Gene expression of growth signaling pathways is up-regulated in CD133-positive medulloblastoma cells

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Abstract. Medulloblastoma (MB) is the most common malignant brain tumor in children. Cancer initiating cells (CICs) have been proposed to be involved in the development of brain tumors including MB. Prominin-1 antigen (CD133) is a candidate surface molecular marker for CICs. In the present study, CD133-positive cells were isolated from human Daoy MB cells and their gene expression was compared with that of control Daoy cells. DNA microarray analysis revealed that there were 398 up-regulated genes (>2-fold increase) and 318 down-regulated genes (<50% decrease) in the CD133-positive cell-enriched fractions. Up-regulated genes included neuregulin-1, cyclin D1, cyclin-dependent kinase 6, vascular endothelial growth factor, inhibin β A, promyelocytic leukemia gene, MYC, and hairy enhancer of split-1, which are components of growth signaling pathways. Molecular studies suggest that developmentally regulated signals important for stem cell maintenance are also involved in MB tumorigenesis. Moreover, these molecules can serve as novel targets for MB treatment.

Introduction

Medulloblastoma (MB) is the most common malignant brain tumor in childhood and is thought to arise from precursor cells in the cerebellar granule cell lineage (1). MB patients are now divided into stratification groups according to age, degree of resection and disease dissemination, and are treated depending upon risk. Although the use of multidisciplinary approaches and stratification management of the disease have improved prognosis, 50% of patients, particularly in the high-risk group, experience disease recurrence, dissemination to the cerebrospinal fluid space, and/or a high incidence of sequelae (2).

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The concept regarding the existence of cancer stem cells or cancer initiating cells (CICs) is currently a focal point. The hypothesis that cancerous cells originate from rare populations of CICs that are more tumorigenic than other cancer cells has gained increasing credence (3). CICs are thought to persist in tumors as a distinct population that can cause tumor recurrence and distant metastasis. The existence of CICs in MB has also been reported (4,5). Prominin-1 antigen (CD133) was identified in hematopoietic stem cells (6,7) and neuroepithelial stem cells (8) and has generally been used as a marker for CICs (9). Although some investigators assert that CD133 is not an adequate marker of CICs since both CD133-positive and -negative cells are able to initiate tumors (10), it is also true that CIC-like cells that exhibit self-renewal and multipotential properties are restricted in the CD133-positive cell fractions. In the present study, CD133-positive cells were isolated from the human Daoy MB cell line using magnetic-activated cell sorting (MACS) beads and the transcript profiles of CD133-positive Daoy MB cells were investigated using DNA microarray analysis in order to obtain a better understanding of the molecular properties of CICs involved in MB tumorigenesis.

Materials and methods

Cell culture. The human Daoy medulloblastoma (MB) cell line was purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Inc., St. Louis, MO, USA) with 10% fetal bovine serum (FBS; Sigma-Aldrich, Inc.), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37˚C under 5% CO_2.

Flow cytometry. Cells were detached in phosphate-buffered saline (PBS) containing 0.25% trypsin and 0.02% EDTA for 3 min at 37˚C under 5% CO_2, and the reaction was stopped by adding complete medium (DMEM with 10% FBS). Following centrifugation at 1000 rpm for 5 min, the cells were washed and resuspended in bovine serum albumin (BSA)/PBS buffer (PBS with 0.1% BSA and 2 mM EDTA). Half of the cells were incubated with FcR blocking reagent (Miltenyi Biotec Inc., Auburn, CA, USA) and anti-CD133-PE (Miltenyi Biotec Inc.) for 10 min at 4˚C, and the remaining cells were incubated with IgG-PE (BD Biosciences, San Jose, CA, USA) as controls. After washing, the cells were resuspended in BSA/PBS buffer

Table I. RT-PCR primer sequences of the genes of interest.

Gene symbol	Genebank	Sense	Antisense
NRG1	NM013959	TTGGTGCTGCTTTCTTGTTG	CGGAGCCTCACACACCTATT
CCND1	BC000076	TCCTCTCCAAAATGCCAGAG	TGAGGCGGTAGTAGGACAGG
CDK6	NM001259	AGGGTGCAGTCAAAACAACC	TCCCATCCACTTCAAAGGAG
VEGF	AF091352	TGCAGATTATGCGGATCAAA	GCGAGTCTGTGTTTTTGCAG
INHBA	M1343	AGACGCTGCACTTCGAGATT	CCCTTTAAGCCCACTTCCTC
JAG1	U73936	AGCTGGCTTACACTGGCAAT	AAGTGGGAGCTCAAAGACCA
MYC	NM002467	CTCCTGGCAAAAGGTCAGAG	TCGGTTGTTGCTGATCTGTC
HES1	NM005524.2	CTCTCTTCCCTCCGGACTCT	AGGCGCAATCCAATATGAAC
PML	AF230411	GCAGCAGTGAGTCCAGTGA	GCTCTGCCTGCACTTCTTT
NFASC	NM015090.2	TGCCTTGCTTTTGAGGAGAT	GGCTGTGGTCAGGGAAACTA
APOE	NM000041	CCAATCACAGGCAGGAAGAT	AGCGCAGGTAATCCCAAAAG
ASTN	AB0006627	ACAACACCCTCCTGGATCTG	AAGGAGTCCATTGCACCAAC
BMP2	NM001200	GGAGAATGCCCTTTTCCTCT	ACAACCCTCCACAACCATGT
NEFL	NM006158.2	TCTGTTTGCTTGCAGAGTGG	GCTAACCACCGAAGGTTCAA
MAP2	U89330	AAGAAGGTCGCCATCATACG	GGCGGATGTTCTTCAGAGAG
GAPDH		TGCACCACCAACTGCTTAG	GAGGCAGGGATGATGTTC

and analyzed using the Beckman Coulter Epics XL system (Beckman Coulter, Inc., Chaska, MN, USA). The data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Cell sorting. CD133-positive Daoy cells were sorted using the CD133 cell isolation kit (Miltenyi Biotec Inc.). Briefly, cells were suspended in BSA/PBS buffer, incubated with FcR blocking reagent and CD133 microbeads (Miltenyi Biotec Inc.) for 30 min at 4˚C. To determine the sorting efficiency, the cells were incubated with anti-CD133/2-PE for 10 min. Following washing and centrifugation, the cells were resuspended in BSA/PBS buffer, loaded onto a magnetic separation column (Miltenyi Biotec Inc.) and placed in a magnetic cell separator. The column was rinsed, and the magnetically labeled cells were flushed out with elution buffer and collected. These cells were used in the subsequent experiments.

DNA microarray analysis. Total RNAs were isolated from the CD133-positive Daoy cells (sorted and control) using TRIzol™ (Invitrogen, Carlsbad, CA, USA). Synthesis and labeling of cRNAs and hybridization of biotin-labeled cRNA probes to the Human Genome U133A 2.0 expression Chip arrays (Affymetrix, Santa Clara, CA, USA) were performed according to the manufacturer's protocol. The imaging screens were scanned and analyzed using the Affymetrix Microarray Suite and GeneSpring GX (Agilent Technologies, Santa Clara, CA, USA).

Semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analyses. Total RNAs were prepared and used as templates for cDNA synthesis with random hexanucleotide primers and SuperScript reverse transcriptase II (Invitrogen). Real-time PCR analyses were performed using a QuantiTect SYBR-Green PCR kit (Takara, Kyoto, Japan) and a LightCycler System (Roche, Basel, Switzerland). The PCR primer sequences were determined using WWW primer tool, Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) (Table I). The transcript abundance of the genes of interest was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard. At least 3 independent analyses were performed for each sample and for each gene.

Results

CD133-positive Daoy MB cells were highly enriched by MACS. Flow cytometry showed that 3-5% of Daoy cells expressed prominin-1 antigen (CD133) and CD133/2 antigens. After MACS was applied, the CD133-positive cells were highly enriched (>60%). These cells were then used in the DNA microarray gene expression analyses.

Transcript analysis in CD133-positive MB cells. Transcript analysis using DNA microarrays was performed, and the acquired data were filtered according to the gene expression level. In comparison with the control Daoy cells, the CD133-positive cell-enriched fractions exhibited a >2-fold increase in the expression of 398 genes, and a <50% decrease in the expression of 318 genes. A number of molecules involved in the growth signaling pathways, which play important roles both in MB oncogenesis and stem cell proliferation, were up-regulated in the CD133-positive cell-enriched fractions. These molecules included neuregulin-1 (NRG1; which showed a 6.818-fold increase), cyclin D1 (CCND1; 5.636), cyclin-dependent kinase 6 (CDK6; 3.564), vascular endothelial growth factor (VEGF; 3.186), inhibin β A (INHBA; 3.115), Jagged 1 (JAG1; 2.702), promyelocytic leukemia gene (PML; 2.538), MYC (2.479), and hairy enhancer of split-1 (HES1; 2.078) (Table II). On the other hand, neural differentiation markers or developmentally

Table I. Gene changes in the CD133-positive Daoy cells.

Symbol	Genebank	Map	Fold change	Gene name
Up-regulated genes				
RGS16	U94829	1q25-q31	10.87	Regulator of G-protein signaling 16
NRG1	**NM_013959**	**8p21-p12**	**6.818**	**Neuregulin 1; a ligand for the NEU/ERBB2**
CCND1	**BC000076**	**11q13**	**5.636**	**Cyclin D1**
JUN	BC002646	1p32-p31	5.161	V-jun sarcoma virus 17 oncogene homolog (avian)
CASP2	BC002427	7q34-q35	5.081	Caspase 2, apoptosis-related cysteine peptidase
EGR1	NM_001964	5q31.1	3.963	Early growth response 1
MET	AA005141	7q31	3.886	Met proto-oncogene (hepatocyte growth factor receptor)
Cep290	AF317887	12q21.33	3.883	Centrosome protein cep290
CDK6	**NM_001259**	**7q21-q22**	**3.564**	**Cyclin-dependent kinase 6**
MAX	NM_002382	14q23	3.531	MYC associated factor X
DKK1	NM_012242	10q11.2	3.277	Dickkopf homolog 1 (*Xenopus laevis*)
VEGF	**AF091352**	**6p12**	**3.186**	**Vascular endothelial growth factor**
INHBA	**M13436**	**7p15-p13**	**3.115**	**Inhibin, β A (activin A, activin AB α polypeptide)**
PYGO1	AL049925	15q21.1	3.104	Pygopus homolog 1 (*Drosophila*)
JAG1	**U73936**	**20p12.1-p11.23**	**2.702**	**Jagged 1 (Alagille syndrome)**
HDAC9	NM_014707	7p21.1	2.651	Histone deacetylase 9
KHSRP	AI933301	19p13.3	2.643	KH-type splicing regulatory protein (FUSE binding protein 2)
NPAT	U58852	11q22-q23	2.564	Nuclear protein, ataxia-telangiectasia locus
GADD45B	NM_015675	19p13.3	2.563	Growth arrest and DNA-damage-inducible, β
PML	**AF230411**	**15q22**	**2.538**	**Promyelocytic leukemia**
GREM1	NM_013372	15q13-q15	2.508	Gremlin 1, cysteine knot superfamily, homolog
MYC	**NM_002467**	**8q24.12-q24.13**	**2.479**	
CCNT1	NM_001240	12pter-qter	2.41	Cyclin T1
TGFBR1	NM_004612	9q22	2.408	Transforming growth factor, β receptor I
EGFR	U95089	7p12	2.299	Epidermal growth factor receptor
CCNE2	AF112857	8q22.1	2.162	Cyclin E2
SMAD5	AF010601	5q31	2.125	SMAD, mothers against DPP homolog 5 (*Drosophila*)
HES1	**BE973687**	**3q28-q29**	**2.078**	**Hairy and enhancer of split 1 (*Drosophila*)**
SMAD3	NM_005902	15q21-q22	2.016	SMAD, mothers against DPP homolog 3 (*Drosophila*)
Down-regulated genes				
NFASC	**AI821777**	**–**	**0.0608**	**Neurofascin homolog (chicken)**
IGF1	AI972496	12q22-q23	0.166	Insulin-like growth factor 1 (somatomedin C)
IGFBP5	AW007532	2q33-q36	0.175	Insulin-like growth factor binding protein 5
SEMA3E	NM_012431	7q21.11	0.197	Semaphorin 3E
APOE	**NM_000041**	**19q13.2**	**0.296**	**Apolipoprotein E**
BBP	AA012917	1p32.1	0.356	TM2 domain containing 1
VCAM1	NM_001078	1p32-p31	0.359	Vascular cell adhesion molecule 1
SLIT3	AB011538	5q35	0.328	Slit homolog 3 (*Drosophila*)
RARRES2	BC000069	7q36.1	0.365	Retinoic acid receptor responder (tazarotene induced) 2
ASTN	**AB006627**	**1q25.2**	**0.392**	**Astrotactin**
BMP2	**AA583044**	**20p12**	**0.397**	**Bone morphogenetic protein 2**
TNC	BF434846	9q33	0.404	Tenascin C (hexabrachion)
UNC5B	AA127885	10q22.2	0.406	Unc-5 homolog B (*C. elegans*)
NEFL	**AL537457**	**8p21**	**0.418**	**Neurofilament, light polypeptide 68 kDa**
RBP1	NM_002899	3q23	0.448	Retinol binding protein 1, cellular
CDH11	AU144378	–	0.459	Cadherin 11, type 2, OB-cadherin (osteoblast)
RAI16	NM_022749	8p21.3	0.461	Retinoic acid induced 16
CASP4	AL050391	11q22.2-q22.3	0.48	Caspase 4, apoptosis-related cysteine peptidase
MAP2	**U89330**	**2q34-q35**	**0.49**	**Microtubule-associated protein 2**

The genes of interest are indicated in boldface type.

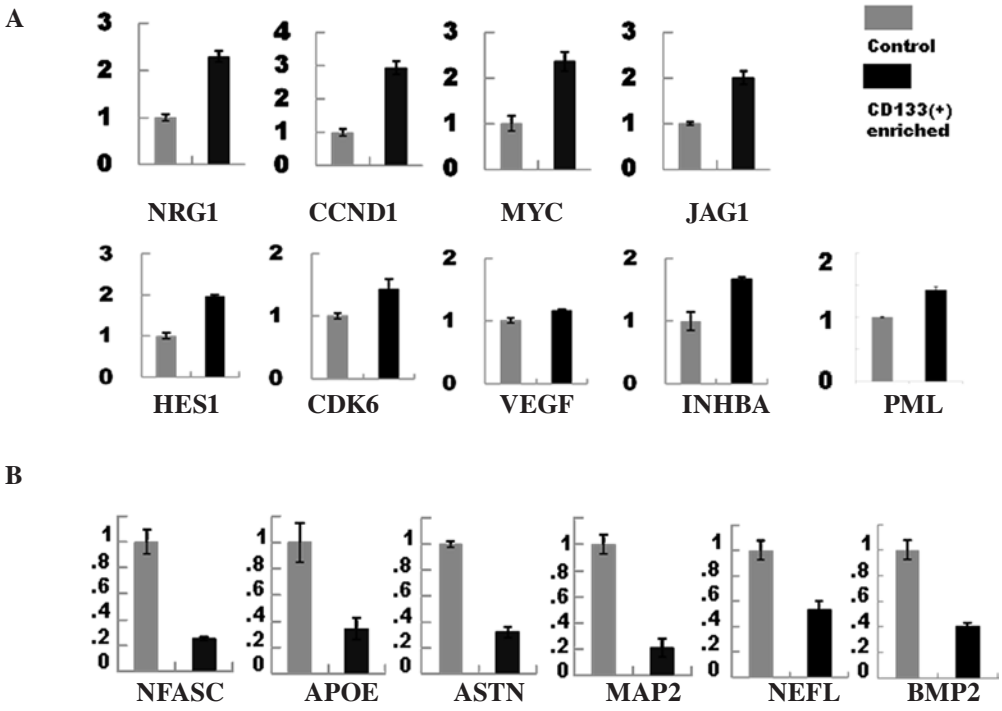

Figure 1. Expression analyses of the genes of interest using semi-quantitative RT-PCR analyses of the up-regulated (A) and down-regulated genes (B).

regulated genes, expressed in the granule cell lineage, such as neurofascin (NFASC; 0.0608), apolipoprotein E (APOE; 0.296), astrotactin (ASTN; 0.392), neurofilament light polypeptide 68 kDa (NEFL; 0.418), and microtubule-associated protein 2 (MAP2; 0.49) were down-regulated (Table II). Semi-quantitative RT-PCR analyses were then performed in the selected genes (up-regulated genes, Fig. 1A; down-regulated genes, Fig. 1B) and the gene expression changes were confirmed to be significant.

Discussion

In the present study, we first isolated CD133-positive cells in the human Daoy medulloblastoma (MB) cell line. The percentage of CD133-positive cells was approximately 3-5%, which was in accordance with previous studies (0.5-10%) (11,12). After MACS was applied, the percentage of CD133-positive cells was noted to be greater than 60%. These enriched cell fractions were subsequently subjected to transcript analysis using DNA microarrays.

Transcript analysis using DNA microarrays identified various molecules that were components of the growth signaling pathways, which play important roles both in MB oncogenesis and stem cell proliferation. The genes which exhibited up-regulated expression included the activator of MAP kinase signal (RGS16), a ligand of ERBB (EGF signal component; NRG1), Wnt signal targets CCND1 and c-myc, a ligand of Notch signal (JAG1), and its target (HES1) (13-15) (Table II). c-myc is known to play a key role in stem cell self-renewal and was used to produce induced pluripotent stem cells (16). The Wnt and Notch pathways are involved in the maintenance of stem cell properties and in MB oncogenesis (17-19). The remaining up-regulated genes included INHBA, an inhibitor of differentiation factors, such as activin and TGF β, and VEGF which plays a role in the neovascularization of tumors (20,21). These genes may be involved in tumor recurrence or distant metastasis.

In contrast, the genes whose expression decreased to less than 50% included the neural markers (MAP2 and NEFL) (22,23), developmentally regulated genes in the cerebellar granule cell lineage (NFASC, UNC5B, ASTAN, SLIT3 and APOE) (24-27), and molecules involved in retinoic acid-induced apoptosis in neuroblastoma (RBP1, BMP2, RARRES2 and CASP4) (28,29). Down-regulation of these genes may result in the inhibition of differentiation and maintenance of undifferentiated properties of CICs or may contribute to the inhibition of cell death, thereby providing infertility to CICs.

An understanding of the molecular pathway involved in MB oncogenesis has been advanced by analyses of the Turcot- and Gorlin-inherited syndromes which are associated with the development of MB. The Wnt and sonic hedgehog (SHH) signal pathways are involved in MB oncogenesis in the Turcot and Gorlin syndromes, respectively (30-33). In addition, the Notch, epidermal growth factor receptor ERBB, and platelet-derived growth factor (PDGF) signaling pathways are involved in MB oncogenesis or prognosis (33-37). These pathways play crucial roles in the proliferation and/or differentiation of the cerebellar granule cell lineage where MB originates. Furthermore, molecular studies have shown that developmentally regulated signals, such as Wnt, SHH and Notch, play important roles in self-renewal, proliferation and/or the multipotency of stem cells, and are also involved in MB oncogenesis (17-19). These molecular studies and the results of the present study indicate that further understanding of the molecular properties and fundamental signaling pathways of CICs involved in MB oncogenesis may lead to the

development of new, more effective, and less toxic treatment modalities for MB, thereby improving the quality of life of children with MB.

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