

Resistance of Stem-Like Cells From Neuroblastoma Cell Lines to Commonly Used Chemotherapeutic Agents

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Background. Cancer stem cell theory suggests that the presence of tumor initiating stem-like cells in cancers may be responsible for cancer progression and relapse. CD133 cell surface marker expression has been used to identify stem-like cells in cancer cell lines. Our goal was to identify such cells in neuroblastoma cell lines and to study the cytotoxicity of common anticancer drugs for those cells. **Materials and Methods.** CD133+ cells from SK-N-SH and SK-N-BE cell lines were isolated using magnetic microbeads. Cytotoxicity of four anticancer drugs was studied on CD133+ and CD133– populations. The percentage of live, apoptotic, and dead cells in each population after drug treatment was estimated by MTT and PI/Annexin-binding assays. Western blot analyses were used to identify differences in the expression of kinases. **Results.** Eight to 10% of SK-N-SH and 3–5% of SK-N-BE cells were CD133+. These cells were

more resistant than CD133– cells to all four chemotherapeutic agents tested in the MTT assay. Decreased apoptosis was observed in CD133+ cells compared to CD133– cells by PI/Annexin V-binding assay. Western blot analysis showed that CD133+ cells expressed less MKP-1. Phosphorylated forms of both ERK and P-38 kinases were expressed at higher levels in CD133+ cells than in CD133– cells. **Conclusions.** This study suggests that CD133+ cells are more resistant to anticancer drugs than CD133– cells. Differences in the expression and phosphorylation of kinases could be partially responsible for this difference. Targeting CD133-expressing cells could be a strategy to develop more effective treatments for neuroblastoma. *Pediatr Blood Cancer* 2010;54:361–368.

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Key words: cancer stem cells; drug resistance; neuroblastoma

INTRODUCTION

The cancer stem cell theory states that tumors contain a subset of cells that are capable of self-renewal and differentiation, can propagate tumor growth, and are resistant to apoptosis [1]. Recent evidence shows that cancer tissue contains a hierarchical organization of cells including stem cells similar to normal tissue. These stem-like cancer cells are similar to normal stem cells [2,3] and can be responsible for cancer progression and metastasis. Normal stem cells can be transformed into cancer stem cells after genetic modifications such as mutations in oncogenes, tumor suppressor genes, and repair genes or due to epigenetic alterations such as histone modification and abnormal methylation [4]. Recently, several cancers including those of the blood [5,6], breast [7], brain [8], and colon [9] were shown to have stem-like cells. Presence of these stem-like cells in solid tumors was first reported in human breast tumor samples [7].

Subsequently, several other human solid tumors have been studied for the presence of tumor initiating stem-like cells.

CD133 has been shown to be a marker of cancer stem cells. It is a five transmembrane domain glycoprotein expressed by untransformed hematopoietic and neural progenitors [8,10]. It has been reported that cancer cells expressing stem cell markers are chemoresistant and could thus be responsible for clinical relapse [11]. Mahller et al. [12] have reported the presence of pluripotent tumor initiating cells in neuroblastoma cell lines that are CD133 and nestin positive and are susceptible to a targeted oncolytic virus. Glioblastoma-derived CD133+ and CD133– cells have been shown to have differential growth characteristics and molecular profiles [13]. Singh et al. [8] have shown that human brain tumors contain CD133+ stem-like cells that are capable of growing tumors in immunodeficient mice. Nineteen to 29% of cells in glioblastomas and 6–21% of cells of medulloblastomas are reported to be CD133+ and tumorigenic [3].

Neuroblastoma is the most common extra-cranial solid tumor of childhood. It causes, approximately, 15% of deaths attributable to malignant conditions in children [14]. Despite multimodal treatment, the outcome of high-risk neuroblastoma is still dismal with a

EFS <50% [12]. One of the major causes of poor outcome is drug resistance which leads to treatment failure. Based on the above reports, we hypothesize that CD133+ cells isolated from neuroblastoma cancer cell lines are more resistant to commonly used chemotherapeutic agents namely cisplatin, etoposide, doxorubicin, and carboplatin than CD133– cells. In this study, CD133-expressing stem-like cells were positively isolated from the neuroblastoma cell lines SK-N-SH and SK-N-BE, and the effect of commonly used chemotherapeutic agents was studied on CD133+ and CD133– populations. Here we report that CD133+ cancer cells are more resistant to chemotherapeutic agents than CD133– cells.

MATERIALS AND METHODS

Cell Lines

Human neuroblastoma cell lines SK-N-BE and SK-N-SH were obtained from American Type Culture Collection, Rockville, MD and SH-SY-5Y, osteosarcoma cell lines U2OS and P16T, and Ewing sarcoma cell line EWS-1 were obtained from the Children's Hospital of Michigan Cell Culture Laboratory frozen-stored cell bank. After thawing, the cells were cultured in RPMI medium (RPMI; Fisher Scientific, Pittsburg, PA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA) and 1% antibiotic/antimycotic

Additional supporting information may be found in the online version of this article.

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Conflict of interest: Nothing to report.

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Received 16 July 2009; Accepted 1 October 2009

solution, in a humidified atmosphere containing 5% CO₂ at 37°C. Cells were trypsinized and passaged at a ratio of 1:3 at confluence or washed in culture medium and counted for experiments.

Immunophenotyping by FACS Analysis

Trypsinized cells were washed in ice-cold PBS and stained for CD133 expression as previously explained by Barami et al. [15]. Briefly, the cells were resuspended in PBS + 30% adult bovine serum at a concentration of 5×10^6 cells/ml and added to twelve 75 mm tubes at 100 µl/tube containing pre-aliquoted monoclonal antibodies CD133 PE and CD45 FITC. After incubating cells with antibody in the dark for 30 min at room temperature, the cells were washed with 1 ml of cold PBS and resuspended in 0.5 ml of PBS + Fix (PBS + 2.5% Coulter Fixative). A two-color analysis was performed using a Coulter XL Flow Cytometer (Coulter Corp., Miami, FL) gated on the viable cell population as inferred from forward and side scatter (FS and SS) analyses. Cells were incubated with isotype-matched controls for all samples.

Isolation of CD133+ Cell Population

Confluent layers of SK-N-SH or SK-N-BE cells were trypsinized and washed with PBS. CD133+ cells were positively selected from the cell suspension using MACS cell isolation kit (Miltenyi Biotechnologies, Auburn, CA). Briefly, the cells were resuspended in PBS containing 0.5% bovine serum albumin and 2 mmol/L EDTA and incubated with CD133/1 antibody labeled magnetic microbeads for 30 min at 4°C. Subsequently, CD133+ cells were isolated by magnetic cell separating columns. The purity of the isolated cell population was estimated by flow cytometry using anti-CD133 or isotype control antibody (Miltenyi Biotechnologies).

Equal numbers of EWS-1 cells with high percentage of CD133+ cells were incubated either with anti-CD133 antibody-labeled magnetic microbeads or with media for 30 min at 4°C to serve as positive control. Subsequently, the cells were washed and treated with various doses of chemotherapeutic agents and percentage live cells estimated by MTT assay as described below.

Treatment With Chemotherapeutic Agents

CD133+ and CD133- cells from cell lines were seeded in 96-well tissue culture plates at a density of 10×10^3 cells/well and incubated for 24 hr before the addition of various drugs. The cells were incubated with various doses of Cisplatin (0–50 µg/ml; SICOR Pharmaceuticals, Irvine, CA), etoposide (0–200 µg/ml), and doxorubicin (0–200 ng/ml; Bedford Laboratories, Bedford, OH), and carboplatin (0–200 µM; SICOR Pharmaceuticals, CA), for 24, 48, or 72 hr. At the end of the incubation period the cell survival was directly estimated by MTT assay or the cells were trypsinized and used for Annexin V/PI analysis or lysed for Western blot analysis.

MTT Assays

MTT assays were performed as previously described [16]. Briefly, at the end of the incubation period with cisplatin, etoposide, carboplatin, or doxorubicin, the cells were incubated with MTT

solution, and subsequently acid isopropanol was added to dissolve formazan crystals. Absorbance was measured using a V_{max} microplate reader (Molecular Devices, Sunnyvale, CA) at 590 nm, and percentage of cell survival was calculated.

Detection of Apoptosis Using Annexin V/PI Staining

The protocol described by Ozgen et al. [17] was modified and used for the detection of apoptosis. Cells from control (with no drug added) and experimental 96-well plates were trypsinized and resuspended at a concentration of 1 or 2×10^6 cells/ml in RPMI + 10% FBS. Apoptosis was estimated using the Apoptosis Detection Kit (R&D Systems, Minneapolis, MN). Briefly, 50 µl of cell suspensions was stained by adding 10 µl of diluted Annexin V-FITC (AnxV) and 10 µl of 250 µg/ml of propidium iodide (PI). After 15 min of incubation in the dark, the reaction was diluted with 0.4 ml of binding buffer and then analyzed using a Coulter XL Flow Cytometer (Coulter Corp.). Cells were gated to include the viable cell populations based on FS and SS character, and these gated events were analyzed on two-color histograms to identify Annexin-V-expressing cells. Drug sensitivity and treatment variables were assessed by the addition of Flow Count Fluorospheres (Coulter Corp.) to the final 0.4 ml of added binding buffer. The reactions were performed with drug-treated and control cells that had been initially cultured at identical cell densities and then harvested and resuspended in equivalent volumes. Absolute, relative viable, dead, and apoptotic counts were determined by tracking the Flow Count beads in the FL2 channel.

Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting

Whole-cell lysates were prepared as described [16]. Cell lysates (30–100 µg) were electrophoresed through 4–10% SDS-PAGE. The separated proteins on gels were transferred to PVD membranes (Schleicher & Schuell, Inc., Keene, NH) using Bio-Rad mini gel electrotransfer system. Non-specific binding was blocked with blocking buffer containing 10% fat-free milk powder in phosphate-buffered saline (PBS) for 1 hr at room temperature, followed by incubation with primary antibodies in blocking buffer with 5% fat-free milk powder overnight at 4°C and room temperature for 1 hr. The membrane was then washed three times with PBS, and incubated with secondary antibody for 1 hr at room temperature and washed three times. The bound antibody was detected using Chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's protocol.

Statistical Analysis

Data are expressed as mean ± SD. The mean percentages of live or dead cells between groups were compared using parametric independent samples *t*-test. Appropriate assumptions (normality and/or homogeneity of variances) were checked and verified. Statistically significant differences were considered achieved with a *P*-value < 0.05.

RESULTS

Approximately, 85% of cells in the Ewings sarcoma cell line EWS-1 were CD133+ (Fig. 1A) while neuroblastoma cell lines SK-

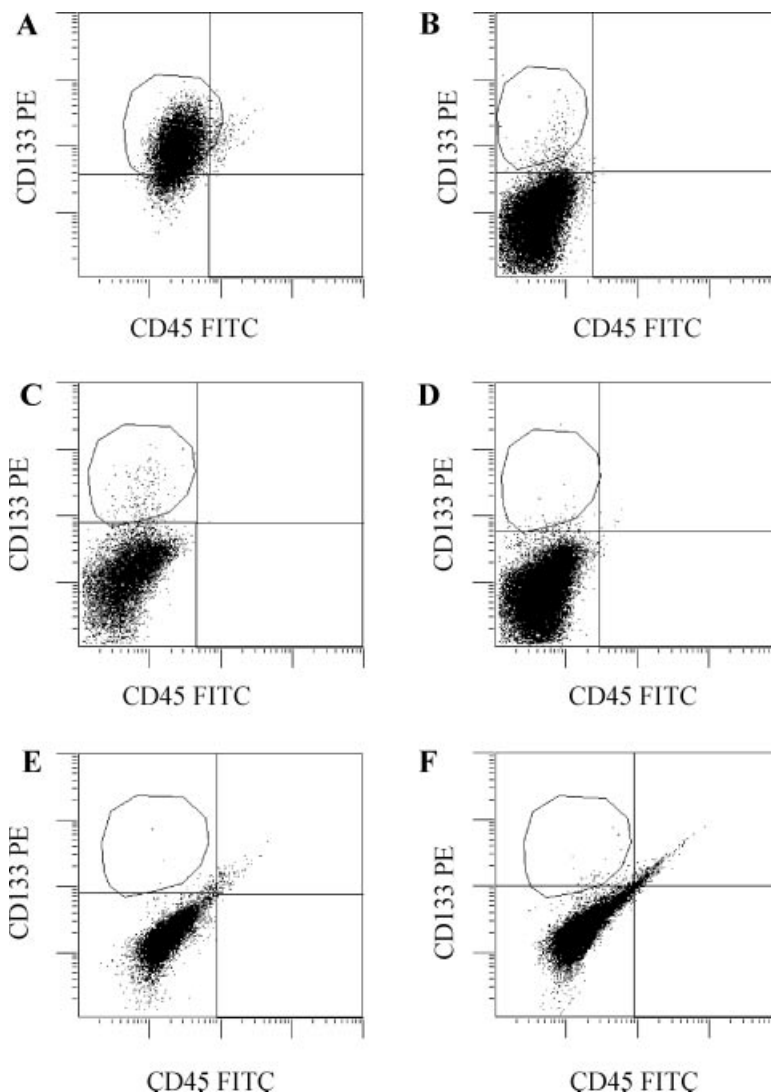


Fig. 1. Percentage of CD133 expressing cells in cell lines: (A) EWS-1: ~85%; (B) SK-N-SH: 8–10%; (C) SK-N-BE: 3–5%; (D) SH-SY-5Y: <0.5%; (E) U20S: 0.5%; and (F) P16T: <0.5%.

N-SH and SK-N-BE contained 8–10% and 3–5% CD133+ cells, respectively (Fig. 1B,C). Neuroblastoma cell line SH-SY-5Y and osteosarcoma lines U20S and P16T had less than 0.5% CD133+ cells (Fig. 1D–F).

CD133-expressing cells were positively isolated from cell line SK-N-SH using magnetic microbeads and the purity of the population was subsequently confirmed by immunophenotyping. Greater than 85% of the isolated cells expressed CD133.

CD133+ and CD133– cells from the cell line SK-N-SH treated with various concentrations of chemotherapeutic drugs resulted in a dose-related reduction in the percentage of live cells as estimated by MTT assay at 24, 48, and 72 hr (Fig. 2A–D). Further in all cases, CD133+ population had a significantly higher percentage of live cells compared to CD133– population. At 24 hr of treatment, although there was a higher percentage of live cells in CD133+ population, statistically significant difference was not observed between the CD133+ and CD133– groups with cisplatin, etoposide, doxorubicin, and carboplatin treatment as estimated by the MTT assay. However, after 48 hr of treatment with cisplatin at concen-

trations 6.25–50 $\mu\text{g/ml}$, CD133+ population had a significantly higher percentage of live cells than CD133– population. Similarly, at etoposide concentrations of 12.5–200 $\mu\text{g/ml}$ and doxorubicin at 100–200 $\mu\text{g/ml}$ CD133+ cells had significantly greater percentages of live cells. At 72 hr, at all doses tested of cisplatin (3.125–50 $\mu\text{g/ml}$) etoposide (3.125–200 $\mu\text{g/ml}$), doxorubicin (3.125–200 ng/ml), and carboplatin (12.5–200 $\mu\text{M/ml}$), a significantly higher percentage of live cells were estimated in CD133+ populations (Fig. 2).

Estimation of the percentage dead or apoptotic cells in both groups treated with low and high doses of drugs using Annexin V/PI detection kit showed that CD133+ had a significantly lower percentage of dead cells relative to control compared to the CD133– group consistent with the result obtained by the MTT assay (Fig. 3). These results suggest that CD133+ cells are more resistant to drug-induced apoptosis compared to CD133– cells.

CD133+ and CD133– cells from the cell line SK-N-BE treated with various concentrations of cisplatin resulted in a dose-related reduction in the percentage of live cells as estimated by MTT assay at 24 hr. Further in all cases, CD133+ population had a significantly

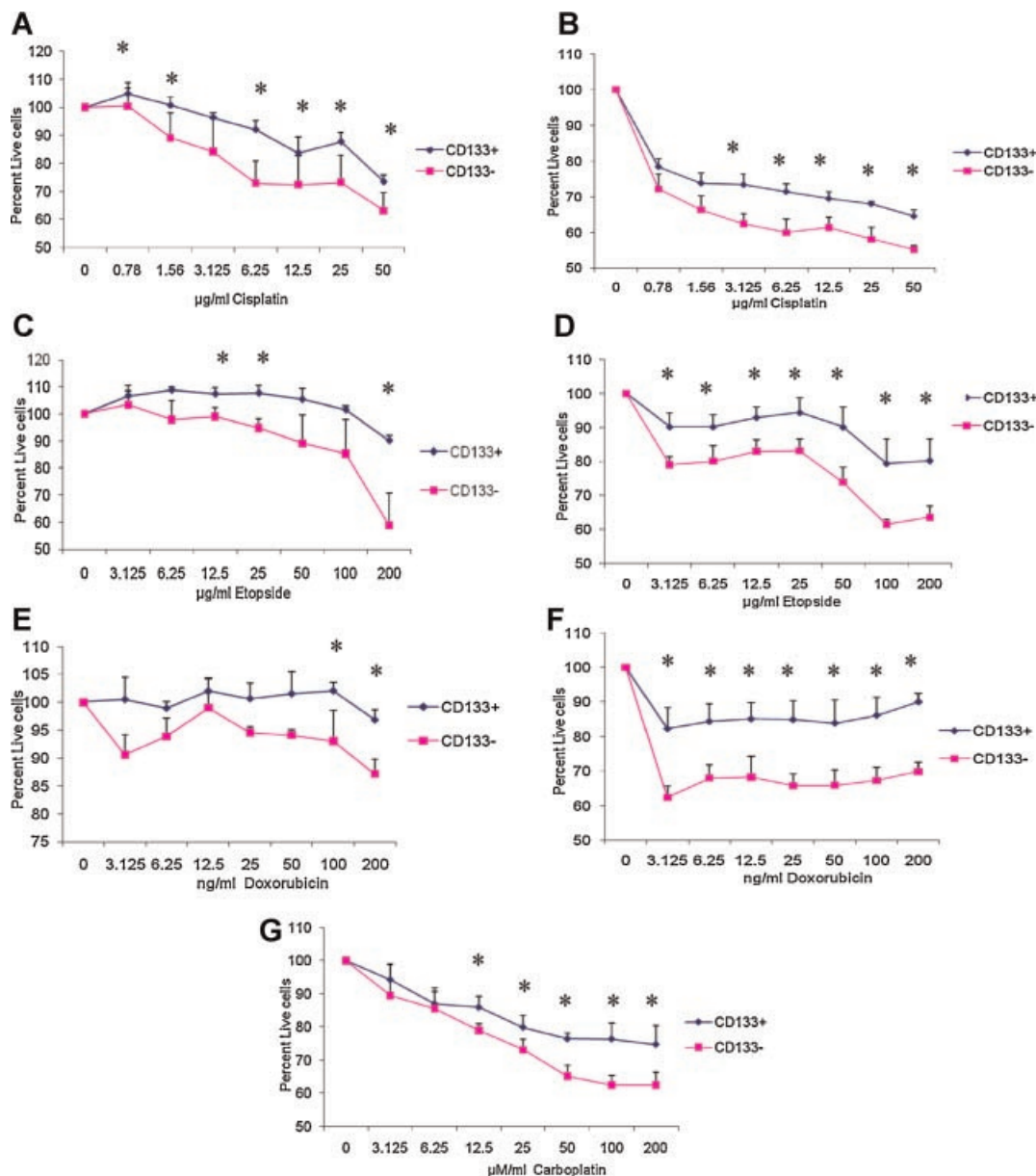


Fig. 2. Percentage of live cells in CD133+ and CD133- populations of cell line SK-N-SH after exposure to chemotherapeutic agents. **A:** Cisplatin—48 hr; **(B)** cisplatin—72 hr; **(C)** etoposide—48 hr; **(D)** etoposide—72 hr; **(E)** doxorubicin—48 hr; **(F)** doxorubicin—72 hr; **(G)** carboplatin—72 hr. Values are expressed as a percentage of untreated controls and are mean \pm SD ($n = 6$; $*P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

higher percentage of live cells compared to CD133- population (Fig. 4).

EWS-1 cell line with $>85\%$ CD133+ cells was used as a control to estimate the possible role of the microbeads used to isolate the CD133+ cells in the observed resistance. The presence of microbeads caused no difference in cell viability compared to cells without beads at all doses tested with cisplatin, etoposide, doxorubicin, and carboplatin (data shown as Supplemental Information Online, Fig. 6).

Western blot analysis was performed to study the levels of phosphorylated and total forms of select MAP kinases involved in apoptotic and cell survival mechanisms (Fig. 5). CD133+ cells constitutively expressed lower levels of MKP-1 compared to

CD133- population. Phosphorylated P-38 and P-ERK were expressed at significantly higher levels in CD133+ cells while there was no difference in the expression of phosphorylated form of P-JNK and the expression of total P-38, ERK, or JNK kinases between CD133+ and negative groups.

DISCUSSION

According to the cancer stem cell theory, cancers develop from a small number of stem-like cells that are capable of self-renewal as well as differentiation. These cells are resistant to conventional drug therapies and have a high drug efflux capacity [11,18,19]. Cancer stem cells have been identified in acute myeloid leukemia [5]

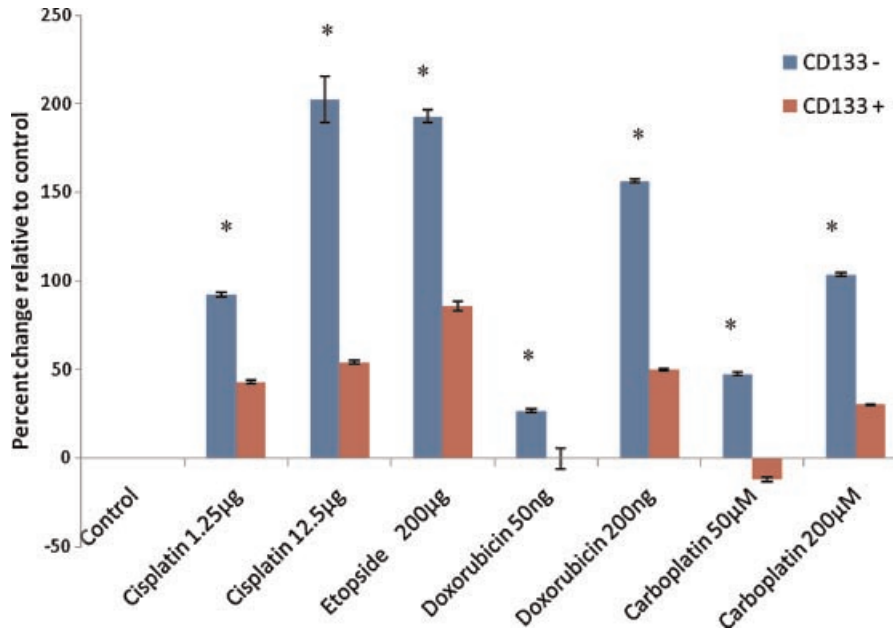


Fig. 3. Percentage of dead and apoptotic CD133+/- cells after treatment with chemotherapeutic drugs as determined by the Annexin V/PI-binding assay. Values are expressed as a percentage of dead and apoptotic cells in untreated controls and are mean ± SD (n = 6; *P < 0.005). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

expressing stem cell markers CD34+ and CD38-. They could generate original tumors with different phenotypes. Side population (sp) cells that export Hoechst 33342 dye have been used to identify stem-like cells in certain cancers, that generated sp and non-sp cells ex vivo [11,18].

CD133 is a stem cell marker expressed by normal neuronal stem cells [20]. Singh et al. [8] have shown that CD133+ cells isolated from human brain tumors could develop tumors in mice while CD133- cells were not tumorigenic. Identifying stem-like cancer cells from existing cancer cell lines could lead to development of effective targeted therapies. Here we utilized the marker CD133 to identify the presence or absence of stem-like cells in various cell

lines derived from human solid tumors. The tumorigenicity and differentiation potential of these CD133+ cells are yet to be determined. However, identifying differential responses of CD133+ and CD133- cells to chemotherapeutic agents could give a better insight into the effect of anticancer medications on cancer cell lines. Ewing sarcoma cell line EWS-1 contains a very high percentage (>85%) CD133+ cells while neuroblastoma cell line SK-N-SH has 8–10% CD133+ cells. We suggest that SK-N-SH cell line can serve as a model to study the differences in the cytotoxic effects of various anticancer drugs between CD133+ and CD133- cells.

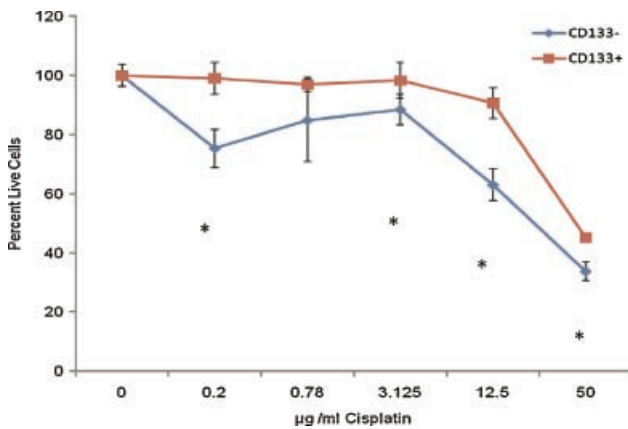


Fig. 4. Percentage of live cells in CD133+ and CD133- populations of cell line SK-N-BE after exposure to cisplatin for 24 hr as determined by the MTT assay. Values are expressed as a percentage of untreated controls and are the mean ± SD (n = 5; *P < 0.05). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

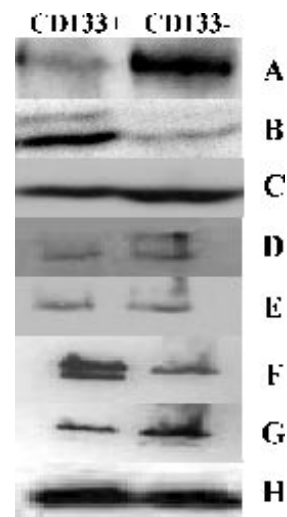


Fig. 5. Expression of kinases in CD133+ and CD133- populations of neuroblastoma cell line SH. A: MKP-1, (B) phospho-P-38, (C) P-38, (D) phospho-JNK, (E) total JNK, (F) phospho-ERK, (G) total ERK, and (H) β-actin. The results are representative of two separate experiments.

Several mechanisms have been proposed to explain the resistance of cancers to chemotherapeutic agents, including altered gene expression [21], hypoxia in the tumors [22], and migration of tumor cells to low drug penetration sites [23]. Keshelava et al. [24] have reported that the resistance of neuroblastoma cell lines is dependent on the patient's phase/stage of therapy when the cell lines were derived. However, in recent years, cancer stem cells have been an important focus in the study of drug resistance mechanisms [25–29]. We investigated the sensitivity of CD133+ and CD133– cells from the cell line SK-N-SH to four drugs commonly used for neuroblastoma treatment: cisplatin, etoposide, doxorubicin, and carboplatin.

Keshelava et al. [24] have also shown that for cell lines derived from children with neuroblastoma both doxorubicin and carboplatin had a very high range of sensitivity compared to cisplatin and etoposide. For this study we chose a wide range of drug concentrations to cover both low-dose and high-dose treatments and the concentrations correspond to the dosages used to treat patients [30–34]. MTT assay has been used as a predictor of drug sensitivity in vitro for several types of tumors [35–37]. Here we determined that CD133+ cells isolated from cell line SK-N-SH are more resistant to cisplatin, etoposide, carboplatin, and doxorubicin at 72 hr. CD133+ cells had significantly higher percentages of live cells at all doses of the drugs tested.

Although anti-CD133-labeled magnetic microbeads have been extensively used for CD133+ cell isolation [38–43], we eliminated the possibility of the beads interfering in the observed differences by studying the effect of the chemotherapeutic drugs on CD133+ rich EWS-1 cell line, in the presence and absence of the beads. The presence of microbeads conferred no apparent change to the chemosensitivity of the cells (data available as a Supplement Online).

CD133+ cancer stem cells from glioblastomas cell lines show significant resistance to chemotherapeutic agents temozolomide, carboplatin, paclitaxel, and etoposide compared to CD133-negative cells [44] similar to our observation with SK-N-SH and SK-N-BE derived CD133+ cells. Mahller et al. [12] have reported the presence of pluripotent tumor initiating cells in neuroblastoma cell lines that are CD133 and nestin positive, had increased ABCG2 expression, and were resistant to doxorubicin. Shervington and Lu [45] have shown that CD133-expressing glioblastoma cells co-express multidrug resistance genes such as *mdr1* and *bcl-2*, and Liu et al. [44] have associated high drug resistance of CD133+ cells to expression of BCRP1 and MGMT. An in-depth gene profiling of CD133+ and CD133– fractions of SK-N-SH cell line could identify the differences in gene expressions and help develop gene targeted therapies.

Mitogen-activated protein kinases (MAP kinases) play important roles in cell-cycle progression, differentiation, cell survival, and apoptosis. Three major groups comprise the family of MAP kinases: the extracellular regulated kinases (ERKs), the C-jun N terminal kinases (JNKs), and the p38MAP kinases [46,47]. An initial screening of the constitutive expression of kinases and phosphatase MKP-1 by Western blot analysis showed that CD133+ cells expressed higher levels of the phosphorylated forms of P-ERK and P-P38 while the expression of kinase-phosphatase MKP-1 is higher in the CD133– cells.

Four mechanisms have been proposed for the resistance of solid tumors to platinum-based drugs: (1) reduced platinum accumulation, (2) intracellular inactivation of cisplatin, (3)

enhanced repair of DNA damage, and (4) blocking the induction of apoptosis and promoting cell survival [48]. Differential expression of MAP kinases (P-38, JKN, ERK, and P13K) has been implicated in the cell survival mechanism [48]. Although the traditional role of activated P-38 is in cell differentiation, growth inhibition, and apoptosis [49], Thornton and Rincon [47] have reported that P-38 also contributes to cell survival by induction of G2/M cell-cycle checkpoint in tumor and non-tumor cells. Kinases ERK1/2 have been implicated in the tumor cell survival signaling as reviewed by Balmanno and Cook [50]. Although total ERK1 (p44) is prominently expressed in both CD133+ and CD133– populations, conspicuously low levels of ERK2 (p42) are seen in both groups. However, the phosphorylated forms of both ERK 1 and ERK2 are expressed in significantly higher levels in CD133+ cells possibly playing a role in the increased survival of CD133+ cells. JNKs are activated by various external stimuli such as environmental stresses, cytokine response, oxidant stress, and DNA damage [51]. Interestingly, we find no significant difference in the activated form of JNK between CD133+ and CD133– cells. While the kinase cascade plays an important role in the activation of downstream kinases by phosphorylation, regulated dephosphorylation of MAPKs is crucial to determine the magnitude and duration of activation, thus determining the physiological outcome [52]. Mitogen-activated protein kinase phosphatase 1 (MKP-1) has been shown to dephosphorylate ERK, JNK, and P-38 in the cell nucleus [52]. Higher expression of MKP-1 in CD133– cells relates to the increased dephosphorylation of P-38 and ERK seen in CD133– cells. Differential activations of various MAPKs determines the cell fate by triggering downstream regulators toward apoptotic, cell survival, or differentiation pathways. Here we show that the CD133+ stem cell fraction of the SK-N-SH line has constitutively higher levels of phosphorylated P-38 and ERK1/2. This could be one of the pathways by which cancer stem cells show higher resistance to chemotherapeutic drugs. A detailed study of the MAPK expression in CD133+/- cells after treatment with anticancer agents would allow us to determine the role of kinases in chemoresistance and could lead to more effective therapies.

In summary, this study shows that CD133+ cells in neuroblastoma cell lines SK-N-SH and SK-N-BE are more resistant to conventional anticancer drugs compared to CD133– cells. Differences in the activation of MAPKs and expression of MKP-1 could be partially responsible for the resistance. Further investigation is warranted to confirm the role of kinases since they may serve as molecules for targeted therapy. Similar studies are needed to be done on primary neuroblastoma tissue samples from patients to ensure the presence, and drug resistance of CD133+ cells. Targeting these CD133+ cell populations using novel agents or novel combinations of existing drugs could be one of the strategies to overcome resistance and develop more effective treatment for neuroblastoma.

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