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A distinct gene expression signature characterizes human neuroblastoma cancer stem cells☆,☆☆

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ABSTRACT

Neuroblastoma, a malignancy of multipotent embryonic neural crest cells, is the most common extracranial solid cancer in childhood and most common cancer in infancy. Cellular phenotype has been shown to be an important determinant of the malignant potential in human neuroblastoma cells and tumors. Whereas neuroblastic (N-type) are moderately malignant and nonneuronal (S-type) cells are nonmalignant, I-type stem cells are highly tumorigenic, irrespective of N-myc amplification status. In the present study, we sought to determine which genes were overexpressed in the I-type cells which might characterize and maintain the stem cell state and/or malignancy of human neuroblastoma cancer stem cells. We used a microarray platform to compare the steady-state expression levels of mRNAs from 13 human neuroblastoma cell lines representing the three cellular phenotypes. Using qRT-PCR and Western blot analyses, we identified seven genes whose expression is consistently elevated exclusively in neuroblastoma cancer stem cells: CD133, KIT, NOTCH1, GPRC5C, PIGF2, TRKB, and LNGFR. Moreover, we show that the genes are phenotype specific, as differentiation of I-type BE(2)-C cells to either an N- or S-type morphology results in significantly reduced mRNA expression. Finally, we show that NOTCH1 plays an important role in maintaining the stem cell phenotype. The identification and characterization of these genes, elevated in highly malignant neuroblastoma stem cells, could provide the basis for developing novel therapies for treatment of this lethal childhood cancer.

1. Introduction

Neuroblastoma is the most common extracranial solid cancer in childhood and the most common cancer in infancy, with nearly 90% of cases occurring in children younger than five years of age (Gurney et al., 1995; Cheung and Dyer, 2013). It is a malignancy of multipotent embryonic neural crest cells. Age, stage, and N-myc amplification are important prognostic factors and are used for risk stratification and treatment assignment. The differences in outcome for patients with neuroblastoma are striking: low- and intermediate-risk patients have an excellent prognosis and outcome whereas those with high-risk disease continue to have poor outlook despite intensive therapy (Matthay et al., 2009). This is especially true for patients older than 18 months at diagnosis with metastatic disease to bone, bone marrow, liver, and lymph nodes. A better understanding of the tumor stem cell may offer alternative strategies for more effective treatment.

Previous studies have shown that distinct cell types are present in human neuroblastoma cell lines and tumors (Shimada et al., 1984; Walton et al., 2004). In vitro, the three cell types differ markedly with regard to their morphology, biochemistry, and malignant potential. The most abundant are neuroblastic (N-type) cells which have small, rounded cell bodies with short neuritic processes and, in culture, attach weakly to the underlying substrate or grow as clumps of floating cells. These cells possess neuronal marker proteins and are mildly tumorigenic. By contrast, non-neuronal, substrate-adherent (S-type) cells grow as a contact-inhibited monolayer with a large cytoplasmic/nuclear ratio. They possess marker proteins identifying them as non-neuronal neural crest-derived cells, such as melanocytes, glial, or smooth muscle cells. Cells with this phenotype are non-tumorigenic (Spengler et al., 1997).

Abbreviations: ACTN4, α-actinin4; BUdR, 5-bromo-2′-deoxyuridine; CFE, colony-forming efficiency; CHGA, chromogranin A; DBH, dopamine-β-hydroxylase; GAPD, glyceraldehyde phosphate dehydrogenase; G418, geneticin; GPRC5C, G-protein-coupled receptor, family C, group 5, member C; HCAM, homing cell adhesion molecule/CD44; Hsp70, heat shock protein 72/73; I-type, intermediate stem cell; Klf4, Krüppel-like factor 4; LNGFR, p75 low affinity nerve growth factor receptor; N-type, neuroblastic; NFL, neurofilament 68; NFM, neurofilament 160; NTRs, neurotrophic receptors; PlGF, placental growth factor; qRT-PCR, quantitative real-time PCR; RA, all-*trans* retinoic acid; RQ, relative quantification; S-type, substrate-adherent non-neuronal; SG2, secretogranin 2; Trk, tyrosine kinase receptor; VIM, vimentin.

☆ Conflict of interest: none.

☆☆ Contribution: RAR, JDW, and N-KVC designed the experiments and wrote the manuscript; RAR, JDW, DH, and H-FG performed the research.

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A third cell type, termed I-like because of a morphology intermediate between N- and S-type cells, has been shown to be a stem-like cell (Walton et al., 2004). These cells possess marker proteins of both N and S phenotypes. Of particular interest, I-type cells are 4- to 5-times more tumorigenic than N-type cells. In N-*myc* non-amplified tumors, increased frequency of these stem-like cells correlated with disease progression and poor survival (Ross and Spengler, 2007). Moreover, the I-type cells were shown by semi-quantitative RT-PCR to express high levels of two known stem cell markers, CD133 and KIT (Walton et al., 2004).

In the present study, we have confirmed and extended these findings by qRT-PCR and Western blotting to identify five genes in addition to CD133 and KIT whose mRNA and protein expression were significantly elevated in the I-type cancer stem cell compared to either N or S cells. The identification of these genes, elevated in the most malignant neuroblastoma cell type, could provide the basis for developing novel therapies focusing on stem cells or stem-like cells.

2. Materials and methods

2.1. Cell culture and differentiation

Six human neuroblastoma I-type [BE(2)-C, SK-N-MM, SK-N-HM, SK-N-LP, CB-JMN, and SH-IN], five N-type [BE(2)-M17, SH-SY5Y, KCN-69n, SK-N-BE(1)n, and LA1-55n], and four S-type [SH-EP1, LA1-5s, SMS-KCNs, and SK-N-BE(2)s] enriched populations or clonal cell lines were included in this study and have been described, in part, previously (Walton et al., 2004). Cells were cultured in a 1:1 mixture of Eagle's Minimum Essential Medium (with non-essential amino acids) and Ham's Nutrient Mixture F12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) without antibiotics. In differentiation studies, the I-type BE(2)-C cell line was grown for 7–21 days in the presence of 10 μM all-*trans* retinoic acid (RA) or 10 μM 5-bromo-2′-deoxyuridine (BUdR), concentrations determined in earlier studies to yield optimal differentiation into either N or S cells, respectively (Walton et al., 2004; Ross et al., 1995).

2.2. Semi-quantitative and real-time reverse transcription polymerase reaction (RT-PCR)

In studies of placental growth factor (PlGF) splice variants, semi-quantitative RT-PCR was used to assess which variant was more highly expressed in I-type cells. Products were separated on 1.6% agarose gels and the amount of product measured by densitometry. Quantitative real-time PCR (qRT-PCR) was performed using a StepOne thermocycler (Applied Biosystems, Foster City, CA) with Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA). Relative mRNA level of a target gene was measured by the $\Delta\Delta C_T$ method. In this method, expression levels of target mRNAs in each cell line were normalized to an endogenous control (glyceraldehyde phosphate dehydrogenase [GAPD]), and then expressed as a fold change to that of a reference calibrator cell line (SH-SY5Y). The thermocycle parameters were: 95 °C for 30 s, followed by 40 cycles of 95 °C for 3 s, and 60 °C for 15 s. The primer sets used are available upon request.

2.3. Immunoblot analyses

Cells in exponential growth phase were lysed by the method of Ikegaki et al. (1986) and proteins separated by standard techniques (Lazarova et al., 1999). Blots were probed with antibodies to prominin 1, Notch1, c-kit, G-coupled protein receptor C5C, placental growth factor, secretogranin 2, and neurofilament 160 (Abcam, Cambridge, MA). Bound antibody was detected by chemiluminescence using secondary antibodies conjugated to horseradish peroxidase. The amount of protein was quantified by scanning densitometry of resulting Kodak XAR films and normalized to heat shock protein 72/73 (Hsp70) [Ab-1] (Oncogene Research Products, Boston, MA). Band specificity was confirmed using protein standards.

2.4. Stable transfection

Plasmids containing NOTCH1 (Sigma-Aldrich Corporation, St. Louis, MO) shRNA constructs were transfected into I-type BE(2)-C cells using Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions; stable transfectants were selected with 100–500 μg/ml Geneticin (G418) (Invitrogen Corporation, Carlsbad, CA). Selected populations were isolated using cloning cylinders and used for further experiments.

2.5. Transformation assay

Anchorage-independent growth ability was measured by growth in soft agar (0.33% Difco Bacto agar) (Walton et al., 2004). Mean colony-forming efficiency (CFE; the number of colonies divided by cell inoculum × 100) was determined in duplicate in two to four independent experiments.

3. Results

3.1. Microarray analyses

Microarray analyses were performed on 13 human neuroblastoma enriched populations or clones to compare differential gene expression in the N, I, and S cells to identify genes up-regulated in I-type cells which might contribute to either their stem cell features and/or their high malignant potential. To confirm the validity of the Affymetrix Human Genome U133 Plus 2.0 (HG133 Plus 2) microarray with regard to cell phenotype, we examined expression levels of markers we had previously shown to reflect cell phenotype in the three cell variants (Walton et al., 2004). Three N-type [neurofilament 68 (NFL), dopamine-β-hydroxylase (DBH), and chromogranin A (CHGA)] and three S-type [vimentin (VIM), Homing cell adhesion molecule/CD44 (HCAM), and α-actinin4 (ACTN4)] markers were analyzed in the 13 cell variants. High level expression of N-type markers was evident in both N or I cell types whereas high level expression of S-type markers was pronounced, for the most part, only in S and I cells (Table 1).

From the microarray, 64 genes were initially identified to be elevated more than 5-fold in I-type cells. Only seven genes were confirmed by qRT-PCR whose expression was consistently higher in all I-type cancer stem cells. They were: CD133, KIT, NOTCH1, GPRC5C, PlGF2, LNGFR, and TRKB. The elevated expression of two of these genes (CD133 and KIT) was previously reported using semi-quantitative RT-PCR (Walton et al., 2004). To expand and validate the differential expression of each of these seven genes in human neuroblastoma I-type cells and to examine their potential roles in cancer stem cell biology, we used

Table 1
Relative mRNA expression levels for N and S cell markers from the microarray.

Gene[a]	Relative expression level (%)		
	N[b]	I	S
N-marker			
NFL	100 ± 28	48 ± 29	6 ± 1
DBH	100 ± 38	54 ± 23	8 ± 1
CHGA	100 ± 13	99 ± 268	3 ± 1
S-marker			
VIM	28 ± 12	65 ± 16	100 ± 17
HCAM	3 ± 1	14 ± 8	100 ± 29
ACTN4	27 ± 8	35 ± 4	100 ± 44

[a] Gene abbreviation: NFL, neurofilament 68 kD; DBH, dopamine-β-hydroxylase; CHGA, chromogranin A; VIM, vimentin; HCAM, homing cell adhesion molecule (CD44); ACTN4, α-actinin4.
[b] Phenotype: N, neuroblastic; I, intermediate stem cell; S, substrate-adherent (nonneuronal).