 cases with high TOP2A expression, eighteen cases with low TOP2A expression, and 24 cases without TOP2A expression. Up to October 30th in 2015,

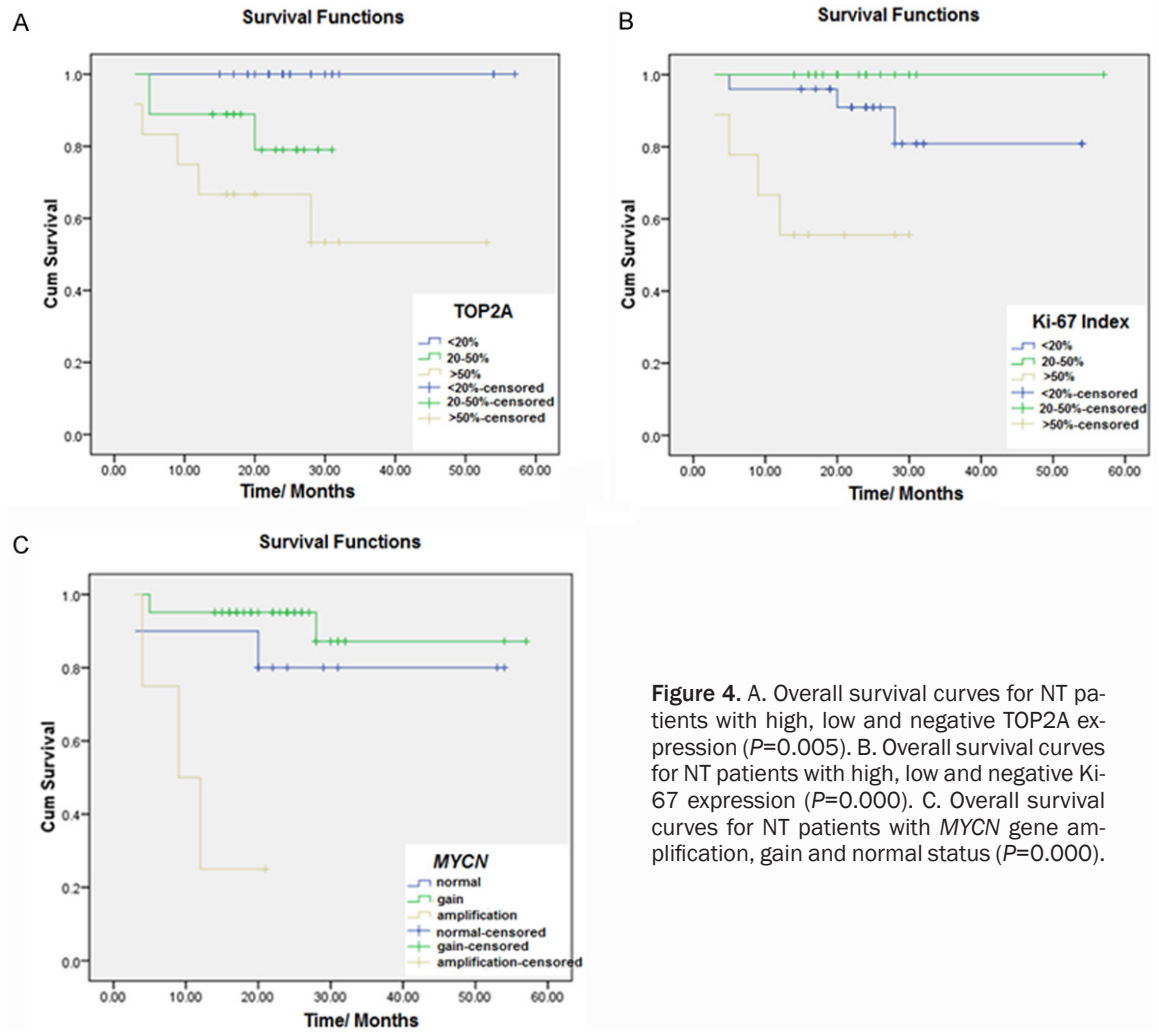

Figure 4. A. Overall survival curves for NT patients with high, low and negative TOP2A expression (P=0.005). B. Overall survival curves for NT patients with high, low and negative Ki-67 expression (P=0.000). C. Overall survival curves for NT patients with *MYCN* gene amplification, gain and normal status (P=0.000).

Table 4. Overall survival of NTs patients categorized by TOP2A expression, Ki-67 index and *MYCN* gene status

	Case (n)	Survival Time (months)	Median Survival Time (months)	OS Rate	*P* value
TOP2A					
>50%	12	3-53	18.5	58.33% (7/12)	0.005*
20-50%	18	5-31	19	83.33% (15/18)	
<20%	24	15-57	24	100% (24/24)	
Ki-67					
>50%	9	3-30	15.3	55.56% (5/9)	0.003*
20-50%	18	14-57	23.2	100% (18/18)	
<20%	25	5-54	25.7	92% (3/25)	
MYCN					
Amplification	4	4-21	11.5	25% (1/4)	0.000*
Gain	41	5-57	25.4	92.68% (38/41)	
Normal	10	3-54	27.6	80% (8/10)	

Note: *P<0.05.

of all 54 patients, forty-six cases were alive and 8 dead, survival time ranged from 3 to 57 months, with average survival time of 23.8 months. In the 12 patients with high TOP2A expression, seven patients were alive (7/12, 58.33%) and 5 dead (5/12, 41.67%), survival time ranged 3-53 months, with median survival time of 18.5 months. In the 18 NT patients with low TOP2A expressed, fifteen patients survived (15/18, 83.33%) and 3 died (3/18, 16.67%), survival time ranged 5-31 months, with median survival time of 19 months.

Table 5. The multivariate Cox regression analysis of NT prognosis

Factors	B	SE	Wald	df	Sig.	Exp (B)
TOP2A	1.652	.645	6.564	1	.010	5.216

Note: TOP2A regression coefficient is set for B, and the corresponding ratio risk is denoted as RR. RR=Exp (B), B=1.652, then RR=5.216, which means RR amplified 5.216 times if TOP2A expression increased each unit, suggesting that TOP2A expression is the unfavorable factor of NTs.

By contrast, all the 24 patients without TOP2A expression were alive (24/24, 100%), and survival time was between 15-57 months, with median survival time of 24 months. Kaplan-Meier analysis showed a statistically significant difference in OS rate between groups of patients with high, low and negative TOP2A expressions (log-rank test P=0.005) (**Figure 4A**; **Table 4**). Cases with high TOP2A expression were associated with worse survival (overall survival (OS) rate 58.33%) in comparison with low (83.33%) or negative (100%) expressed tumors (**Table 4**). Furthermore, survival analyses were additionally performed on the subgroups categorized by other clinico-pathological features, and these results indicated that both high Ki-67 expression and *MYCN* gene status were significantly associated with lower OS rate (*P*=0.003, *P*=0.000) (**Figure 4B**, **4C**; **Table 4**). There was no significant survival difference when age, gender, location, tumor type, clinical stage, risk classification or *TOP2A* gene status were considered. In the multivariate Cox regression analysis, TOP2A expression was an independent unfavorable prognostic factor of outcome in NT patients (*P*=0.010) (**Table 5**).

Discussion

In the current study, we evaluated the prognostic significance of the TOP2A in NTs. To our knowledge, this is the first study on TOP2A protein expression and gene amplification in NTs- a surprising fact, given the role of TOP2A as an important therapeutic target and its location at 17q. As mentioned above, 17q gain is one of the most frequent chromosomal alterations in NB, and this locus is a powerful predicting factor for patient survival and aggressive disease [6]. However, agreed-on strong candidates at this locus are lacking. In this study, we showed that *TOP2A* was a new candidate of prognostic importance within 17q, and high TOP2A expression was associated with worse patient survival, aggressive tumor type, high Ki-67 index and MKI, as well.

In all the 88 specimens analyzed, TOP2A protein expression was found in over half cases (55.09%), and one third of the cases presented high expression of TOP2A, with >50% NT cells being positive. In addition, the present data showed that high TOP2A protein expression was more frequently found in NB cases as compared to GNB and GN cases (*P*=0.006). As is known that NTs constitute a heterogeneous group of tumors, with the increasing differentiation of neoplastic cells and Schwannian stroma amount, neuroblastic tumor would range from undifferentiated NB to the well differentiated GB. Our data documented firstly that high TOP2A expression in NTs appeared to link with the aggressive tumor type-NB, suggesting its potential role in the development of NB. Besides that, a significant association between TOP2A overexpression and Ki-67 expression or MKI in NTs was also observed in this study. These findings were consistent with previous studies on other cancer types [16-18]. An earlier study by Qiao JH et al. reported a strong association of TOP2A protein expression with Ki-67 expression and MKI in invasive breast cancer. The similar observations were also found in hepatocellular and prostate cancers [16, 17]. Ki-67 protein is a human nuclear protein expressed only in proliferating cells during all of the active phases of the cell cycle (G1, S, G2, and M phases) but not in the resting cells (G0), while TOP2A expression peaked in G2/M phase cells and decreased when cells completed mitosis [10, 19]. Both Ki-67 and TOP2A expression can be used as molecular biomarkers of cell proliferation in various cancers. TOP2A expression is strongly correlated with Ki-67 expression in breast cancer [20, 21]. Taken together, we may speculate that TOP2A activity possibly signifies high proliferation and plays a role in the evolution of NT cells, as is known that NTs presented up-regulated proliferation and disturbed neuronal differentiation.

To the best of our knowledge, our study firstly documented the prognostic value of TOP2A in NTs. The follow-up analysis has demonstrated that NT patients with high TOP2A expression experienced significantly worse survival (OS rates 58.33%), and TOP2A expression was an independent unfavorable prognostic factor in NTs. The data were in agreement with recent

studies on other cancer types [22-28]. A study by de Resende MF et al. has reported that prostate cancer patients with higher levels of TOP2A presented shorter biochemical recurrence-free survival (BRFS), and TOP2A was an independent prognostic factor of BRFS in this cancer. It has been reported that ovarian cancer patients with overexpression of nuclear TOP2A protein had a marked decreased OS [23, 24]. Therefore, TOP2A was a strong indicator of poor prognosis in NTs, and detection of TOP2A overexpression may predicate the survival of NT patients.

In the current study, thirty-five percent of NT cases presented *TOP2A* amplification. Notably, *TOP2A* amplification status was more frequently presented in advanced neuroblastomas (stage III and IV), older patients (>18 m) and high risk cases. This significant correlation of *TOP2A* gene status with the INSS stage, age and risk classification strongly suggested the potential role of *TOP2A* in the prediction of clinical outcome. However, we failed to find the correlation between TOP2A immunopositivity and gene amplification. Therefore, we have demonstrated for the first time that TOP2A overexpression in NTs was probably not only resulted from *TOP2A* gene amplification. The discordance between *TOP2A* gene amplification and TOP2A protein expression has been demonstrated by several studies on other cancer types, such as breast cancer, prostate cancer and gastric carcinomas, which reported that TOP2A overexpression was not closely linked with *TOP2A* copy number amplification [29-31]. The possible reason for the inconsistence is still unclear. A study by Isaacs et al. has indicated that TOP2A was highly regulated at transcriptional and translational levels, suggesting that TOP2A overexpression may be secondary to the aberration at the transcriptional or translational level [18, 19]. Additionally, no relationship was detected between *TOP2A* amplification status and patient outcome or survival, which was in line with previous studies on other cancers [31, 32].

Currently, topoisomerase inhibitors, such as anthracyclines and epipodophyllotoxins, represent as the effective adjuvant agents widely used in the treatment of NTs. In chemotherapeutics of cancers, these agents stabilize the cleavable complexes formed by topoisomerases to cause DNA breaks and result in cell death. The sensitivity of tumor cell lines to TOP2A inhibitors is associated with TOP2A expression. Correlation between *TOP2A* amplification or deletion and response to anthracyclines has also been shown in breast cancers and other cancers [10, 11]. However, because of the inevitable heart toxicity and bone marrow inhibition, not all the patients benefit from these agents. The present results indicated that the expression of TOP2A predicted unfavorable prognosis in NTs. Accordingly, it is speculated that the survival time of NT patients with TOP2A overexpression may be extended by the use of TOP2A-targeted chemotherapeutic agents. Furthermore, our data showed that more than half of NT cases present TOP2A immunopositivity, in which 68.66% of NB cases were included. Accordingly, it is assumed that most of the NT (especially NB) patients may benefit from TOP2A-targeted chemotherapy, and the detection of TOP2A overexpression in NT patients might help in selecting appropriate patients for TOP2A-targeted therapies.

In conclusion, our studies identified TOP2A as an unfavorable prognostic marker in NTs. The expression of TOP2A protein was significantly related with aggressive tumor type, Ki-67 index and MKI. *TOP2A* amplification status was more frequently presented in the patients in advanced clinical stage and in high-risk group. TOP2A protein overexpression in NTs was not secondary to gene amplification. Thus, we propose that TOP2A, as a poor prognostic factor of NTs, could help in individualizing therapies to largely reduce side effects.

Disclosure of conflict of interest

None.

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Supplementary Table 1. Individual patients' data

No.	Original Case No.	Gender	Age (Months)	Site	Type	TOP2A Protein	*TOP2A* Gene	Ki-67 Index	MKI	*MYCN* Gene	Clinical Stage	Risk stratification	Hospital	survival condition	Follow-up time/Months
1	107572	Female	2	Adrenal/retro-peritoneal	NB	<20%	No alteration	<20%		Gain	4 s	Low Risk	Beijing Children's Hospital	Living	54
2	136917	Female	75	Adrenal/retro-peritoneal	NB	<20%	No alteration	<20%	<2%	Gain	4		Beijing Children's Hospital		
3	125390	Female	79	Adrenal/retro-peritoneal	NB	<20%	No alteration	<20%	<2%	Gain	4	High Risk	Beijing Children's Hospital		
4	126775	Male	24	Adrenal/retro-peritoneal	NB	<20%	No alteration	<20%		Gain	4	High Risk	Beijing Children's Hospital		
5	107281	Male	9	Mediastinum	NB	<20%	No alteration	20-50%		Gain			Beijing Children's Hospital		
6	127794	Male	10	Mediastinum	NB	<20%	No alteration	<20%		Gain			Beijing Children's Hospital	Living	24
7	130076	Male	16	Mediastinum	NB	<20%	No alteration	<20%	<2%	Gain			Beijing Children's Hospital	Living	22
8	131801	Female	12	Intraspinal	NB	<20%	No alteration	20-50%		Normal	4	High Risk	Beijing Children's Hospital	Living	20
9	122793	Female	17	Intraspinal	NB	<20%	No alteration	20-50%	<2%	Gain			Beijing Children's Hospital	Living	30
10	107118	Female	30	Adrenal/retro-peritoneal	GNB	<20%	No alteration	<20%		Normal			Beijing Children's Hospital	Living	54
11	122648	Female	45	Mediastinum	GNB	<20%	No alteration	<20%	<2%	Normal	2	Intermediate Risk	Beijing Children's Hospital	Living	31
12	137297	Female	61	Mediastinum	GNB	<20%	No alteration	<20%		Gain		Intermediate Risk	Beijing Children's Hospital	Living	15
13	125151	Male	22	Mediastinum	GNB	<20%	No alteration	<20%		Gain	4	High Risk	Beijing Children's Hospital	Living	28
14	22964	Male	48	Mediastinum	GNB	<20%	No alteration	<20%		Gain			Beijing Children's Hospital		
15	136450	Female	32	Adrenal/retro-peritoneal	NB	<20%	Amplification	<20%	2-4%	Gain	4	High Risk	Beijing Children's Hospital	Living	19
16	136056	Female	74	Adrenal/retro-peritoneal	NB	<20%	Amplification	<20%	<2%	Gain	4	High Risk	Beijing Children's Hospital	Living	19
17	133757	Male	15	Adrenal/retro-peritoneal	NB	<20%	Amplification	<20%	<2%	Gain	3	High Risk	Beijing Children's Hospital	Living	22
18	133174	Male	24	Adrenal/retro-peritoneal	NB	<20%	Amplification	<20%		Normal	4	High Risk	Beijing Children's Hospital	Living	22
19	121884	Male	36	Adrenal/retro-peritoneal	NB	<20%	Amplification	<20%	2-4%	Gain		High Risk	Beijing Children's Hospital		
20	1833	Male	36	Adrenal/retro-peritoneal	NB	<20%	Amplification			Gain			Beijing Children's Hospital		
21	125154	Male	45	Adrenal/retro-peritoneal	NB	<20%	Amplification	<20%		Gain	4	High Risk	Beijing Children's Hospital		

22	127823	Male	69	Adrenal/retro-peritoneal	NB	<20%	Amplification	<20%		Gain	4	High Risk	Beijing Children's Hospital	Living	24
23	126924	Male	33	Adrenal/retro-peritoneal	GNB	<20%	Amplification	<20%		Gain	4	High Risk	Beijing Children's Hospital	Living	25
24	107408	Male	42	Adrenal/retro-peritoneal	GNB	<20%	Amplification	<20%		Gain			Beijing Children's Hospital		
25	121660	Male	57	Adrenal/retro-peritoneal	GNB	<20%	Amplification	<20%		Gain	4	High Risk	Beijing Children's Hospital	Living	31
26	128083	Male	60	Adrenal/retro-peritoneal	GNB	<20%	Amplification	<20%	<2%	Gain	4	High Risk	Beijing Children's Hospital	Living	24
27	20142886	Male	64	Adrenal/retro-peritoneal	GNB	<20%	Amplification	<20%		Gain			Children's Hospital of Hunan Province		
28	124808	Male	108	Adrenal/retro-peritoneal	GNB	<20%	Amplification	20-50%		Gain	4	High Risk	Beijing Children's Hospital	Living	28
29	136975	Female	51	Mediastinum	GNB	<20%	Amplification	<20%	<2%	Gain			Beijing Children's Hospital	Living	15
30	127051	Male	44	Mediastinum	GNB	<20%	Amplification	<20%	<2%	Gain	4	High Risk	Beijing Children's Hospital	Living	25
31	105165	Female	24	Intraspinal	GNB	<20%	Amplification	20-50%		Gain			Beijing Children's Hospital	Living	57
32	120217	Male	36	Adrenal/retro-peritoneal	GN	<20%	Amplification	<20%		Gain	4	High Risk	Beijing Children's Hospital	Living	32
33	125304	Female	22	Adrenal/retro-peritoneal	NB	<20%		<20%		Gain	4	High Risk	Beijing Children's Hospital		
34	32819	Female	83	Adrenal/retro-peritoneal	NB	<20%		20-50%		Normal	4	High Risk	Beijing Children's Hospital	Living	24
35	32560	Male	46	Adrenal/retro-peritoneal	NB	<20%				Amplification			Beijing Children's Hospital		
36	135241	Female	36	Mediastinum	NB	<20%		20-50%		Gain			Beijing Children's Hospital	Living	17
37	129464	Female	1	Adrenal/retro-peritoneal	NB	20-50%	No alteration	20-50%		Gain	4s		Beijing Children's Hospital		
38	2442	Female	3	Adrenal/retro-peritoneal	NB	20-50%	No alteration	<20%		Gain			Beijing Children's Hospital		
39	133306	Female	17	Adrenal/retro-peritoneal	NB	20-50%	No alteration	<20%		Gain	4	High Risk	Beijing Children's Hospital		
40	138230	Female	27	Adrenal/retro-peritoneal	NB	20-50%	No alteration	>50%	>4%	Gain	4	High Risk	Beijing Children's Hospital	Death	5
41	2011	Male	25	Adrenal/retro-peritoneal	NB	20-50%	No alteration	20-50%	<2%	Gain			304 Hospital		
42	136204	Male	38	Adrenal/retro-peritoneal	NB	20-50%	No alteration	20-50%		Gain	4	High Risk	Beijing Children's Hospital	Living	16
43	153395	Male	40	Adrenal/retro-peritoneal	NB	20-50%	No alteration			Amplification			301 Hospital		
44	20134345	Male	48	Adrenal/retro-peritoneal	NB	20-50%	No alteration	>50%		Amplification			Beijing Children's Hospital		

45	137119	Female	7	Mediastinum	NB	20-50%	No alteration	>50%	>4%	Gain		Low Risk	Beijing Children's Hospital	Living	16
46	126525	Female	11	Mediastinum	NB	20-50%	No alteration	<20%	<2%	Gain			Beijing Children's Hospital	Living	26
47	134578	Female	15	Mediastinum	NB	20-50%	No alteration	20-50%	2-4%	Gain		Low Risk	Beijing Children's Hospital	Living	17
48	128356	Female	29	Mediastinum	NB	20-50%	No alteration	20-50%	2-4%	Gain	2	2	Beijing Children's Hospital	Living	24
49	107152	Male	2	Mediastinum	NB	20-50%	No alteration	>50%		Normal			Beijing Children's Hospital		
50	138277	Male	5	Mediastinum	NB	20-50%	No alteration	20-50%	2-4%	Gain		Low Risk	Beijing Children's Hospital	Living	14
51	137592	Male	7	Mediastinum	NB	20-50%	No alteration	20-50%		Gain		Low Risk	Beijing Children's Hospital	Living	18
52	129069	Male	9	Mediastinum	NB	20-50%	No alteration	<20%		Gain	4	Low Risk	Beijing Children's Hospital		
53	122899	Male	11	Mediastinum	NB	20-50%	No alteration	20-50%	<2%	Gain	4	Intermediate Risk	Beijing Children's Hospital		
54	129091	Male	12	Mediastinum	NB	20-50%	No alteration	20-50%		Gain	2	Low Risk	Beijing Children's Hospital	Living	23
55	128208	Female	60	Mediastinum	GN	20-50%	No alteration	<20%		Normal			Beijing Children's Hospital	Death	20
56	123982	Female	32	Intraspinal	GN	20-50%	No alteration	<20%		Normal			Beijing Children's Hospital	Living	29
57	131077	Female	9	Adrenal/retro-peritoneal	NB	20-50%	Amplification	20-50%	<2%	Amplification			Beijing Children's Hospital		
58	167772	Female	24	Adrenal/retro-peritoneal	NB	20-50%	Amplification			Gain			Beijing Children's Hospital		
59	152145	Female	31	Adrenal/retro-peritoneal	NB	20-50%	Amplification			Gain			Peking University Third Hospital		
60	123681	Female	38	Adrenal/retro-peritoneal	NB	20-50%	Amplification	<20%		Amplification	4	High Risk	Beijing Children's Hospital		
61	1472	Male	9	Adrenal/retro-peritoneal	NB	20-50%	Amplification	20-50%		Gain			Beijing Children's Hospital	Living	31
62	131475	Male	48	Adrenal/retro-peritoneal	NB	20-50%	Amplification	>50%	2-4%	Amplification	4	High Risk	Beijing Children's Hospital	Living	21
63	124577	Male	56	Adrenal/retro-peritoneal	NB	20-50%	Amplification	<20%		Gain	4	High Risk	Beijing Children's Hospital	Death	5
64	138763	Male	59	Adrenal/retro-peritoneal	NB	20-50%	Amplification	<20%		Gain	4	High Risk	Beijing Children's Hospital	Living	17
65	125246	Male	83	Adrenal/retro-peritoneal	NB	20-50%	Amplification			Gain	4	High Risk	Beijing Children's Hospital	Living	27
66	124897	Female	77	Adrenal/retro-peritoneal	GNB	20-50%	Amplification	20-50%	2-4%	Gain	4	High Risk	Beijing Children's Hospital	Living	26

67	138216	Male	55	Bone Marrow	NB	20-50%		>50%		Gain	4	High Risk	Beijing Children's Hospital	Living	14
68	131408	Female	4	Adrenal/retro-peritoneal	NB	>50%	No alteration	20-50%	2-4%	Gain		Low Risk	Beijing Children's Hospital	Living	20
69	107515	Female	6	Adrenal/retro-peritoneal	NB	>50%	No alteration	>50%		Amplification	3	High Risk	Beijing Children's Hospital		
70	126684	Female	12	Adrenal/retro-peritoneal	NB	>50%	No alteration	<20%		Gain	4	Intermediate Risk	Beijing Children's Hospital		
71	21302	Male	2	Adrenal/retro-peritoneal	NB	>50%	No alteration	20-50%		Normal			Beijing Children's Hospital		
72	127262	Male	22	Adrenal/retro-peritoneal	NB	>50%	No alteration			Amplification	4		Beijing Children's Hospital	Death	4
73	165531	Male	23	Adrenal/retro-peritoneal	NB	>50%	No alteration	>50%		Amplification	4	High Risk	Beijing Children's Hospital	Death	9
74	134515	Male	11	Mediastinum	NB	>50%	No alteration	20-50%		Gain	4	Intermediate Risk	Beijing Children's Hospital	Living	17
75	136680	Male	33	Mediastinum	NB	>50%	No alteration	20-50%	2-4%	Gain	4	High Risk	Beijing Children's Hospital		
76	124729	Female	22	Lymph nodes in the neck	NB	>50%	No alteration	>50%		Gain	3	Intermediate Risk	Beijing Children's Hospital	Living	28
77	126811	Male	60	Lymph nodes in the neck	NB	>50%	No alteration	20-50%		Normal	4	High Risk	Beijing Children's Hospital		
78	124854	Female	33	Adrenal/retro-peritoneal	GNB	>50%	No alteration	<20%		Gain	4	High Risk	Beijing Children's Hospital	Death	28
79	122525	Male	24	Mediastinum	GNB	>50%	No alteration	<20%		Gain	4	High Risk	Beijing Children's Hospital		
80	724430-1	Male	48	Adrenal/retro-peritoneal	NB	>50%	Amplification			Normal			301 Hospital	Living	53
81	158938	Male	72	Adrenal/retro-peritoneal	NB	>50%	Amplification			Gain			304 Hospital		
82	122646	Male	36	Intraspinal	NB	>50%	Amplification	>50%	>4%	Gain	4	High Risk	Beijing Children's Hospital	Living	30
83	165916	Female	41	Adrenal/retro-peritoneal	GNB	>50%	Amplification			Gain			Beijing Children's Hospital		
84	136265	Female	17	Adrenal/retro-peritoneal	NB	>50%		>50%		Amplification	4s	High Risk	Beijing Children's Hospital	Death	12
85	136836	Male	27	Adrenal/retro-peritoneal	NB	>50%		>50%		Normal	4	High Risk	Beijing Children's Hospital	Death	3
86	123161	Male	48	Adrenal/retro-peritoneal	NB	>50%		<20%		Gain	4		Beijing Children's Hospital	Living	32
87	135666	Male	14	Mediastinum	NB	>50%		20-50%	2-4%	Gain			Beijing Children's Hospital	Living	16
88	138569	Male	37	Bone Marrow	NB	>50%		20-50%		Gain	4	High Risk	Beijing Children's Hospital		
89	132067	Female	24	Bone Marrow	NB		No alteration	20-50%		Normal	4	High Risk	Beijing Children's Hospital	Living	20

Note: NT: Neuroblastic tumors, NB: Neuroblastoma, GNB: Ganglioneuroblastoma, GN: Ganglioneuroma; The blank in immunohistochemistry and FISH means no specimens.