Histopathology

Histopathology 2012, **60**, 1144–1155. DOI: 10.1111/j.1365-2559.2012.04191.x

CD133 expression is associated with poor outcome in neuroblastoma via chemoresistance mediated by the AKT pathway

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Date of submission 2 May 2011
Accepted for publication 4 August 2011

Sartelet H, Imbriglio T, Nyalendo C, Haddad E, Annabi B, Duval M, Fetni R, Victor K, Alexendrov L, Sinnett D, Fabre M & Vassal G

(2012) *Histopathology* **60**, 1144–1155

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Aims: Neuroblastoma is a frequent childhood cancer with a heterogeneous prognosis. CD133 expression is an independent prognostic marker for a low survival rate in several cancers. The aim of this study was to determine the prognostic value of CD133 expression in a large cohort of neuroblastoma cases, to define the chemoresistance of neuroblastoma cells expressing CD133, and to determine whether this chemoresistance is regulated by activation of the AKT pathway.

Methods and results: Two hundred and eighty samples of neuroblastoma were screened for CD133 expression. The sensitivity of purified CD133+ neuroblastoma cells isolated from two human cell lines to doxorubicin, vincristine and cisplatin, as single agents or in combination with LY294002, an AKT inhibitor, was evaluated *in vitro*. CD133 expression was found in 100 of 280 tumours. There was a significant association between CD133 expression and the following poor prognosis covariates: age, International Neuroblastoma Staging System stage, *MYCN* amplification, and phospho-AKT (pAKT) expression. Patients with CD133− tumours had significantly better 3-year event-free and overall survival than patients with CD133+ tumours. In a multivariate model, CD133 expression was independently associated with decreased overall survival. $CD133^{high}$ neuroblastoma cells were significantly resistant to chemotherapy as compared with $CD133^{low}$ cells. Treatment of unsorted neuroblastoma cells with the three anticancer drugs significantly enriched the CD133+ subpopulation. $CD133^{high}$ cells expressed significantly higher levels of pAKT than $CD133^{low}$ cells. LY294002 treatment abolished the preferential survival of $CD133^{high}$ cells.

Conclusions: CD133 is associated with *in-vitro* resistance to chemotherapy involving activation of the AKT pathway.

Keywords: AKT pathway, cancer stem cell, CD133, chemoresistance, neuroblastoma

Abbreviations: CSC, cancer stem cell; DAB, 3,3′-diaminobenzidine; FACS, fluorescence-activated cell sorting; INSS, International Neuroblastoma Staging System, NB, neuroblastoma; pAKT, phospho-AKT; pERK, phospho-extracellular signal-regulated kinase; TMA, tissue microarray

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Introduction

CD133, a cell-membrane protein, was originally classified as a marker of primitive haematopoietic and neural stem cells. There is increasing evidence highlighting the role of CD133 as a reliable marker of cancer stem cells (CSCs) in various human tumours, such as prostatic, pancreatic, colonic, hepatocellular and renal tumours.[1,2] CSCs constitute a distinct subpopulation of cancer cells that possess functional characteristics normally associated with stem cells, such as self-renewal and the ability to differentiate into multiple cell types.[3,4] In animal models, CSCs drive tumour progression.[3,4] Published data suggest that CSCs are more resistant to chemotherapy and lead to tumour relapse and metastasis.[5] CD133 has been identified as a marker of a subset of neural stem cells in the adult central nervous system and of glioblastoma stem cells.[6,7] Furthermore, studies have shown that cancer cell CD133 expression in medulloblastoma, a malignant tumour histopathologically resembling neuroblastoma (NB), is associated with an invasive phenotype.[8]

NB is a frequent malignant paediatric tumour, and is often associated with a poor prognosis. Survival of children older than 12 months with an advanced stage of NB is dismal, despite aggressive treatment,[9] as compared with the survival of children younger than 1 year.[10] NB cells secrete catecholamine metabolites that are excreted in the urine, offering a non-invasive diagnostic technique. This observation has led to the consideration of screening for NB in infants with specific catecholamine markers.[11–13] Mass screening for NB has been shown to have no clinical benefit, because mass-screened patients with NBs very rarely died from tumour progression,[14] and the incidence of the aggressive NBs did not decrease in populations where mass screening was applied.[13]

In high-risk cases of NB, a subpopulation of cells with stem cell properties enriched in tumour-initiating capacity has been isolated.[15] These can be used as a model for the development of new therapeutic strategies by identifying molecular determinants of NB.[15] A recent report of 40 NB cases demonstrated that CD133+ CSCs were more prevalent in patients with unfavourable histology than in patients with favourable histology.[16] Also, cell line studies with different markers, including CD133, have revealed the presence of a CSC subpopulation in NB.[17] Moreover, patients with CD133− tumour cells have longer survival than patients with CD133+ tumour cells, pointing to a close relationship between the frequency of CSCs and prognosis.[16]

Activation of the AKT [protein kinase B/AKT] pathway has emerged as a crucial regulator of cellular processes, including apoptosis, proliferation, differentiation, and metabolism.[18–20] Therefore, the AKT pathway is a relevant target for antineoplastic therapies.[21] For example, hepatocellular carcinoma studies have demonstrated that CD133+ CSCs confer chemoresistance by preferential expression of the AKT survival pathway.[22] Perifosine, a specific inhibitor of activation of the AKT pathway, is an effective cytotoxic agent in NB cells *in vitro* and *in vivo*.[23] The activation of this pathway is associated with chemoresistance to several drugs currently used in NB: doxorubicin,[24,25] vincristine,[26,27] and cisplatin.[28,29] Furthermore, RET overexpression in NB associated with AKT pathway activation reversed the CD133-related inhibition of neurite elongation and NB cell differentiation.[30]

The aim of our study was to determine the prognostic value of CD133 expression in a large cohort of NB cases, to define the chemoresistance of NB cells expressing CD133, and finally to determine whether this chemoresistance is regulated by activation of the AKT pathway.

Materials and methods

STUDY DESIGN AND PATIENTS

Study cases were selected on the basis of the following inclusion criteria: (i) a diagnosis of NB between July 1988 and March 2008, (ii) written consent from the patients, and (iii) adequate specimen material for study purposes. Two hundred and eighty patients with NB were included in our study. They were both treated and followed up in two paediatric oncological centres: hôpital Bicêtre and Institut Gustave Roussy (Le Kremlin-Bicêtre, Villejuif, France) and CHU Sainte-Justine (Montréal, Canada). Thirty-one of the 280 NB cases were identified by routine provincial (Quebec, Canada) mass screening. Tumours were classified according to the International Neuroblastoma Staging System (INSS).[31] Treatment protocols were based on risk group defined by patient age at time of diagnosis, INSS stage, and *MYCN* amplification status of the tumour.

SAMPLES AND EVALUATION OF CD133 STATUS

A tissue microarray (TMA) was constructed with four 0.6-mm-diameter representative NB tumour tissue cylinders transferred into a recipient paraffin block with a tissue arrayer. TMA blocks of NBs contained 280 primary tumours and 97 metastases (81 lymph nodes, 14 hepatic metastases, and two cutaneous metastases).

Fifty-five paired control normal samples were included as well (41 adrenal glands and 14 sympathetic ganglia). Immunohistochemistry was performed on paraffin-embedded sections with an Ultraview Universal 3,3′-diaminobenzidine (DAB) detection kit (Ventana Medical Systems, Tuscon, AR, USA). Sections were incubated for 32 min with primary antibodies against phospho-AKT (pAKT) (1:100, rabbit polyclonal, S473-r; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and CD133 [1:100, rabbit polyclonal, Ab19898 (Abcam, Cambridge, MA, USA) and 1:200, mouse monoclonal, 32AT1672 (Santa Cruz Biotechnology); two antibodies were used to ensure the reproducibility of the results]. Sections were counterstained with haematoxylin. Normal mouse or rabbit IgG at the same concentration as the primary antibody was used as a negative control. In TMAs, an orthotopic graft of D283 medulloblastoma cells was used as a positive control, and a schwannoma as a negative control.

Two investigators, blinded for clinical data, evaluated immunostaining independently in representative samples containing at least 100 NB cells. CD133 expression, when present, was always focal or multifocal. For statistical purposes, two defining groups were created: one without expression of CD133 in NB cells (CD133−) and one with expression (CD133+). For quality control purposes, the TMA was constructed with five additional control cases of CD133+ tumours and five control cases of CD133− tumours.

For the pAKT antibody, immunostaining scores were established by semiquantitative optical analysis based on the percentage of positive NB cells in each sample: 0, no positive cells; 1+, 1–25%; 2+, 26–50%; 3+, 51–75%; and 4+, >76.

CELL CULTURE

Two distinct NB cell lines were used: SK-N-SH [a non-*MYCN*-amplified cell line,[32] purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured in accordance with the American Type Culture Collection guidelines]; and NB 10 [a *MYCN*-amplified cell line,[33] established at Saint Jude's Children's Research Hospital (Memphis, TN, USA)]. Cells were maintained in Dulbecco's modified Eagle's medium, 10% fetal bovine serum and 0.2% gentamicin at 37°C in a 5% CO_2 atmosphere, before and after purification of CD133+ CSCs.

CD133 CELL ISOLATION

Magnetic separation of CD133+ cells from the two cell lines was performed as specified by the manufacturer, with a CD133 Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), allowing us to define two populations: $CD133^{high}$ and $CD133^{low}$. The quantities of CD133− and CD133+ NB cells were calculated by fluorescence-activated cell sorting (FACS) analysis with an anti-CD133 antibody directly associated with APC-A (Miltenyi Biotec). Selected cells were then cultured.

CELL PROLIFERATION ASSAY

Chemotherapy-induced cytotoxicity was determined by MTT cell proliferation assay (Cell Titer 96; Fisher Scientific, Rockville, MD, USA), according to the manufacturer's instructions. Absorbance was measured at 570 nm. Assays were performed three times. The mean cell viability was compared with that of positive control cells exposed only to culture medium. Percentage cell viability was defined as the number of drug-exposed viable tumour cells relative to the number of control NB tumour cells.

QUANTIFICATION OF CD133+ CELLS AFTER CHEMOTHERAPY

Both NB cell lines were incubated with the following chemotherapy drugs, previously used at IC50 (doxorubicin, 4 μM; cisplatin, 200 μM; vincristine, 15 μM, IC50s were previously calculated; HS Sartelet, GV Vassal, unpublished data), alone or in combination with LY294002, an AKT inhibitor (Calbiochem, Darmstadt, Germany). The cell lines were also incubated alone with LY294002 or RAD001, an mTOR inhibitor (Novartis, Basel, Switzerland) at IC50. After 24 h of drug exposure, NB cells were incubated with an anti-CD133 antibody directly associated with APC-A (Miltenyi Biotec) and were analysed by FACS.

WESTERN BLOTTING

For western blotting, we used two types of sample: the two NB cell lines (SK-N-SH and NB10), selected or not selected for CD133, and 31 frozen tumour tissue samples from the 280 tumours studied in the TMA (seven stage 1, eight stage 2, seven stage 3, seven stage 4, and two stage 4S). Fifteen micrograms of protein from representative NB cells was extracted and analysed by western blotting. Proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with antibodies against CD133 (1:2000; Abcam), pAKT (1:500; Santa Cruz Biotechnology), phospho-extracellular signal-regulated kinase (pERK)1/2 (1:1000; Cell Signaling, Beverly, MA, USA) and β-actin (Sigma-Aldrich, St. Louis, MO, USA) for 1 h

at room temperature, and this was followed by incubation with secondary antibody, peroxidase-labelled donkey anti-rabbit or anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Western blots were visualized with enhanced chemiluminescence before exposure of the membrane to photosensitive film. Analysis by densitometry using Kodak ID 3.6 software allowed us to calculate the relative expression of pAKT in tumours with or without expression of CD133.

STATISTICAL ANALYSIS

Association tests were performed with Fisher's exact test. Spearman correlation values (rho) were used to compare levels of protein expression observed using the two antibodies against CD133. Survival curves were constructed according to the methods of Kaplan and Meier, and survival curve comparison was performed with a two-sided log-rank test. Event-free survival was considered to be from the time of surgery of the primary tumour as baseline to the time of the first event (local relapse, disease progression, death, or last follow-up); overall survival was considered to be from the time of surgery to the time of death or last follow-up. Multivariate analysis, based on the Cox proportional-hazards regression model, identified any independent prognostic variables. All statistical analyses were performed with SPSS 15.0 (SPSS, Chicago, IL, USA). *P*-values <0.05 were considered to be statistically significant.

Results

CHARACTERISTICS OF PATIENTS

Clinicopathological characteristics of the 280 patients and tumours are detailed in Table 1. With a median follow-up of 67 months (0–218 months), the 3-year event-free and overall survival rates (±SE) for the entire cohort were 72 ± 2% and 79 ± 2%, respectively.

FREQUENCY AND DISTRIBUTION OF CD133

Immunohistochemical expression of CD133 was both cytoplasmic and membranous, and was found exclusively in NB cells (Figure 1A–C). CD133 was detected in 100 of 280 NBs (35.7%) (Table 1). Excellent reproducibility and specificity of immunohistochemical staining with CD133 was confirmed using the two different antibody clones (Ab19898 and 32AT1672) ($P < 0.001$). In addition, the results concerning CD133 expression were confirmed in the ten additional control cases (five of CD133 positive and five of CD133 negative tumors). Western blot analysis corroborated the immunohistochemistry results [in 31/31 (100%) cases], revealing a very low level of CD133 expression

Table 1. Patient comparison profile in regard to CD133 tumour expression

	All patients No. (%)	CD133+		
		No.	%	*P*-value
Total	280	100	35.7	
Age				
Median (range) (months)	18 (0–183)			
<365 days	111 (39.6)	12	10.8	
≥365 days	169 (60.4)	88	52.1	<0.0001
Stage				
1	68 (24.2)	2	2.9	
2	40 (14.2)	2	5	
3	44 (15.7)	8	18.1	
4	106 (37.8)	84	79.2	<0.0001
4S	22 (7.8)	4	18.1	<0.0001
MYCN status				
Amplified	33 (14.7)	22	66.6	
Non-amplified	191 (85.3)	50	26.1	<0.0001
Unknown	56			
Neuroblastoma type				
Standard	249 (88.9)	99	39.7	
Mass screening	31 (11.1)	1	3.2	<0.0001
Shimada histopathological category[48]				
Favourable	145 (51.8)	37	25.5	
Unfavourable	135 (48.2)	63	46.6	0.0003
COG risk group				
Low	107 (38.2)	3	2.8	
Intermediate	44 (15.7)	9	20.4	0.008
High	107 (38.2)	86	80.3	<0.0001
Unknown	22 (7.9)			
Type of sample				
Tumours	280	100	35.7	
Metastases	97	37	38.1	0.71
Control	55	1	1.8	<0.0001

COG, Children's Oncology Group.

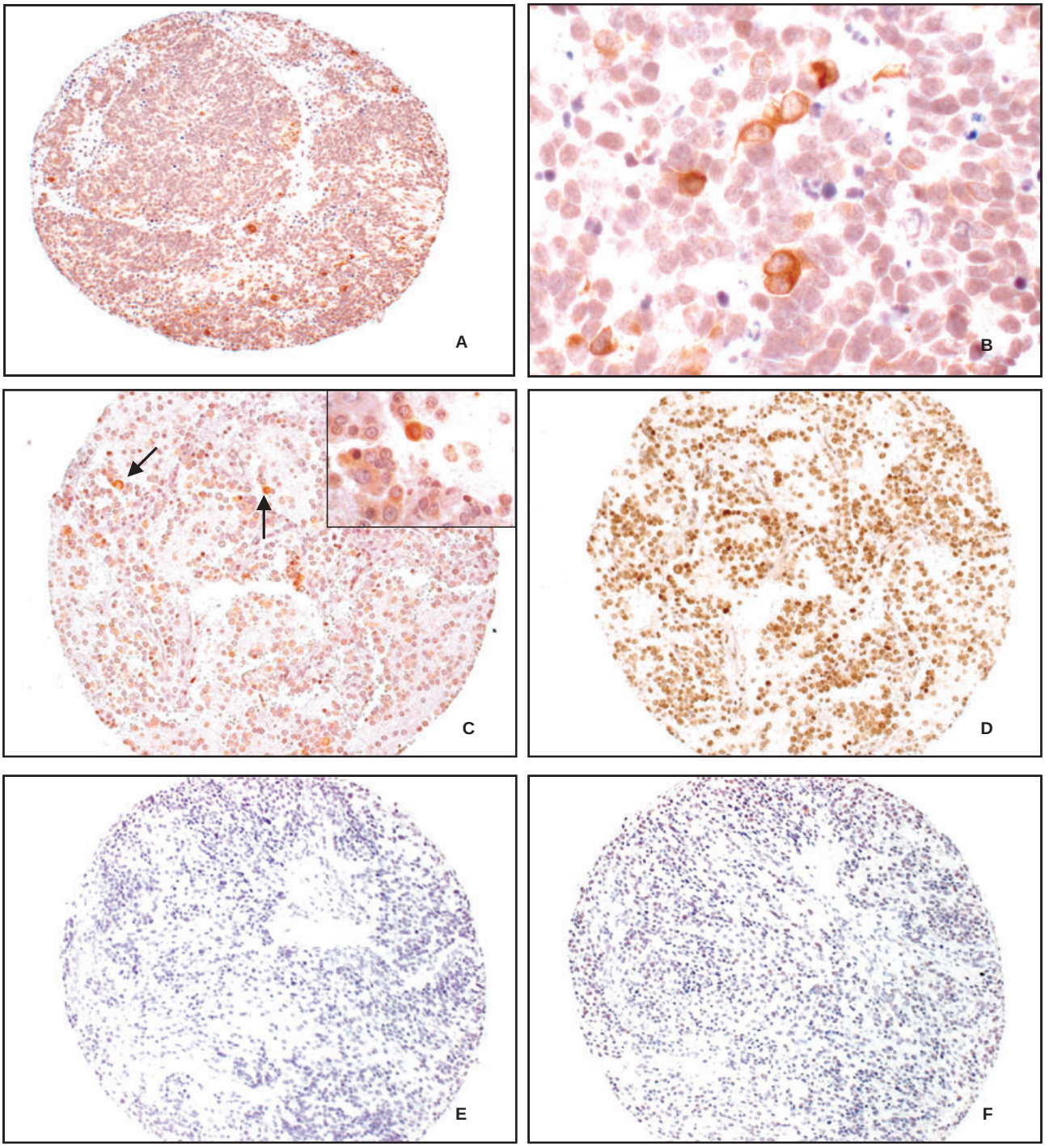

Figure 1. Immunohistochemistry performed on paraffin-embedded tissue sections: CD133 [Abcam antibody (**A**, **B**) and Santa Cruz Biotechnology antibody (**C**, **E**)]; pAKT (**D**, **F**). CD133 expression was shown by neuroblastoma cells but not stromal cells; (cells expressing CD133: in figure **A**, **B** at high magnification, **C** (arrows) and inset **C** at high magnification) the percentage of positive neuroblastoma cells was always <10% (**A**–**C**). Expression was cytoplasmic and membranous (**B** and **C** inset). Phospho-AKT (pAKT) expression was significantly higher in tumours with expression of CD133 than in those without (**C**, **D**, CD133+ tumour; **E**, **F**, CD133– tumour).

in the positive tumour cases (Figure 2). CD133 expression in NB was associated with statistically significant independent adverse prognostic factors: age of 1 year old or older ($P < 0.0001$), INSS stage 4 ($P < 0.0001$), *MYCN* amplification ($P < 0.0001$), unfavourable Shimada histological category ($P < 0.0001$), and high-

risk Children's Oncology Group status ($P < 0.0001$) (Table 1). The level of CD133 expression was significantly higher in tumours than in control samples ($P < 0.0001$), but there was no significant difference between tumours and metastases ($P = 0.71$) (Table 1).

CORRELATION OF CD133 EXPRESSION WITH PATIENT OUTCOME

A univariate analysis of patient outcome demonstrated that CD133 expression was significantly associated with a decreased probability of survival (Figure 3A–F).

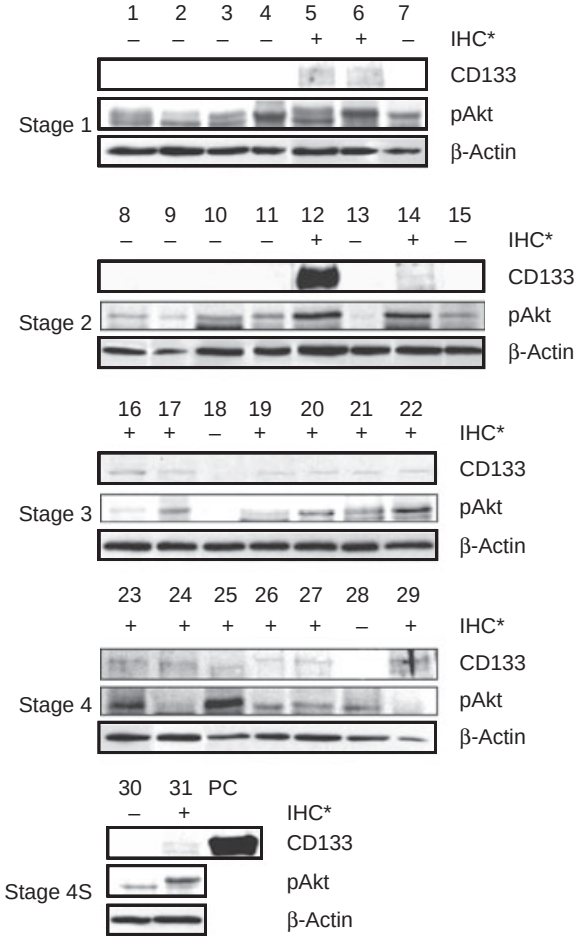

Figure 2. Western blot analysis was performed in parallel on 31 frozen primary tumour samples, with corroborating results from CD133 immunohistochemical tissue microarray studies. Proteins were immunoblotted with antibodies against CD133, phospho-AKT (pAKT), and β-actin, with a lysate of D283 medulloblastoma cells as a positive control. CD133 was positive in 51.6% of neuroblastoma cases (16/31) (perfect concordance between immunohistochemistry and western blot in 31/31 of the parallel cases). pAKT was positive in 87% of cases, with a high concordance with immunohistochemical studies (27/31). *Expression of CD133 in immunohistochemistry: +, positive; –, negative.

Patients whose tumours showed CD133 expression had 3-year event-free and overall survival rates of 43 ± 5% and 51 ± 5%, respectively, as compared with 89 ± 2% ($P < 0.001$) and 96 ± 2% ($P < 0.001$), respectively, for patients whose tumours did not express CD133 (Figure 3A,B). Survival analysis showed that patients whose tumours expressed CD133 had statistically significant worse global survival and event-free survival (Kaplan–Meier plot of overall survival, and event-free survival, $P < 0.0001$; Figure 3A,B).

Analysis of the subgroup of cases without amplification of *MYCN* showed that CD133 expression was highly associated with decreases in both event-free survival ($P < 0.0001$) and overall survival ($P < 0.0001$) (Figure 3C,D). In this subgroup, patients in whom tumours showed CD133 expression had 3-year event-free and overall survival rates of 50 ± 7% and 69 ± 6%, respectively, as compared with 88 ± 3% and 97 ± 1% ($P < 0.001$), respectively, in patients in whom tumours did not express CD133. Within the risk groups defined by the Children's Oncology Group, CD133 expression was associated with shortened event-free survival among intermediate-risk and low-risk groups ($P = 0.01$ for both) (Table 1; Figure 3E,F).

Applying multivariate Cox regression, we found that the presence of CD133 was associated with decreased overall survival (relative risk of 6.9, $P = 0.003$; Table 2). We also found a significant link between CD133 and *MYCN* ($P = 0.039$). When both CD133 and *MYCN* were present, the death rate was 9.9 times higher ($P = 0.039$) than in the group with both latter variables absent. INSS stage 4 disease and age were also independently significant in this model (Table 2).

CD133+ NB CELLS CONFER CHEMORESISTANCE TO CONVENTIONAL CHEMOTHERAPEUTIC AGENTS

SK-N-SH and NB10 cells were sorted into $CD133^{high}$ and $CD133^{low}$ populations (Figure 4), and exposed for 24 h to varying concentration of doxorubicin, vincristine, and cisplatin, three chemotherapeutic agents commonly used in the treatment of NB. $CD133^{high}$ cells were found to be more viable than their matched $CD133^{low}$ counterparts (Figure 5A–F).

Moreover, treatment of tumour cell lines with vincristine, cisplatin, doxorubicin, LY294002 and RAD001 specifically targeted tumour cells lacking the CD133 phenotype and significantly enriched the CD133+ subpopulation in both cell lines and for all of the drugs used (Figure 6A,B).

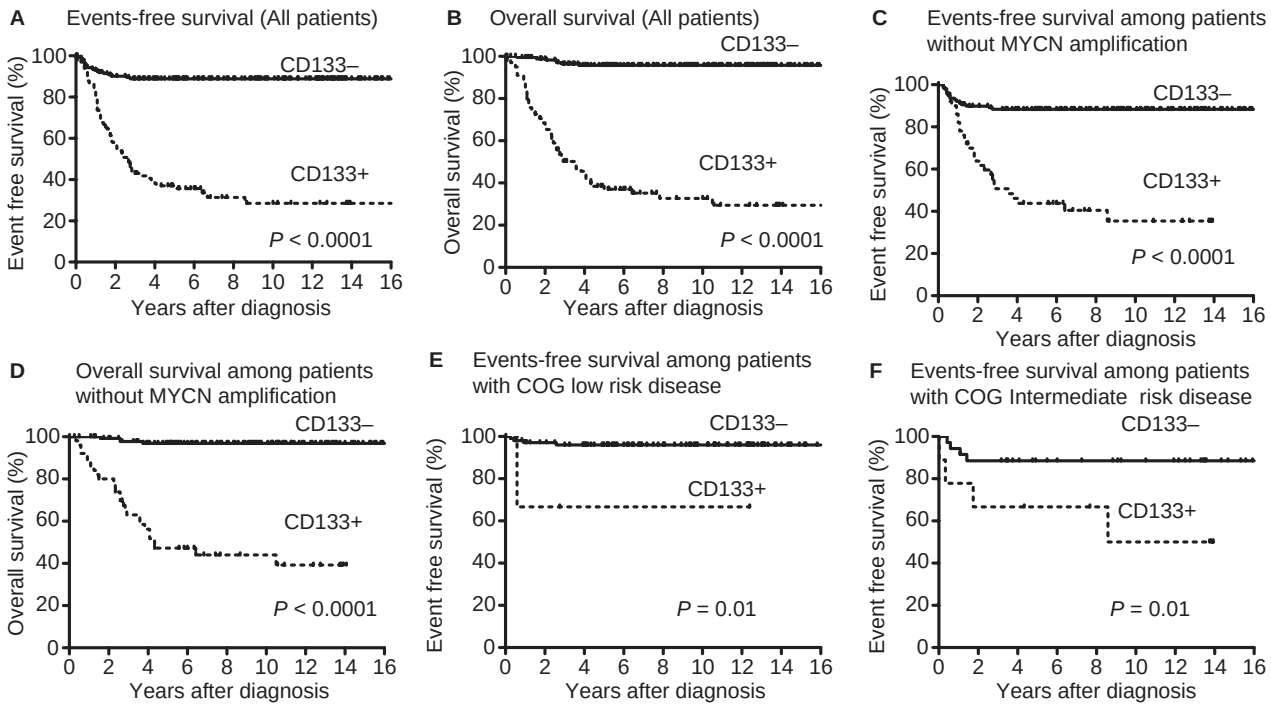

Figure 3. Event-free and overall survival according to CD133 expression. The rates of event-free and overall survival are shown for all patients (**A**, **B**) and for those patients whose tumours did not have *MYCN* amplification (**C**, **D**). The rate of event-free survival is shown for those patients with low-risk disease (**E**), and for those with intermediate-risk disease (**F**), as defined by the Children's Oncology Group (COG). Two-sided *P*-values were calculated with the log-rank test.

Table 2. Multivariate Cox regression

		95% CI		
	Relative risk	Lower	Upper	*P*
CD133	6.907	1.967	24.254	0.003
Age	3.073	1.024	9.218	0.045
MYCN	8.281	1.782	38.493	0.007
Stage	3.701	1.367	10.024	0.010

CI, Confidence interval.
Relative risk: relative risk in survival.
CD133: tumours expressing CD133 as compared with tumours not expressing CD133.
Age: patients under 1 year of age as compared with patients over 1 year of age.
MYCN: *MYCN* amplified as compared with *MYCN* not amplified.
Stage: stage 4 as compared with other stages (1, 2, 3, 4S).

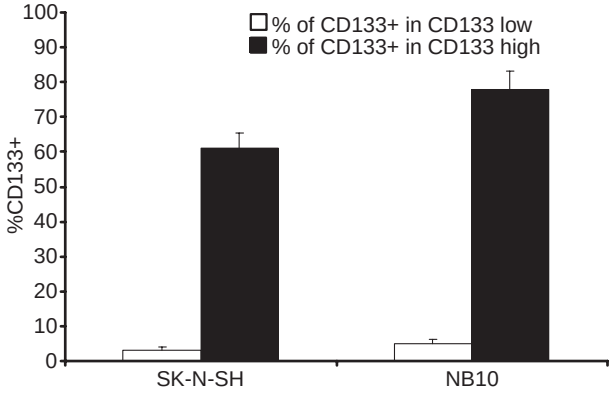

Figure 4. Fluorescence-activated cell sorting study of the percentage of positive CD133 neuroblastoma cells after selection.

IMPORTANCE OF AKT ACTIVATION IN CD133+ TUMOURS

In our TMA immunohistochemical study, pAKT was expressed in 97% of tumours, with a semiquantitative scoring median of 2. Using the Fisher's exact test, we demonstrated that the expression of pAKT was significantly higher in CD133+ tumours than in CD133– tumours ($P<0.01$) (Figure 1C–F). Similarly, densitometry analysis of western blots showed that the relative expression of pAKT was significantly higher in tumours expressing CD133 ($P < 0.04$) (Figure 7). Western blotting using the two cell lines showed a higher level

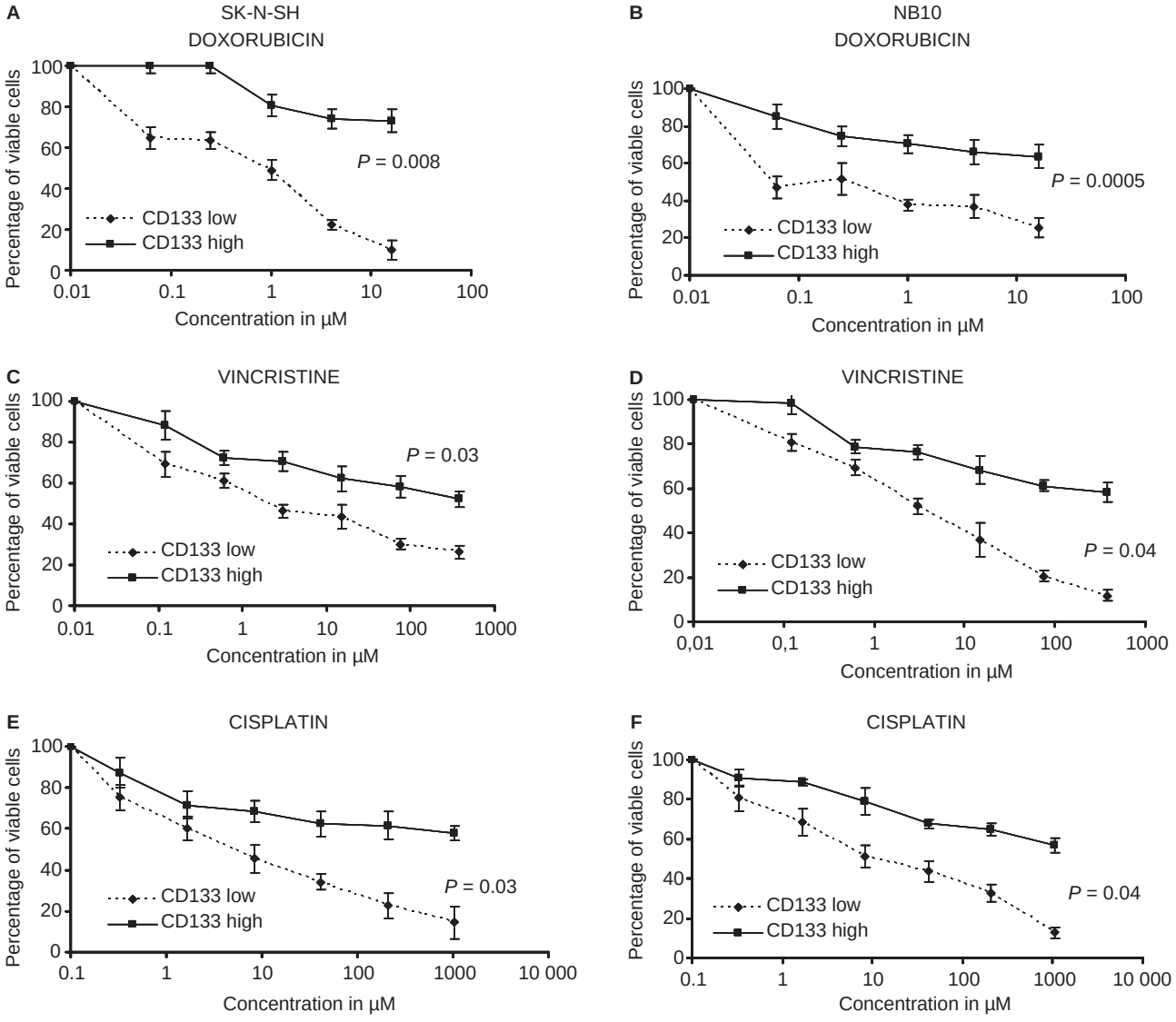

Figure 5. CD133+ neuroblastoma cells confer chemoresistance to conventional chemotherapeutic agents. The effect of chemotherapeutic agents on neuroblastoma cells from SK-N-SH and NB10 cell lines, sorted according to CD133 expression ($CD133^{low}$ and $CD133^{high}$), determined with the standard MTT assay at various concentrations of doxorubicin (0.06–16 µM) (**A**, **B**), vincristine (0.1–380 µM) (**C**, **D**), and cisplatin (0.3 µM to 1.04 mM) (**E**, **F**). Cells were incubated for 24 h. Statistical differences between $CD133^{high}$ and $CD133^{low}$ groups were calculated using Fisher's exact test.

of pAKT expression in $CD133^{high}$ cells than in $CD133^{low}$ cells in both cell lines, but no difference for pERK (Figure 8).

AKT INHIBITION RESTORED *IN-VITRO* CHEMOSENSITIVITY TO CD133+ CELLS

Inactivation of the AKT pathway using a specific AKT inhibitor (LY2940202) at IC50 restored *in-vitro* chemosensitivity of CD133+ cells to vincristine, cisplatin, and doxorubicin (Figure 9A–F). In addition, combination treatment of NB cell lines with LY2940202 at IC50 and either vincristine, cisplatin or doxorubicin did not modify the proportion of the CD133+ subpopulation in either cell line (Figure 6A,B).

Discussion

Tumour cells expressing CD133, with the ability to initiate tumour growth, are referred to as cancer-

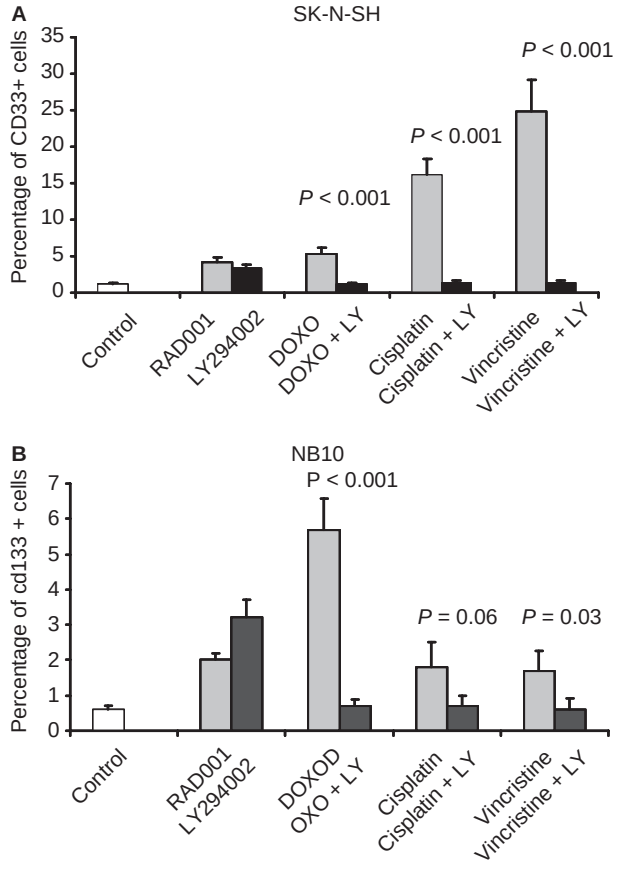

Figure 6. Quantification of CD133+ cells after chemotherapy. Both NB cell lines [SK-N-SH (**A**) and NB 10 (**B**)] were incubated for 24 h with chemotherapeutic agents previously used at IC50 [doxorubicin, 4 μM; cisplatin, 200 μM; vincristine, 15 μM; LY294002 (an AKT inhibitor), 20 μM; and RAD001 mTOR inhibitor (Novartis, Basel, Switzerland), 10 μM]. The studies using doxorubicin, cisplatin and vincristine, were repeated with addition of LY294002 at IC50 to the medium. Control represented untreated cells. Neuroblastoma cells were incubated with an anti-CD133 antibody directly associated with APC-A, and were analysed by fluorescence-activated cell sorting.

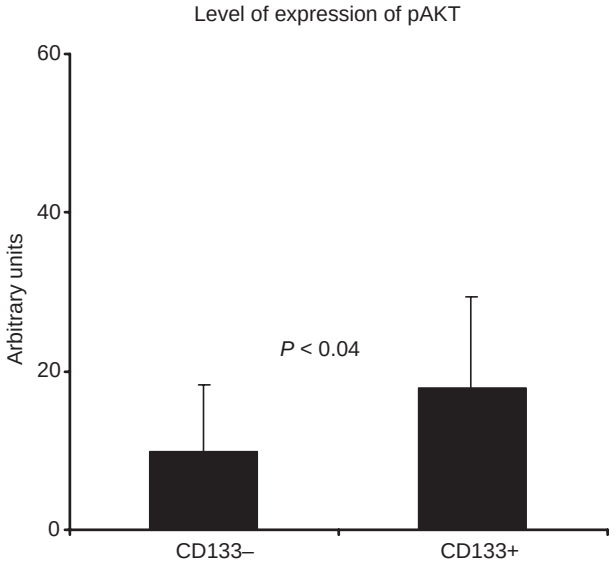

Figure 7. Densitometry analysis of western blots showed that phospho-AKT (pAKT) expression was significantly higher in patients expressing CD133, confirming the immunohistochemical analysis.

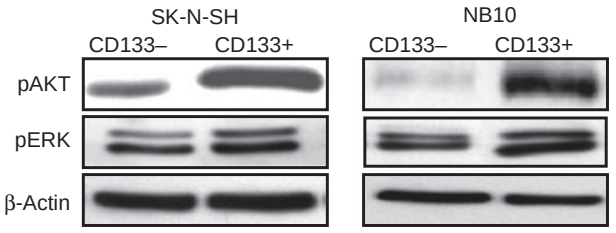

Figure 8. Western blots using SK-N-SH and NB10 cells, with and without CD133 selection. Proteins from the two cell lines were immunoblotted with antibodies against pAKT, pERK and β-actin for 1 h at room temperature.

initiating cells or CSCs, and have been identified in various tumours, including colon and breast carcinoma.[34,35] Recent studies have demonstrated that CD133 expression is an independent prognostic marker for a low survival rate in colorectal cancer, hepatocellular carcinoma, and glioblastoma.[34,36,37] CD133 expression is significantly correlated with the presence of metastases in paediatric melanoma.[38] In human central nervous system neoplasms, CD133 expression is associated with small round blue cell tumour morphology.[39]

The remarkable heterogeneity of outcome in NB, varying from spontaneous regression or maturation to metastatic tumours with poor prognosis, defied explanation until molecular genetic and biochemical analysis of these tumours began to shed light on the disparate clinical behaviours. Many genetic features of NBs, such as ploidy status, oncogene amplification, and allelic loss, have now been shown to correlate with clinical outcome.[40] Amplification of *MYCN* is associated with rapid tumour progression and a poor prognosis in patients with both earlier and advanced stages of disease;[41] however, the reason why *MYCN* amplification is associated with a more aggressive phenotype is still uncertain. In this study, we have shown that CD133 is also strongly and independently associated with poor outcome in patients with NB. The predictive impact of CD133 is significant: it is an independent predictor of worse progression-free survival in patients without *MYCN* amplification and in patients with low-risk and intermediate-risk disease according to the Children's Oncology Group classification. Several

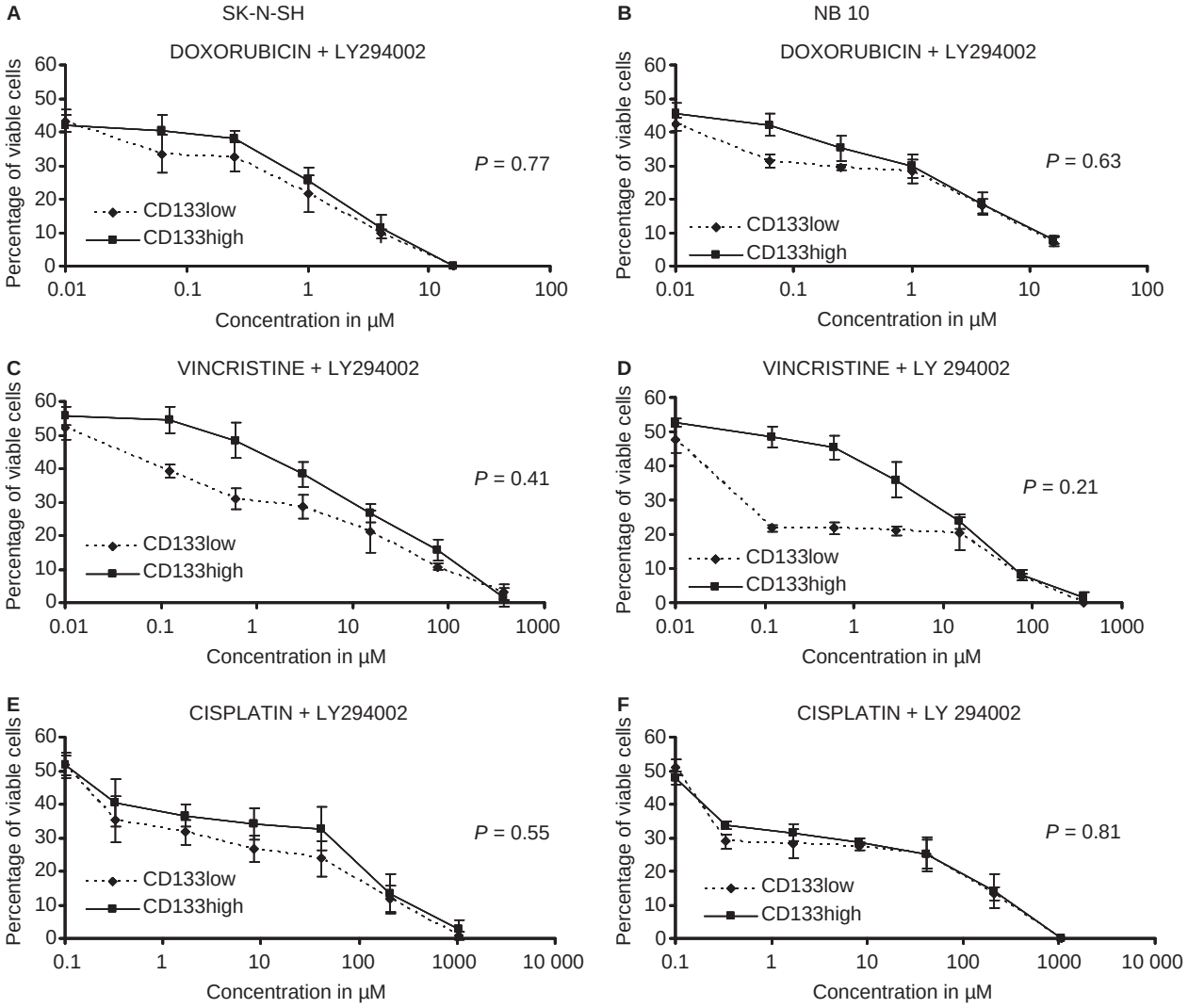

Figure 9. AKT inhibition restores *in-vitro* chemosensitivity to CD133+ cells. Cells were incubated with doxorubicin, vincristine and cisplatin at the same concentrations as in Figure 5, in combination with LY294002 at IC50 (20 μM).

in-vitro studies have demonstrated that hypoxia could enhance expression of CD133,[42,43] but we found that the expression of hypoxia-inducible factor 1α, a hypoxic marker, showed no correlation with that of CD133 (data not shown).

CD133 expression in NB cells is also associated with chemoresistance *in vitro* to traditional chemotherapeutic agents. In our study, we found that CD133+ cells isolated from two NB cell lines (one with amplification of *MYCN*, and the other without) showed increased resistance to doxorubicin, vincristine and cisplatin at various concentrations as compared with their autologous CD133− counterparts. Another group has recently reported similar findings.[44] Chemoresistance was further demonstrated by enrichment of the CD133+ subpopulation in unselected NB cells when they were exposed to chemotherapeutic agents at IC50. Also, CD133 was associated with poor prognosis and chemoresistance in NB, the property of CSCs of cells expressing CD133 were not demonstrated in the present study and require further investigation in NB.

While the specific signalling pathways by which CD133+ cancer cells evade the effects of chemotherapy currently remain speculative, several possible aetiological mechanisms have been described. In glioblastoma, the association of the high expression of epidermal growth factor receptor, of inhibitors of apoptosis protein and the presence of CD133 led to resistance to concomitant chemoradiotherapy.[5,45] Preliminary studies have attributed the increased resistance shown by CD133+ cells exposed to chemotherapeutic agents to elevated expression of specific ATP-binding cassette

drug transporters.[46,47] CD133+ hepatocellular carcinoma cells contribute to chemoresistance through preferential activation of the AKT pathway and its associated mechanism of suppression of apoptosis.[23] In our study we found overexpression of the activated form of AKT in CD133+ tumours, compared with CD133− tumours, regardless of *MYCN* amplification, both in immunohistochemistry and in western blot studies. This was confirmed by western blot studies using cell lines, which demonstrated a high level of expression of pAKT, but not of pERK, in $CD133^{high}$ cells. With these preliminary data, we hypothesized that chemoresistance of CD133+ NB cells is related to activation of the AKT pathway and we therefore examined the ability of an AKT inhibitor (LY2960202) to prevent CD133+ cell chemoresistance. AKT inhibitors are known to specifically induce cell death in NB.[23] Coincubation of the AKT inhibitor with doxorubicin, cisplatin or vincristine almost completely inhibited the preferential survival effect induced by CD133+ cells, especially at high concentrations (concentration higher than IC50), providing additional evidence that the AKT pathway plays a role in the increased survival and resistance of CD133+ NB cells. Moreover, the combination of LY2940202 with chemotherapeutic agents at IC50 removed the enrichment of the CD133+ subpopulation of cells in unselected NB cells.

In summary, our study of NB from 280 patients revealed that CD133 expression is present in about a third of primary NBs, is strongly associated with a poor outcome in univariate analysis, and is independently predictive of worse overall survival. The identification of CD133 expression represents a potential aid in guiding the prognosis and treatment of patients with tumours without *MYCN* amplification. Specific therapies targeted at inhibition of the AKT pathway in CD133+ NB cells may provide a novel method with which to sensitize NB cells to chemotherapy and reduce NB recurrence.

Acknowledgements

We would like to thank The Tristan Baker Fund and Leucan and Charles Bruneau foundation for their support of our work, and Dr C. Bernard and I. Harvey for their help.

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