Expression and clinical significance of stem cell marker CD133 in human neuroblastoma

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Background: **Recent evidences indicate that CD133, a kind of transmembrane protein, can be used as a marker to isolate stem cells from tumors originating from neural crest. This study was undertaken to explore the expression and clinical significance of stem cell marker CD133 in neuroblastoma (NB).**

Methods: **Immunohistochemical staining was used to detect the expression of CD133 in 32 patients with NB and 8 patients with ganglioneuroblastoma (GNB). The relationships were analyzed among CD133 expression, international neuroblastoma staging system (INSS) stages, pathological classification, and postoperative survival time of NB patients.**

Results: **The expression rates of CD133 in NB and GNB were 46.9% (15/32) and 37.5% (3/8) respectively, mainly in cytoplasm of neuroblastoma cells. The expression rates of stage 1-2, stage 3-4 and stage 4S were 30.7%, 57.9% and 37.5%, respectively. The differences in various stages were significant (*P*<0.05). The positive rate of CD133 in patients with unfavorable histology (52.4%) was significantly higher than that in patients with favorable histology (36.8%) (*P*=0.007). The survival time of CD133 negative patients was significantly longer than that of CD133 positive patients (*P*=0.026).**

Conclusions: **CD133 which might be correlated with the development and progression of NB can serve as one of the important indicators for prognosis of NB .**

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Introduction

Neuroblastoma (NB) is an embryonic neoplasm originating from neural crest (NC) with cellular heterogeneity as one of its oncobiological characteristics. Recent studies have indicated that cancer stem cells in tissues and cell lines of NB could be refurnished by themselves or be differentiated multi-directionally, and that they were closely related to the high malignancy of NB.[1] CD133, as a kind of transmembrane protein with the molecular weight of 120 kD, has been used as a cell marker to isolate the stem cells of NC and tumors.[2] In this study, immunohistochemical staining was used to detect the expression of CD133 in tissues obtained from NB patients at different international neuroblastoma staging system (INSS) stages and with different pathological classification. The relationships were analyzed among CD133 expression, INSS stages, pathological classification, and postoperative survival time of NB patients.

Methods

Tissue samples

Tissue samples were taken from 40 patients (neuroblastoma, NB, 32; ganglioneuroblastoma, GNB, 8) who underwent surgery during 1997-2006 at the Union Hospital of Tongji Medical College, Huazhong University of Science and Technology in Wuhan, China. This study was approved by the Ethics Committee of Tongji Medical College. Informed consents were obtained from all the patients. Ten patients underwent total resection, 11 partial resection and 19 biopsy. Twenty-two patients had the tumor located at the adrenal gland, 11 at posterior abdominal membrane, and 7 at mediastinal septum. The age of the patients ranged from 5 months to 8 years, with an

average age of 2 years and 7 months. There were 24 male and 16 female patients and the ratio of male to female was 1.5:1. According to the INSS, 6 patients belonged to stage 1, 7 to stage 2, 8 to stage 3, 11 to stage 4 and 8 to stage 4S. The mitosis karyorrhexis index (MKI) and pathological types were evaluated according to the method by Shimada et al.[3] Nineteen patients showed favorable histology (FH) and 21 unfavorable histology (UFH). Twenty-two of the NB patients and 4 of the GNB patients were followed up for 6 months to 5 years. The survival time was defined as the interval that ranged from the day of surgery to the last day of follow-up or the day of relapse, metastasis or death. The censored value was zero.

Immunohistochemical staining

Tissue samples were fixed in 10% formalin and embedded in paraffin, and then cut into 4-5 μm slices. The streptavdin-peroxidase-biotin (SP) immunohistochemical method was used to detect the expression of CD133. Briefly, after the slices were dewaxed and hydrated, antigens were repaired using a bathing method. When citric acid buffer (pH 6.0) was heated up to 95ºC-99ºC, the slices were bathed in it for 30 minutes and then cooled at room temperature for 20 minutes. The slices were then taken out and washed with phosphate buffered saline. 3% H_2O_2 was used to block intrinsic peroxidase activities of repaired slices. After incubation with normal serum, rAb for CD133 (Santa Cruz Biotechnology, CA) was incubated with slices at 4ºC overnight. The second antibody was from SP reagent kit. Stained with diaminobenzidine (DAB), the slices were dyed using hematoxylin. Instead of CD133 primary antibody, rabbit serum was used as a negative control. Under the light microscope, CD133 positive immunological staining presented brown granules located in cytoplasm. Five to ten high multiple microscope fields were randomly selected to calculate the numbers of positively stained cells in 1000 tumor cells. Positive index (PI) = (number of positive staining cells/1000)×100%. The results of staining were determined by the following standard: when PI was less than 5%, the staining was considered negative (-); between 6% and 25%, it was weak positive (+); between 26% and 50%, it was moderate positive (++); more than 50%, it was strong positive (+++).

Statistical analysis

The SPSS 12.0 statistical software was used for the Chi-square test and Fisher's exact test. Kaplan-Meier analysis was used for data analysis and drawing of survival curve. According to the log-rank variability, $P<0.05$ was considered statistically significant.

Results

Expression of CD133 in NB tissues

In the tumor tissues of 40 patients, 18 showed positive CD133 expression, giving a positive rate of 45.0%. Among them, 7 patients were weak positive, 6 were moderate positive and 5 were strong positive. CD133 was mainly expressed in the cytoplasm of tumor cells. The numbers of CD133-positive tumor cells in different patients varied (Figs. 1-4). The expression of CD133 could be detected in some stromatic fibrous connective tissues and vascular endothelial cells (Fig. 5).

Correlation of CD133 expression with INSS stages of NB

Dispersed or local CD133 expression was observed in 30.7% (4/13) NB samples in stage 1-2, 57.9% (11/19) NB samples in stage 3-4, and 37.5% (3/8) in stage 4S. The differences of CD133 expression in various stages were significant ($P<0.05$) (Table 1).

Table 1. The expression of CD133 in different INSS stages of NB

INSS stage	Total	Expression of CD133		Positive rate (%)
		Positive (*n*)	Negative (*n*)	
Stage 1-2	13	4	9	30.7
Stage 3-4	19	11	8	57.9*
Stage 4S	8	3	5	37.5*†

*: compared with stage 1-2, $P<0.05$; †: compared with stage 3-4, $P<0.05$.

Table 2. The expression of CD133 in different pathological types of NB

Group	Expression of CD133		*P* value
	Positive (*n*)	Negative (*n*)	
Histological type			
NB	15	17	0.012
GNB	3	5	
Shimada type			
FH	7	12	0.007
UFH	11	10	

Table 3. The relations among survival time, histological types, INSS stages, pathological types of NB and CD133 expression

Variables	Stages/types	*n*	Survival time (mon)	*P* value
Histological types	NB	22	23.42±2.32	
	GNB	4	26.35±3.52	0.64
INSS stages	1-2	10	30.25±3.24	
	3-4	9	20.55±3.24	0.02*
	4S	7	25.12±1.14	0.01*
Pathological types	FH	15	25.13±2.34	
	UFH	11	15.24±1.12	0.013
CD133 expression	Negative	12	23.22±2.25	
	Positive	14	14.53±1.26	0.026

*: compared with stage 1-2, $P<0.05$.

Original article

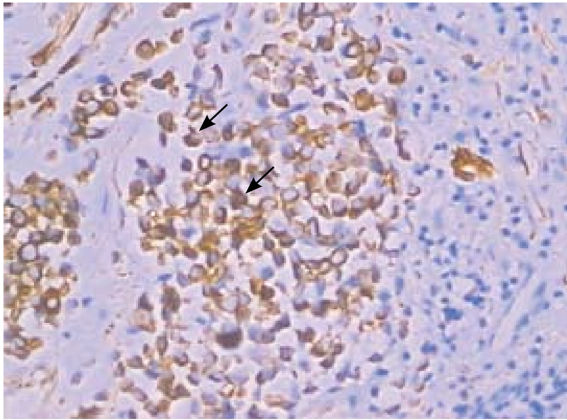

Fig. 1. Strong positive expression of CD133 in NB samples of stage 4 (DAB, original magnification × 200).

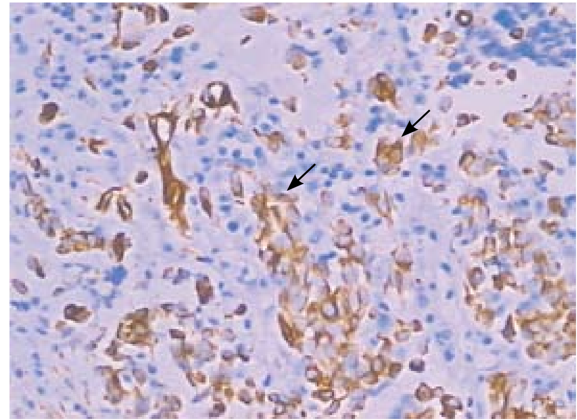

Fig. 2. Moderate positive expression of CD133 in NB samples of stage 3 (DAB, original magnification × 200).

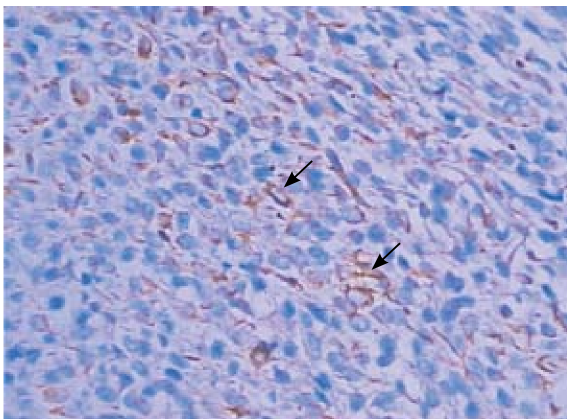

Fig. 3. Local weak positive expression of CD133 in NB samples of stage 2 (DAB, original magnification × 200).

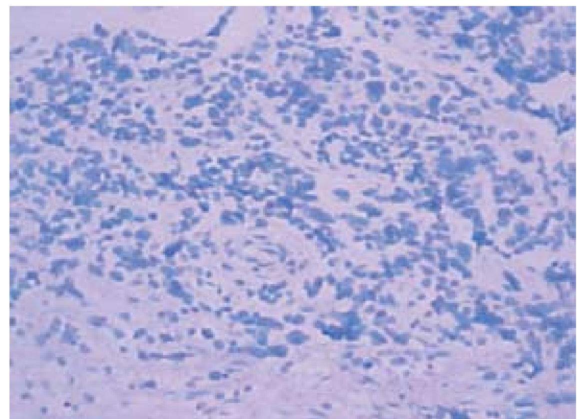

Fig. 4. Negative expression of CD133 in NB samples of stage 1 (DAB, original magnification × 200).

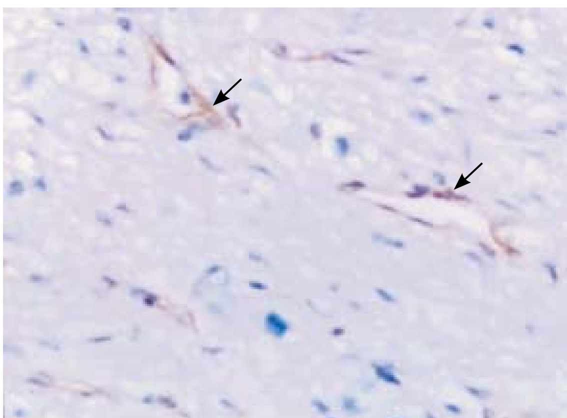

Fig. 5. Weak positive expression of CD133 on some vascular endothelial cells in NB samples (DAB, original magnification × 400).

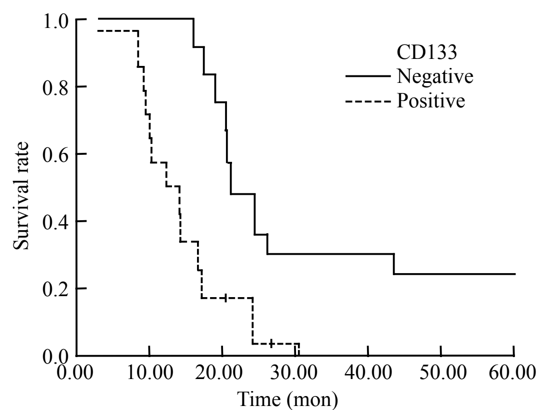

Fig. 6. Survival curve of NB patients with different CD133 expressions.

Correlation between CD133 expression and pathological types of NB

The positive expression of CD133 in the samples of GNB was 37.5% (3/8), and in NB was 46.9% (15/32). The positive expression rate of CD133 in UFH was 52.4% (11/21), and in FH was 36.8% (7/19). Fisher's exact probability analysis was performed to compare the differences of CD133 expression in NB and GNB groups, FH and UFH groups respectively. CD133 positive rates of NB and UFH were higher than those of GNB and FH. The *P* values were 0.012 and 0.007, respectively (Table 2).

Correlation between CD133 expression and postoperative survival time of NB

Twenty-two patients with NB and 4 patients with GNB were followed up. There was no relationship between the median survival time and histological type, but the median survival time was significantly correlated with INSS stages and pathological types. The median survival time of CD133 negative patients was longer than that of CD133 positive patients (Table 3, Fig. 6).

Discussion

Cancer stem cells are subgroup cells with the capabilities of illimitable proliferation, differentiation and clone formation in tumors. There are close relations among cancer stem cells, relapse and metastasis of tumors and drug resistance. Recently, Hirschmann-Jax et al[1] used Hoechst 33 342 staining method and flow cytometry screening to identify the cancer cells that obtained from 23 patients with relapsed, undifferentiated or poorly differentiated NB. They found that 0.8%-51% of collateral stem cells existed in 15 samples which expressed several stem cell surface antigens. This finding confirmed that cancer stem cells existed in NB.[1] Accordingly, we can update the recognition of genetics, development and metastasis of NB if further research of NB cancer stem cells could be evolved. It is also helpful in the prevention and treatment of NB.

CD133, a kind of transmembrane protein with a molecular weight of 120 kD, is considered to be a hematopoietic stem cell marker mainly expressed on $CD34^+$ cell subpopulation in hematopoietic tissues, such as bone marrow, fetal liver, cord blood and peripheral blood before or after mobilization.[4] mRNA transcript of CD133 could be detected in the kidney, pancreas, placenta, liver, lung, brain and myocardium by Northern blot analysis.[5] CD133 expression also exists in retinal glioblastoma cell lines Y79 and WERI-Rb-1, and dysembryoma cell line NT-2.[5] CD133 has been used to mark human NC stem cells. Uchida et al[6] separated $CD133^+/CD34^-/CD45^-$ immunophenotype cells from fetal brain directly, and confirmed that these cells had the basic traits of nerve stem cells, self-replication and the potency of multidirectional differentiation into neuron or gliocyte. Singh et al[7] verified with immunohistochemistry and flow cytometry that CD133 expressed in malignant medulloblastoma and tricholeukocyte astrocytic glioma, which are commonly seen in children. They also confirmed that brain cancer stem cells could be isolated from cell suspension by CD133 antibody immunomagnetic beads or immunofluorescence cell separation. The cancer stem cells did not express differentiated cell marker of the nerves, but another nerve stem cell marker, nestin. Although these CD133 positive stem cells only constituded a minor part of tumor cells, they could form tumor clones when suspension was cultured, and had the abilities of self-renewal and differentiation.[7] Injecting 100 CD133 positive brain stem cells into mouse brain with nonhyperpimelic diabetes mellitus and serious immunodeficiency led to tumor generation which was similar to that of the primary tumor. But there was no tumor generation if injecting 10^5 CD133 negative cells.[8] Recent studies demonstrated the existence of CD133 positive cancer stem cells within glioblastoma, which displayed a strong effect on tumor resistance to chemotherapy.[9] These results indicated that CD133 could be used as the stem cell marker of tumors originating from NC.

Based on the prolonged treatment with either hypoxia or antiblastic etoposide, Marzi et al[10] and Biagiotti et al[11] established the protocols able to purge the stem cell compartment from SH-SY5Y neuroblastoma clone. They found that these stem cells were endowed with immunocytochemical marker CD133.[10] However, there are few reports about the relationship among CD133, INSS stages and prognosis of NB. In our study, CD133 expression existed in NB and the positive rate increased with the progress of INSS stages and degradation of differentiation degrees. Obviously, NB cells can be divided into three types according to cell morphology, i.e., type N (neuroblast type), type S (smooth adherence type), and type I, whose morphological and growth traits are intercalated between the two types mentioned above. Biochemistry and immunochemistry demonstrated that type N cells present the traits of sympathetoblasts and type S cells contain non-neuronal composition in fetal NC such as Schwann cell, menalocyte, and SM precursor cell. While type I cells commonly express the markers of both type N and type S, type I cells have the strongest activities of malignant proliferation and tumor generation in these three types of cells, with the traits of cancer stem cells.[12] Walton et al[12] compared 17 NB cell lines of different types and found that only type I cells expressed CD133, while type N cells and type S cells expressed slightly or even did not express it. The results indicated that some of the CD133 positive cells in our NB samples might be type I cells with the traits of stem cells. In order to investigate whether CD133 could be used as the marker of cancer stem cells, CD133 positive cells are necessary to be isolated from NB tissues by flow cytometry and their biological traits *in vitro* should be determined. In this

study, CD133 expressed in some stromatic fibrous connective tissues and vascular endothelial cells, but the mechanism was not clear. As a membrane antigen of human stem cells or ancestral cells, CD133 might express in vascular endothelial ancestral cells and stromatic stem cells.[13,14] The activation of vascular endothelial ancestral cells participated in angiogenesis and produced a marked effect on the growth and metastasis of tumors. Stromatic stem cells can influence the biological activities of tumor cells via regulating the tumor's microenvironment.[14] We suppose that CD133 might label stromatic stem cells, but further research is needed to prove this hypothesis. Above all, the expression and survey of CD133 are significant not only for evaluating the prognosis of NB patients, but also for isolating NB stem cell subgroups and understanding the mechanisms of NB development.

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Ethical approval: This study was approved by the Data Inspectorate of China and by the Ethical Committee of Tongji Medical College, Huazhong University of Science and Technology, China.

Competing interest: No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

Contributors: Tong QS proposed the study and wrote the first draft. Zheng LD analyzed the data. All authors contributed to the design and interpretation of the study and to further drafts. Tong QS is the guarantor.

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