Clinical Report

Journal of
INTERNATIONAL
MEDICAL RESEARCH

Journal of International Medical Research
2018, Vol. 46(3) 1209–1220

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DOI: 10.1177/0300060517732256
journals.sagepub.com/home/imr

CD133 expression and *MYCN* amplification induce chemoresistance and reduce average survival time in pediatric neuroblastoma

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Abstract

Objectives: Neuroblastoma (NB) is the most common pediatric solid tumor derived from the sympathetic nervous system. *MYCN* is amplified in nearly half of patients with NB, and its association with rapid disease progression and poor outcome is controversial. Characterization of cancer stem cells (CSCs) in NBs has been rarely studied. This study was performed to determine whether *MYCN* and CD133+ CSCs are associated with chemotherapy resistance and the survival time of patients with NB.
Methods: Fifty patients with an unequivocal pathological diagnosis of NB were recruited. *MYCN* expression levels were measured before therapy. CSCs were derived and their multipotency tested by directed differentiation. The patients' responses to chemotherapy and average survival time were compared among the groups as follows: CD133+, CD133−, *MYCN* amplification ≥5 times (i.e. *MYCN*≥5), *MYCN*<5, CD133+ plus *MYCN*≥5, and CD133− plus *MYCN*<5.
Results: CD133+ CSCs differentiated into neuron-like cells. CD133+ patients had a significantly poorer response to chemotherapy than did CD133− patients. CD133+ plus *MYCN*≥5 patients had a significantly shorter average survival time than did CD133− plus *MYCN*<5 patients.
Conclusions: CD133+ CSCs are chemoresistance. CD133 expression and *MYCN* amplification can be used together as a prognostic indicator of disease outcome.

Keywords

Cancer stem cells, CD133, chemoresistance, *MYCN*, neuroblastoma, survival time

Date received: 17 July 2017; accepted: 24 August 2017

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Introduction

Neuroblastoma (NB) is the most common extracranial pediatric solid tumor. NB tumor cells, which are derived from the embryonic neural crest-derived cells, differentiate into nerve cells of the sympathetic nervous system during normal development.[1,2] It is widely assumed that NBs are embryonic tumors originating from a developmental defect that prevents normal cell differentiation and induces continuous replication.[3,4] NBs most frequently originate in the adrenal medulla and paraspinal or periaortic regions, where the sympathetic nerve tissues are present from the neck to the pelvis.[5,6]

The prevalence of NBs is approximately 1 in 7000 live births; the incidence is about 10.54 cases per 1 million per year among children younger than 15 years.[7] About 90% of affected patients are younger than 5 years at diagnosis, and 37% are infants; the median age at diagnosis is 19 months.[8] Most NBs are sporadic, and only 1% to 2% of affected patients have family history of NB. The most common symptom of NB is an abdominal mass; other signs include proptosis and periorbital ecchymosis, abdominal distention, bone pain, pancytopenia, fever, hypertension, anemia, paralysis, and watery diarrhea.[9,10] The clinical course is heterogeneous, ranging from spontaneous regression in neonates to life-threatening metastatic disease in older children.[5] Medical treatments include observation, chemotherapy, surgery, immunotherapy, and autologous peripheral blood stem cell transplantation. Although the 5-year survival rate has significantly improved during the past several years, high-risk NBs remain among the most difficult tumors to cure, with a long-term survival rate of only 40%, despite intensive multimodal therapy.[6,11,12]

Accumulating evidence in recent years has demonstrated that tumors are characterized by a hierarchical organization regulated by a minority of cells called cancer stem cells (CSCs).[13–15] CSCs constitute <1% of tumor cells and have the exclusive ability to self-sustain and self-renew, thus generating heterogeneous lineages of cancer cells of a tumor.[16] The existence of self-renewing and multipotent CSCs in NBs was partially addressed by Walton et al.[17] and Ross et al.[18] Hansford et al.[19] demonstrated that cells that had dissociated from aggressive NB tumors grew like spheres, differentiated into neurons, and formed NBs when orthotopically injected into severe combined immunodeficiency/beige mice. CD133 is a cell-surface glycoprotein harboring five transmembrane domains.[20] Knock-down of CD133 in NB cells effectively retards cell growth.[21] Upregulation of CD133 decreases sensitivity to cytostatics *in vitro*.[22] Whether CD133+ cells have effects on responses to chemotherapy and average survival times requires further research. To the best of our knowledge, no studies have addressed these issues.

MYCN amplification (defined as >10 copies per diploid genome) occurs in 16% of the International Neuroblastoma Risk Group (INRG) analytic cohort and 40%–50% of high-risk NBs.[23,24] Most of the unfavorable clinical and pathobiological features of NB are associated, to some degree, with *MYCN* amplification. However, one controversial study found that *MYCN* is not related to NB progression.[25,26] Whether *MYCN* amplification is associated with NB progression therefore requires further research.

The purpose of this study was to demonstrate whether *MYCN* amplification and the presence of CD133+ cells in NBs correlated with chemoresistance and average survival time. We recruited patients with NB and collected peripheral blood samples to measure *MYCN* amplification before therapy. After surgery, CSCs were derived, and the number of CD133+ cells was calculated. The correlation of *MYCN* amplification

and CD133+ CSCs with disease severity and overall survival was evaluated.

Materials and methods

Patient recruitment

This study was reviewed and approved by The Second Hospital of Hebei Medical University Ethics Committee before the study began. Written informed consent was obtained from all patients recruited. The minimum criterion for a diagnosis of NB was one of the following: (1) an unequivocal pathological diagnosis obtained by light microscopic examination of tumor tissue in accordance with the International Neuroblastoma Pathology Classification or (2) the combination of a bone marrow aspirate or trephine biopsy specimen containing unequivocal tumor cells and a urine specimen containing increased levels of catecholamine metabolites.[27] Patient recruitment started in January 2014 and ended in May 2016.

The International Neuroblastoma Staging System stratifies NB into the following stages according to its anatomical presence at diagnosis: stage I, IIa, IIb, III, IV, and IVS.[1,3,4] The INRG Staging System (INRGSS) classifies NB into the following pretreatment groups: very low, low, intermediate, and high risk.[28] Here we recruited patients with stage IIa to IV NB and an INRGSS classification of very low risk to high risk. *MYCN* expression levels were measured before any therapy. Total RNA samples were prepared from the peripheral blood of six healthy volunteers and were kindly provided by Dr. Zhi-Zhao Ma. The volunteers (three females and three males, aged 10–42 years) granted informed consent for the acquisition of their samples.

The CSCs were derived and their multipotencies tested by directed differentiation (see below). The response to chemotherapy and average survival time of these patients were gathered and compared among the following groups: CD133+, CD133−, *MYCN* amplification ≥5 times (i.e. *MYCN*≥5), *MYCN*<5, CD133+ plus *MYCN*≥5, and CD133− plus *MYCN*<5. *MYCN* amplification and CD133 expression were compared among these six groups.

Therapy

MYCN amplification was calculated before any therapy. For patients with very low-risk NB, intensive observation was performed every 2 months after surgery; for those with low-risk, intermediate-risk, and high-risk NB, surgery was performed before or during chemotherapy. For patients aged >18 months, radiotherapy was administered after chemotherapy. The criteria for chemotherapy were as follows: no uncontrolled infection or organ dysfunction, an alanine transaminase level <3.0-fold higher than the upper limit of the reference range, a total bilirubin level <1.5-fold higher than the upper limit of the reference range, an absolute neutrophil count >1.0 to 1.2×10^9/l, and a platelet count $>75 \times 10^9$/l.

The chemotherapy protocol for patients with low- to intermediate-risk NB was as follows: vincristine (VCR) (1.5 mg/m^2) + carboplatin (CBP) (550 mg/m^2) + adriamycin (ADR) (30 mg/m^2) + cyclophosphamide (CTX) (1.0 g/m^2) for the first cycle, and VCR (1.5 mg/m^2) + CBP (550 mg/m^2) + etoposide (VP-16) (160 mg/m^2) + CTX (1.0 g/m^2) for the second cycle. These two cycles were rotated, and 9-cis retinoic acid (9-cis-RA) (150–160 mg/m^2) was administered for 6 months. The chemotherapy protocol for patients with high-risk NB was as follows: VCR (1.5 mg/m^2) + cisplatin (CDDP) (25 mg/m^2) + VP-16 (100 mg/m^2) + CTX (1.0 g/m^2), ifosfamide (IFOS) (1.5 mg/m^2) + CBP (550 mg/m^2) + docetaxel/trastuzumab/pertuzumab (THP) (30 mg/m^2). These two cycles were rotated, and 9-cis-RA (150–160 mg/m^2) was

administered for 6 months. The drug concentrations for patients with a body weight <12 kg were as follows: VCR (0.05 mg/kg), CBP (16 mg/kg), ADR (1 mg/kg), CTX (33 mg/kg), VP-16 (5 mg/kg), 9-cis-RA (5.33 mg/kg per day), CDDP (0.85 mg/kg), IFOS (50 mg/kg), and THP (1 mg/kg).

MYCN amplification

Total RNA was extracted from the peripheral blood of patients with NB and healthy volunteers using RNeasy columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The total RNA (1 μg) was reverse-transcribed into cDNA (Clontech, Mountain View, CA, USA). A SYBR Green Real-time PCR Kit (Applied Biosystems, Foster City, CA, USA) was used to quantify gene expression levels. Data were analyzed using SDS Relative Quantification Software version 2.2.2 (Applied Biosystems). Ct values were exported into Excel software for data analysis. *MYCN* amplification levels were compared between patients with NB and healthy volunteers. The *MYCN* forward primer was TCTCTCCGAGCAGCGCA, and the reverse primer was TGGCAGC AGGAGACCC.

Tissue dissection and preparation of single-cell suspensions

Tumor samples were collected, and CSCs were immediately derived. The tumor tissue was isolated and minced with scissors, then rinsed with Hank's balanced salt solution (HBSS) (without Ca^{2+} or Mg^{2+}) (product no. 14170112; Life Technologies, Carlsbad, CA, USA) with 20% fetal bovine serum (FBS) (product no. 10439001; Life Technologies) at least three times to remove blood. Equal amounts of the minced tissue were separated into three 15-ml centrifuge tubes. The tissue pieces were incubated at 37°C for 15 min in 10 ml of dissociation solution: HBSS (without Ca^{2+} or Mg^{2+}) supplemented with 0.015M HEPES (Life Technologies), 5.4 mg/ml D-glucose, 0.25% trypsin (product no. 15050057; Life Technologies), 80 U/ml DNase (Sigma-Aldrich, St. Louis, MO, USA), 0.7 mg/ml hyaluronidase (Sigma-Aldrich), and 2 mg/ml kynurenic acid (Sigma-Aldrich). This was followed by gentle trituration and incubation in the same solution at 37°C for another 10 min. Excess solution was removed and the tissue further mechanically dissociated to a single-cell suspension.

Neurosphere cultures

Single cells were plated at 100 cells per well in 6-well ultra-low-attachment multiwall polystyrene plates (Falcon; Becton Dickinson, Franklin Lakes, NJ, USA) in serum-free expansion medium composed of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 Nutrient Mixture (Life Technologies) supplemented with 40 ng/ml epidermal growth factor (R&D Systems, Minneapolis, MN, USA), 20 ng/ml basic fibroblastic growth factor (R&D Systems), 1% B27 supplement (Life Technologies), 2 μg/ml heparin (Sigma-Aldrich), and 100 units/ml penicillin/streptomycin. The cultures were maintained at 37°C in a humid atmosphere with 95% O_2 and 5% CO_2. Fifty percent of the culture medium was changed with fresh medium containing fresh growth factors every other day. After 7 days, the neurospheres were processed for fluorescence-activated cell sorting (FACS) analysis or passaged by mechanical dissociation and resuspended.

Differentiation of CSCs into neurons

The six-well plates were coated with 20 mg/ml poly-L-ornithine (product no. P4957; Sigma-Aldrich) for at least 2 h in the

incubator. The poly-L-ornithine solution was then removed, and the plate was rinsed once with water. Laminin (5 mg/ml) (product no. L2020; Sigma-Aldrich) was added to the plate, which then remained in the incubator for 1 h. Phosphate-buffered saline (PBS) was then used to rinse the plate once immediately before use. The neurospheres were mechanically dissociated and digested with 0.25% trypsin for 10 min. Single cells were then plated on coated six-well plates and mixed with glial cell culture medium (kindly provided by Dr. Zhi-Zhao Ma) with DMEM and L-glutamine at a ratio of 1:1, plus 1% FBS, 100 nM all-trans-retinoic acid (product no. R2500; Sigma-Aldrich), 20 ng/ml brain-derived neurotrophic factor (product no. 203702; Millipore, Billerica, MA, USA), and 20 ng/ml neurotrophin-3 (product no. GF308; Millipore).

FACS

The cells were mechanically dissociated to obtain a single-cell suspension in a solution containing 2 mM EDTA (Sigma-Aldrich) and 0.5% FBS (Sigma-Aldrich) in PBS. The cells were harvested by centrifugation ($500 \times g$, 5 min, 4°C) and incubated with a PE-conjugated anti-CD133 antibody (#85-12-1339-73, eBioscience) for 2 h on ice. They were then incubated or 2 h on ice with secondary antibody conjugated to phycoerythrin, washed with PBS twice, filtered through a 70-µm cup filter (Falcon; Becton Dickinson), and kept on ice until FACS analysis. COS-7 cells were used as a negative control, and liver tumor cells with high CD133 expression (kindly provided by Dr. Jun Li, Hepatological Surgery Department, The Second Hospital of Hebei Medical University, Shijiazhuang, Hebei Province, China) were used as a positive control. Cell sorting was performed using a FACS Vantage TS flow cytometer (Becton Dickinson).

Immunohistochemistry

The cells were fixed with 4% paraformaldehyde for 10 min at room temperature. After blocking with 5% normal goat serum in PBS, the cells were incubated with primary antibody (neuronal class III β-tubulin antibody, product no. MAB1637; Chemicon) overnight at 4°C. After washing three times with PBS, the cells were incubated with secondary antibody (Alexa Fluro 647-AffniPure Goat anti-Mouse IgG, product no. 115-605-205; Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at room temperature. The labeled slides were dehydrated in alcohol and xylene before being cover-slipped with mounting media.

Statistical analysis

Data are expressed as the mean ± standard deviation. A paired Student t test (two tailed) was performed to analyze the data using SPSS v16.0 for Windows (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered statistically significant.

Results

Patients

This study included 50 pediatric patients with NB (28 males, 22 females; ages, 3 months to 8 years). The characteristics of the patients are shown in Table 1.

Computed tomography and pathological images of a representative patient with NB

Computed tomography (CT) images of the NB in a representative patient are shown in Figure 1(a) and (b). Figure 1(a) is a CT image before routine chemotherapy, and Figure 1(b) is a CT image after chemotherapy of the same patient. The solid tumor almost disappeared after chemotherapy. Figure 1(c) and (d) are pathological

Table 1. Patients' characteristics

Parameters	Patients, n (%)
Sex	
Male	28 (56)
Female	22 (44)
Age (months)	
Range	12–168
Median	24
INSS stage	
IIa	8 (16)
IIb	16 (32)
III	15 (30)
IV	11 (22)
INRGSS stage	
Very low	4 (8)
Low	20 (40)
Intermediate	17 (34)
High	9 (18)
MYCN amplification	
≥5-times	21 (56)
<5-times	27 (40)
Undetectable	2 (4)

INSS, International Neuroblastoma Staging System; INRGSS, International Neuroblastoma Risk Group Staging System

images of the NB tumor stained by hematoxylin and eosin. On microscopy, the tumor cells are typically small, round, and blue and exhibit rosette patterns.

Formation of neurospheres from NB-derived CSCs

Single tumor cells were plated onto low-attachment plates and cultured in serum-free medium with growth factors. Figure 1 (e) shows the third day after plating, and Figure 1(f) shows the seventh day after plating. The neurospheres grew larger, demonstrating that CSCs were present inside the neurosphere.

Differentiation of CSCs into neuron-like cells

Multipotent CSCs can differentiate into somatic cells. Whether the CSCs derived from the NB tissue differentiate into neurons determines their identity. As shown in Figure 2(a) and (b), once the neurospheres attached to the bottom of the plate, the cells migrated out of the cell cluster. When the medium was changed to neuron-supporting medium for 2 weeks, neuron-like cells appeared as shown in Figure 2(c). These cells were β-tubulin-positive as shown in Figure 2(d), indicating that they were neurons.

FACS analysis of CD133+ cells among the CSCs

COS-7 cells were used as the negative control as shown in Figure 3(a), and liver tumor cells with a high CD133 expression level were used as the positive control as shown in Figure 3(b). The neurospheres were harvested and stained with a CD133 antibody. FACS analysis shows that there were 0.1% CD133+ cells in the CSCs derived from the NB tissue (Figure 3(c)). Whether and how these CSCs influence cancer pathophysiology and response to chemotherapy requires further research.

Correlation of CD133+ CSCs and MYCN amplification with patients' responses to chemotherapy and average survival time

Achievement of a complete response, a very good partial response, and a partial response measures patients' responses to chemotherapy. Patients with CD133+ NB had a significantly lower likelihood of achieving a complete response ($P<0.01$), a very good partial response, and a partial response ($P<0.05$) than patients with CD133− NB (Figure 4). However, CD133 expression did not significantly influence average survival time. *MYCN* amplification >5-times influenced the response to chemotherapy similar to the effects of CD133 expression. However, >5-fold *MYCN* amplification was associated with

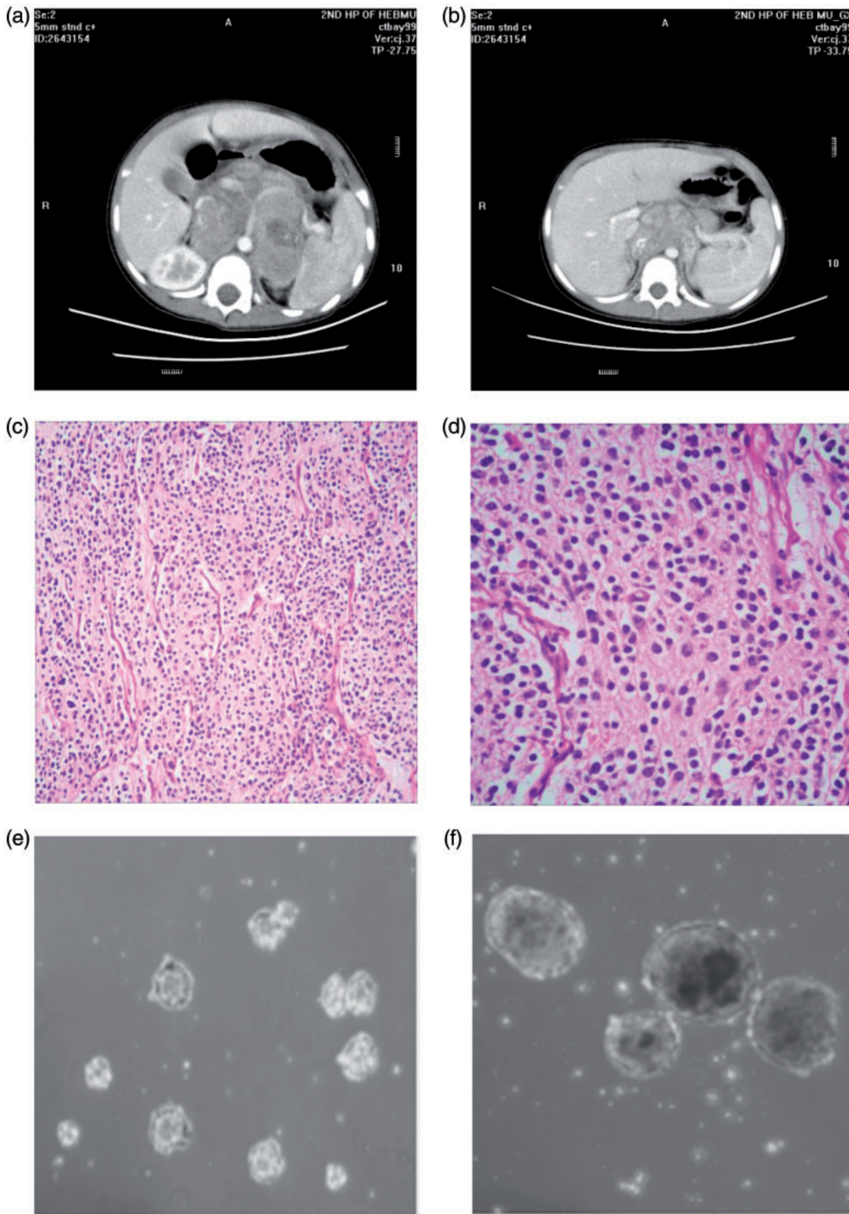


Figure 1. Computed tomography and pathological images of a neuroblastoma (NB) of a representative patient. (a) Computed tomography image before routine chemotherapy. (b) Computed tomography image after chemotherapy of the same patient. The solid tumor almost disappeared after chemotherapy. (c, d) Pathological images of the NB stained using hematoxylin and eosin. Microscopic examination showed that the tumor cells were typically small, round, and blue and exhibited rosette patterns. (e, f) Images of neurospheres on days 3 and 7 after the tumor was dissociated and grown as single cells.

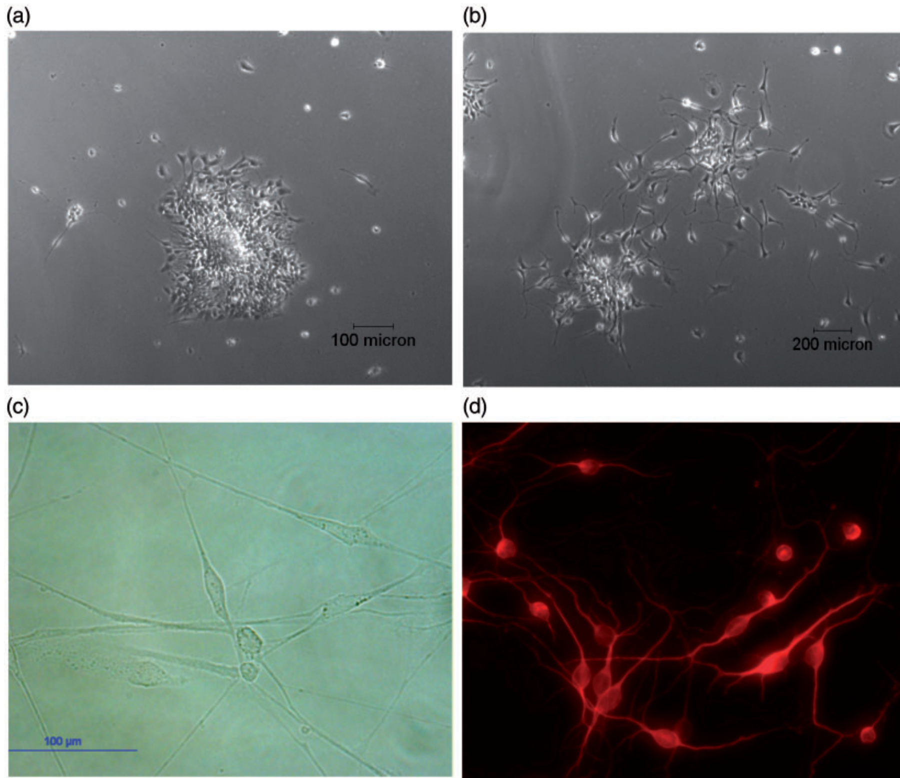
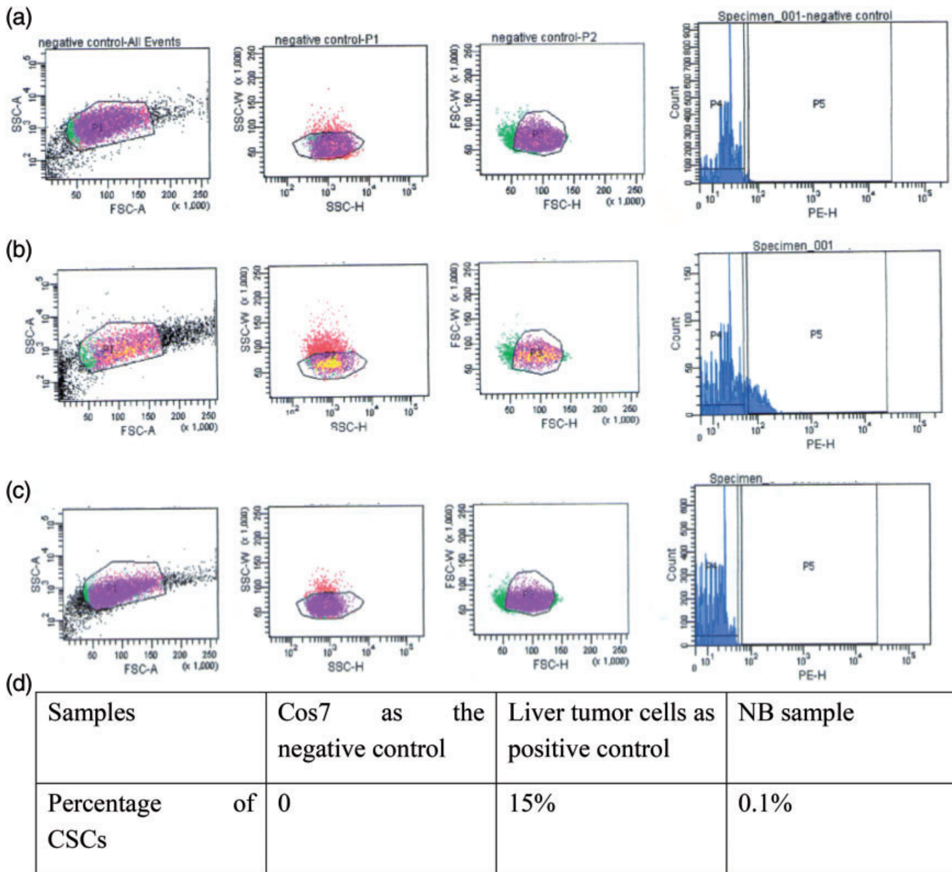


Figure 2. Differentiation of cancer stem cells (CSCs) into neuron-like cells. (a, b) Floating neurospheres attached to the coated plate bottom and migrated out of the cell cluster. (c) In neuron growth medium, these CSCs differentiated into neuron-like cells. (d) The neuron-like cells reacted with the β-tubulin antibody, indicating that these cells were neurons.

significantly decreased average survival time compared with <5-fold *MYCN* amplification (P<0.05). Patients with CD133+ NB with >5-fold *MYCN* amplification experienced a significantly shorter average survival time than patients with CD133− NB with <5-fold *MYCN* amplification (P<0.01).

Discussion

NB, which is the most common pediatric solid tumor, exhibits a heterogeneous clinical course ranging from spontaneous regression in neonates to life-threatening metastatic disease.[5] Many patients with NB experience relapse after achieving a very good response to chemotherapy.[6,11,12] This suggests that self-renewing, multipotent CSCs exist within NB tumor tissue and that these cells are chemoresistant. In the present study, we demonstrate the presence of CD133+ CSCs within NB tumor tissue and show that these cells differentiate into neuron-like cells. The proportion of CD133+ cells was approximately 0.1%, which may be explained by the affinity of the CD133 antibody for its epitope and the neurosphere culture conditions. Improved culture conditions and a high level of technical expertise to



Samples	Cos7 as the negative control	Liver tumor cells as positive control	NB sample
Percentage of CSCs	0	15%	0.1%

Figure 3. FACS analysis of CD133 expression. (a) COS-7 cells served as the negative control (b) Liver tumor cells served as the positive control. (c) Cancer stem cells derived from a NB. (d) Abundance of CSCs. There were 8.5% CD133+ cells among the liver tumor cells. There were only 0.1% CD133+ cells among the CSCs derived from the NB.
PE, phycoerythrin; FSC-A, forward-angle scatter cell area; SSC-H, side-angle scatter cell height.

reproducibly and efficiently perform cell separations are required for future studies.

The *MYCN* amplification level is a predictor of disease progression.[23,24] Here we demonstrated that CD133 expression could be used as an indicator of the response to chemotherapy. Patients with CD133+ NB had a significantly worse response than those with CD133− NB ($P<0.05$). *MYCN* and CD133 together could be used as an indicator of the average survival time of patients with NB. We only recruited 50 patients, and larger study groups are required for future studies. Further, we excluded patients with NB who had undergone autologous peripheral blood stem cell transplantation, immunotherapy, or both because of the uncertainty of these treatments. We plan to closely observe the clinical effects of these two treatments and recruit these patients into our future studies.

The histone deacetylase inhibitor valproic acid increases the number of CD133+ cells that exhibit low sensitivity to cytostatics in patients with NB.[22] The epigenetic effect of valproic acid suggests

Parameters	Patients	Complete response (%)	Very good partial response (%)	Partial response (%)	Average survival (months) (Average±SD)
CD133+	12	1 (8)	2 (17)	9 (75)	36.3±6.9
CD133-	38	8 (21)**	19 (50)*	11(29)*	41.5±9.8
MYCN≧5	21	0	7 (33)	14(67)	24.6±4.7
MYCN<5	27	9 (33)**	12(45)	6(22)*	40.2±6.9*
CD133+ and MYCN≧5	10	1(10)	2(20)	7(70)	12.4±2.8
CD133- and MYCN<5	25	8(32)**	12(48)*	5(20)**	42.7±8.3**

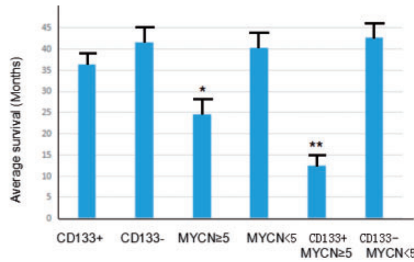


Figure 4. Correlation of CD133 expression and *MYCN* amplification with response to chemotherapy and average survival time of patients with NB. $^{*}P<0.05$, $^{**}P<0.01$. CD133+, *MYCN*≥5, or both were associated with a significantly decreased response to chemotherapy compared with CD133−, *MYCN*<5, or both ($P<0.05$). CD133+ and *MYCN*≥5 were associated with a significantly reduced average survival time compared with the other patterns ($P<0.01$). SD, standard deviation.

that the variability of CD133 expression is related to the acetylation of histones and methylation of the *CD133* promoter. This means that the CD133 gene promoter might be considered a target for drug exploration. In a future study, we will explore the effects of valproic acid antagonists on the survival of CSCs.

In summary, we show here that CD133+ cells exist within NB tissue and that these cells self-renew and are multipotent stem cells. CD133+ CSCs are

chemoresistant. CD133 expression and *MYCN* amplification can be used together as a prognostic factor for predicting disease outcomes.

Declaration of conflicting interests

The authors declare that there is no conflict of interest.

Funding

This study was funded by the Hebei Province Health Department Fund for Excellence Clinical Doctor.

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