Phenotypic Characterization of Retinoblastoma for the Presence of Putative Cancer Stem-like Cell Markers by Flow Cytometry

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Purpose. Retinoblastoma (Rb) is an intraocular tumor that grows rapidly and poses a threat to sight and life. Similar to other tumors, there is increasing speculation that the Rb tumor also contains cancer stem-like cells that could influence the prognosis and response to therapy. This study was undertaken in an attempt to identify putative stem-like cells by characterizing different subpopulations of cells in retinoblastoma.

Methods. Freshly isolated tumor cells obtained from unfixed eye specimens ($n = 7$) were analyzed for the presence of CD44, ABCG2, CXCR4, CD133, and CD90 using flow cytometry. RT-PCR was performed to analyze the expression of human Syntaxin1A, PROX1, CD133, and NSE in the sorted subpopulation of tumor cells.

Results. Two different subpopulations of cells were observed in seven samples. The small cells, assigned FSC^{lo}/SSC^{lo} (forward scatter low/side scatter low, ranging from 1.7% to 17.7%) were characterized as positive for CD44 and negative for CD133, CXCR4, and CD90. The large cells were designated as FSC^{hi}/SSC^{lo} (ranging from 2.7% to 35.1%) and characterized as positive for all markers. RT-PCR analysis revealed that sorted cells of FSC^{lo}/SSC^{lo} subpopulation expressed the retinal progenitor cell markers PROX1 and Syntaxin1A.

Conclusions. Retinoblastoma, on flow cytometric analysis, revealed two distinct subpopulations with variable expression of stem cell and retinal progenitor markers. In these populations, the FSC^{lo}/SSC^{lo} subpopulation appeared to be more primitive, since they expressed stem cell (CD44) and retinal progenitor markers (PROX1 and Syntaxin 1A) combined with a relatively lower percentage of differentiated markers. Moreover, the FSC^{hi}/SSC^{lo} subpopulation showed a higher percentage of differentiated markers (CD90 and CD133). (*Invest Ophthalmol Vis Sci.* 2009;50:1506–1514) DOI:10.1167/iovs.08-2356

Retinoblastoma (Rb) is the most common childhood cancer, caused by inactivation of both alleles of the *Rb1* gene, either as hereditary or as nonhereditary forms, according to Knudson's two-hit hypothesis.[1] It is one of the few tumors for which the genetic cause has been well established. Emerging treatment protocols have produced marked improvement in survival. Preimplantation genetic testing is being actively pursued in clinical practice.[2] However, there are many unclear aspects relating to the cell biology of the tumor and presence of chemoresistant cancer stem-like cells that must be addressed.[3]

Recent evidence suggests the presence of cancer stem-like cells in various malignancies, such as acute leukemia and neural, breast, and ovarian tumors.[4–10] Previous reports have demonstrated that stem cells expressing Oct4, Nanog, ABCG2, ALDH1, MCM2, and low Hoechst 33342 cells are present in human Rb. However, these studies were based on fixed tissues and Rb cell lines.[11–13] Moreover, evaluation of other tumors has been performed on the basis of phenotypic expression of stem cell markers, formation of clones, and ability to form tumors in animal models.[7,14] The marker expression profile that is commonly expressed in leukemia include $CD133^+$ $CD34^+/CD38^-$, $CXCR_4^+$ and in breast tumors $ESA^+CD44^+CD24^{-/low}lin^-$ markers.[8,9,14] In pediatric brain tumors, tumor-derived progenitors form neurospheres that can self-renew when passaged at clonal density. These neurospheres express many genes characteristic of neural and other stem cell markers, including CD133, nestin, SOX2, musashi-1, Bmi-1, and Oct4.[7,15]

In this study we report that there are two subpopulations expressing different markers in Rb based on scatter properties. Furthermore, we have sorted the two subpopulations and analyzed for the retinal progenitor (PROX1 and Syntaxin1A) and differentiated markers (CD133 and NSE). Our results indicate a higher expression of retinal progenitor cell markers in the FSC^{lo}/SSC^{lo} subpopulation. Flow cytometry and gene expression analyses indicated the presence of cells expressing primitive markers in Rb, thus suggesting the possibility of a hierarchy of stem cells within a tumor.

Materials and Methods

Tissue Collection

All samples were collected with the approval of the institutional review board at LVPEI and in accordance with the Declaration of Helsinki. Fresh unfixed eyeballs of patients with diagnosed Rb, without any prior treatment, were included in the study. The enucleated eyeballs were obtained in sterile conditions. After the fresh tissue was placed in plain RPMI or DMEM F12 medium, singly suspended cells were analyzed by flow cytometry. The callotes were fixed in 10% buffered formalin and processed for

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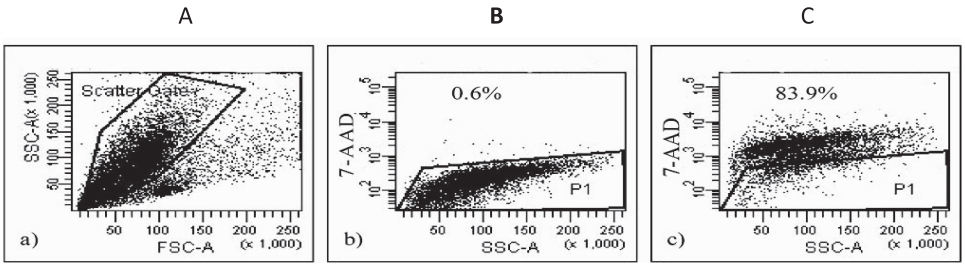

FIGURE 1. (**A**) Scatter gate selection included small cells with high granularity, which represented dead cells; hence, these were eliminated from final analysis; (**B**) control cells without 7-AAD dye; (**C**) cells stained with 7-AAD.

8routine histologic examination. The slides were reviewed by an experienced ocular pathologist (GKV) specifically for features of differentiation; histologic risk factors such as involvement of optic nerve, choroid, anterior segment; and other associated features. Specimens from cases with high risk factors were further evaluated for systemic spread by examining bone marrow and cerebrospinal fluid. The clinical profiles of the patients were obtained from their medical records.

Flow Cytometry

One million cells were stained by incubating with 50 mL diluted primary or direct conjugated antibodies at 4°C for 45 minutes. The cells were washed thrice with PBS containing 0.1% sodium azide. For unconjugated primary mouse IgG antibodies, cells were incubated for 45 minutes with secondary anti-mouse IgG FITC. Stained cells were analyzed by flow cytometry. Isotype controls for the corresponding antibodies were used. The markers analyzed were mouse anti-human CD133 (50 μg/mL at a dilution of 1:10), FcR blocking reagent 10 μL/million cells (Miltenyi Biotech, Gladbach, Germany), PE-conjugated anti-human CD44 (0.2 mg/mL), APC-conjugated anti-human CXCR4 (0.5 mg/mL), FITC-conjugated anti-human CD90 (0.5 mg/mL), APC-conjugated mouse IgG2a κ-isotype control (50 μg/0.5 mL), PE-conjugated rat IgG2b isotype control (0.2 mg/mL), FITC-conjugated mouse IgG1 κ-isotype control (0.2 mg/mL), FITC-conjugated anti-mouse IgG1 (0.5 mg/mL; eBioscience, San Diego, CA), and mouse anti-human ABCG2 (250 μL culture supernatant; Abcam, Cambridge, UK). 7-Amino actinomycin D (50 μg/mL; BD Biosciences, San Jose, CA) staining was performed to eliminate dead cells from analysis. All antibodies were used at 1:100 dilution. The cells were analyzed with flow cytometry (FACS Diva software; BD Bioscience) on a customized system (BD-FACS Aria; BD Biosciences) using 488-nm blue laser and 633-nm red laser. Drop delay was calculated using fluorescent beads (BD Accudrop; BD Biosciences). The sample of case 6 was sorted based on the expression of CD44 and CXCR4 markers.

Elimination of Doublets and Dead Cells from the Final Analysis

On average, 12.3×10^6 cells were obtained from tumor samples. This included many dead cells (ranging from 50.1% ± 2.9% to 93.1% ± 0.7%), which were characterized by their small size, high granularity, and positivity for 7-AAD dye. These were eliminated from the final analysis by appropriate gating (Figs. 1A–C). A fraction of cells ranging from 6.8% ± 0.7% to 49.8% ± 2.9% was selected from the scatter gate; these are shown in doublet discrimination plots (SSC-w versus SSC-h and FSC-w versus FSC-h). Subsequently, the samples were evaluated for dead cells and marker expression in viable cells (viability ranged from 57.3% ± 1.4% to 98.5% ± 0.2%). Percentages of scatter gate, non–scatter gate, FSC gate, viable, and dead cells across all samples are given in Table 1.

Reverse Transcriptase-PCR

Sorted cells (Q4 and Q2 quadrant) were counted on a hemocytometer. Total RNA was isolated from an equal number of cells (TRIzol; Invitrogen Life Technologies Corp., Carlsbad, CA). RNA was reverse transcribed using reagents from the first-strand cDNA synthesis kit (Invitrogen Life Technologies Corp.). GAPDH was used as an internal control and reaction mixture without template as a negative control. Product size and primers for amplification of human Syntaxin1A, PROX1,

TABLE 1. Percentage of Cells That Were in Scatter Gate, Non–Scatter Gate, FSC Gate, Dead, and Viable Cells

Case No.	Cells in Scatter Gate (%)	Cells in Non–Scatter Gate (%)	Cells after Doublet Discrimination FSC Gate (%)	% Viable Cells in FSC Gate	% Dead Cells in FSC Gate
Case 1	6.8 ± 0.7	93.1 ± 0.7	6.3 ± 0.6	67.1 ± 12	32.1 ± 10.8
Case 2	19 ± 0.2	80.9 ± 0.2	13.6 ± 0.2	65.4 ± 3.4	34.6 ± 3.4
Case 3	26.9 ± 1.1	73 ± 1.1	23.6 ± 1.2	61.7 ± 6.7	38.3 ± 6.7
Case 4	7.4 ± 1.2	92.6 ± 1.2	6.6 ± 0.9	57.3 ± 1.4	42.7 ± 1.4
Case 5	35.1 ± 3.6	64.8 ± 4.6	34.3 ± 3.6	98.5 ± 0.2	1.5 ± 0.2
Case 6	30 ± 4.2	69.6 ± 3.9	29.4 ± 4.1	84.6 ± 11.7	15.3 ± 11.7
Case 7	49.8 ± 2.9	50.1 ± 2.9	48.7 ± 2.9	96.3 ± 0.9	3.6 ± 0.9

Results of multiple experiments are expressed as the mean ± SD.

TABLE 2. Primer Sequences and Product Sizes of the Genes Used

Subject No.	Gene Name	Forward Primer	Reverse Primer	Product Size (bp)
1	*Syntaxin1A*	ctgcagtcagtccctcaag	ctgccgaatactgcatctg	170
2	*PROX1*	caagttgtggacactgtggt	gcagactggtcagaggagtt	272
3	*CD133*	cctctggtggggtatttctt	aggtgctgttcatgttctcc	460
4	*NSE*	catcgacaaggctggctacacg	gacagttgcaggccttttcttc	329
5	*GAPDH*	gccaaggtcatccatgacaac	gtccaccaccctgttgctgta	498

TABLE 3. Clinical and Histologic Features of the Seven Rb Cases

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Age	7 y	7 y	4 y	1 1/2 y	4 y	3 y	7 y
Sex	F	F	F	M	M	M	M
Laterality	U	U	U	U	U	U	U
Eye	OD	OS	OS	OD	OS	OD	OS
Heredity	Sporadic	Sporadic	Sporadic	Sporadic	Sporadic	Sporadic	Sporadic
Duration of symptoms	10 d	10 d	3 mo	9 mo	3 d	2 mo	10 d
Clinical features	H/O pain redness and watering	White reflex	White reflex	Leukokoria	H/O pain redness and watering	Progressive loss of vision	H/O pain redness and watering
Retinoblastoma group (ICIR)	Group E	Group D	Group E	Group E	Group E	Group E	—
Histopathologic Risk Factors							
Differentiation	Poorly differentiated	Poorly differentiated	Poorly differentiated	Well differentiated with Flexner-Wintersteiner and Holmer-Wright rosettes	Poorly differentiated	Poorly differentiated	Poorly differentiated
Choroid involvement	Massive choroid involvement	−	Minimum choroid involvement	Minimum choroid involvement	−	−	Minimum choroid involvement
Anterior segment	+	−	−	−	−	−	−
Optic Nerve	−	−	Anterior layers of the cribrosa involved	−	Prelaminar involvement of cribrosa	−	−
Sclera	−	−	−	−	−	−	−
Others	Angle and iris deposits	Focal areas of sub-RPE deposits seen	NVI	NVI	−	−	−
Bone marrow/CSF involvement	Negative	−	ND	ND	−	−	Negative
		−	1 wk	1 mo	−	−	−
Outcome	8 weeks on adjuvant chemotherapy, 4 cycles were completed Two more to be given. Patient is doing well.	Doing well	Doing well	Doing well	Doing well	Doing well	On adjuvant chemotherapy, 2 cycles were completed. Patient is doing well.

U, unilateral; ND, not done; +, positive; −, negative; CSF, cerebrospinal fluid; NVI, neovascularization of iris; H/O, history of.

TABLE 4. Clinicophenotypical Correlation of Markers in Seven Cases of Rb

Case	Age/Sex	Clinicopathological Features and High Risk Factors	% of $ABCG_2$ Expression	% of CD90 Expression	% of CD133 Expression	% of $CXCR_4$ Expression	% of CD44 Expression
Case 1	7 y/F	OD, PD Choroid and anterior segment involved. Iris and sub-RPE deposits noted	1.14	69.3 ± 1.6	73.30	64.5 ± 8.5	86.6 ± 1.7
Case 2	7 y/F	OS, PD Focal areas of sub-RPE deposits seen	0.44	46.8 ± 3.1	91.10	82.1 ± 12.5	83.9 ± 8.4
Case 3	4 y/F	OS, PD Anterior layers of cribrosa involved. Neovascularization of iris with ectopion of uvea, with sub-RPE deposits seen	1.2	94.8 ± 0.5	90.80	92.4 ± 4.2	97.8 ± 1.6
Case 4	1.5 y/M	OD, WD Sub-retinal tumor deposits noted	0.08	85.70	87.10	46.3 ± 15.6	36.5 ± 8.6
Case 5	4 y/M	OS, PD	0	51.5 ± 6.25	42	3.6 ± 0.8	5.9 ± 1.9
Case 6	3 y/M	OD, PD Anterior layers of cribrosa involved	0.2	46.4 ± 9.4	25.5	18 ± 4.4	98.1 ± 0.2
Case 7	7 y/M	OS, PD Superficial choroidal involvement is seen	0	45.4 ± 9.6	13.0	42.6 ± 4.2	88.1 ± 0.5

PD, poorly differentiated; WD, well differentiated.

CD133, NSE, and GAPDH are given in Table 2. The PCR conditions for human Syntaxin1A, PROX1, CD133, and NSE were 1 cycle of 3 minutes at 94°C; 32 cycles of 1 minute at 94°C, 30 seconds at 57°C, and 30 seconds at 72°C; and 1 cycle of 7 minutes at 72°C. GAPDH was amplified under similar conditions for 25 cycles.

Statistics

Results of multiple experiments are reported as the mean ± SD.

RESULTS

Clinical Profile

The clinical features of the seven cases are summarized in Tables 3 and 4. The mean age of patients included in the study was 4.7 ± 2.2 years (range, 1.5–7) comprising three girls and four boys. All the tumors were sporadic cases; with right eye involvement in three and left eye in four cases. Four cases presented with a history of white reflex/leukokoria, two with a history of pain and redness, and one with progressive visual loss. Six of the seven patients had an advanced form of Rb, with five having an ICIR (International Classification of Intraocular Retinoblastoma) group E and one having an ICIR group D tumor.

Clinical Outcome

Cases 1 and 7 had high risk factors for choroid and anterior segment involvement on histopathologic examination. Both patients were advised to undergo a bone marrow/cerebrospinal fluid (BM/CSF) evaluation. The result showed no tumor cells in BM/CSF. During the last follow-up, four cycles of a three-drug regimen of vincristine, etoposide, and carboplatin had been administered to the patient in case 1 and 2 cycles to the one in case 7. The remaining five patients were doing well at the last follow-up.

Histologic Examination

The summary of clinical and histologic findings is provided in Tables 3 and 4. Except for case 4, which was a well differentiated tumor, the other cases were poorly differentiated and case 6 manifested features of anaplasia (Fig. 2). High risk factors of massive choroid involvement were noted in case 1, while cases 3, 4, and 7 showed minimum choroid involvement (with sub-RPE deposits). Tumor in optic nerve (up to cribrosa) was seen in case 3 and 6. The resected margin of the optic nerve was however free in all the cases.

Phenotypic Analysis of Tumor Cells

We determined the expression of putative stem cell (ABCG2, CD44, and CXCR4) and differentiated retinal cell markers (CD90 and CD133) in the seven tumors by using flow cytometry (Fig. 3).

ABCG2. ABCG2 was positive in five of seven tumors, and its expression ranged from 0.08% to 1.2% of the selected population. In case 4, a well-differentiated tumor, it constituted 0.08% of all cells. Among the poorly differentiated tumors, there was no significant difference observed in the percentage of these cells, irrespective of massive choroid or lamina cribrosa involvement.

CD90. All the tumors had a high and variable expression of CD90 ranging from 45.4% ± 9.6% to 94.8% ± 0.5%. CD90 expression was high in case 3, which exhibited involvement of the anterior layers of the cribrosa (94.8% ± 0.5%). There was no disparity in its expression in well-differentiated or poorly differentiated tumors.

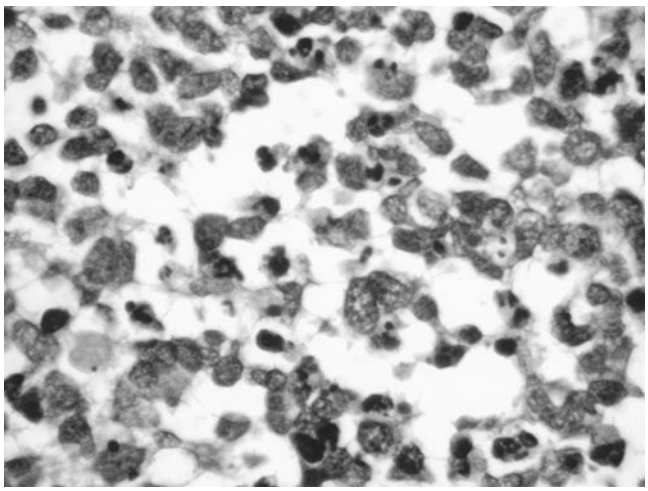

FIGURE 2. Histopathology section of case 6 showing cells with pleomorphic features.

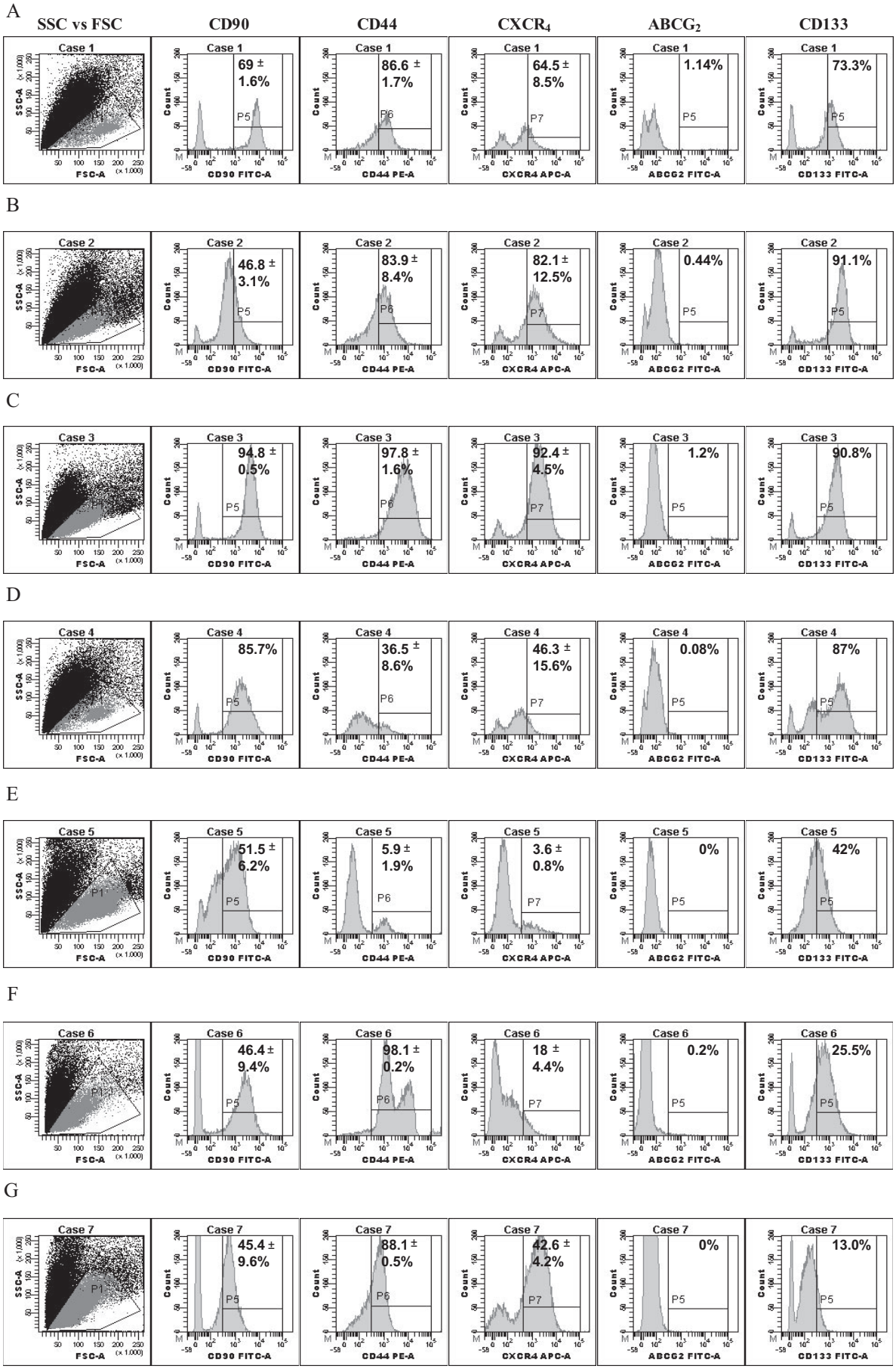

FIGURE 3. (**A–G**) The expression profile of CD90, CD44, CXCR4, ABCG2, and CD133 in 7 cases of Rb.

TABLE 5. Combination of Markers Expressed in Percentage in the Seven Tumor Samples

Combination of Markers	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
$CD44^+CXCR_4^+$	55.5 ± 3.7	75.8 ± 14.9	92.2 ± 1.6	7.3 ± 5.4	0.15 ± 0.05	20.2 ± 6.5	35.9 ± 2.9
$ABCG_2^+CD44^+$	1.032	0.435	0.8	0.028	0	0.2	0
$CD133^+$ $CD44^+$	51.9	81.9	95.3	20.13	6.5	34.9	21.3
$CD90^+$ $CD44^+$	62.8	47.6	91.9	15.9	0.9	33.3	34.7
$ABCG_2^+$ $CXCR_4^+$	0.32	0.435	0.79	0.028	0	0	0
$CD133^+$ $CXCR_4^+$	71.9	81.8	95.6	60.084	8.8	11.7	18.3
$CD90^+$ $CXCR_4^+$	49.2	48	91.24	28.95	3.1	0.7	31.5
$CD44^+$ $ABCG_2^-$	81.9	80.3	98.15	19.13	5.7	95.7	88.3
$CD44^+$ $CD133^-$	25.2	11.1	4.31	10.81	5.4	64.3	65.5
$CD44^+$ $CD90^-$	29.8	34.7	4.8	12.28	5.1	60.7	53.1
$ABCG_2^+$ $CD44^-$	0.14	0.013	0	0.055	0	0	0
$CD133^+$ $CD44^-$	19.8	0.311	0.015	62.36	47.5	0.4	0.1
$CD90^+$ $CD44^-$	6.9	3.03	1.34	71.57	51.5	3.1	2.4
$ABCG_2^+$ $CXCR_4^-$	0.85	0.03	0.154	0	0	0.2	0
$CD133^+$ $CXCR_4^-$	0.26	0.73	0.093	20.95	41.9	24.6	3.3
$CD90^+$ $CXCR_4^-$	21.4	4.05	2.17	58.37	53.1	36.4	10.6
$ABCG_2^-$ $CXCR_4^+$	65.6	85.8	91.8	29.3	2.3	7	34
$CD133^-$ $CXCR_4^+$	3.06	3.37	1.45	0.42	1.3	1.1	20.8
$CD90^-$ $CXCR_4^+$	0.1	23.92	0.78	0.12	0.7	0	8.9
$ABCG_2^+CD44^+CXCR_4^+$	0.6	0.35	1.08	0.002	0	0.1	0
$CD133^+CD44^+CXCR_4^+$	39.63	88.35	93	7.34	0.14	12.5	21.9
$CD90^+CD44^+CXCR_4^+$	30.78	37.3	84.7	6.4	0.07	17.9	29.9

CD133. CD133 showed variable expression in all the tumors ranging from 13.0% to 91.1%, with no specific relationship with differentiation or involvement of the optic nerve or choroid.

CD44. Expression of CD44 ranged from 5.9% ± 1.9% to 98.1% ± 0.2% with the lowest expression in poorly differentiated tumor case 5 and highest in case 3 and 6, which exhibited a marked involvement of anterior layers of lamina cribrosa.

CXCR4. Expression of CXCR4 was variable in all tumors, ranging from 3.6% ± 0.8% to 92.4% ± 4.2% of all cells. The lowest expression was observed in case 5 and highest in the tumor with cribrosa involvement. The percentage of expression of markers along with histologic risk factors for different tumors is shown in Tables 3 and 4.

Combination of Markers Expressed in Rb

The percentage of cells coexpressing ABCG2, CD44, and CXCR4 ranged from 0% to 1.08%. Cells expressing CD133, CD44, and CXCR4 ranged from 0.14% to 93%. Those expressing CD90, CD44, and CXCR4 ranged from 0.07% to 84.7%. Other combinations of markers had variable expression, as shown in Table 5.

Subpopulations of Cells in Rb

We observed two different subpopulations present in the Rb that varied in surface marker expression and scatter properties. The data of all the seven cases are provided in Tables 6 and 7. Two subpopulations include FSC^{lo}/SSC^{lo}, FSC^{hi}/SSC^{lo} and are termed as P2 and P3, respectively (Fig. 4B). The P2 population (ranging from 1.7% to 17.7%) exhibited more cells positive for CD44 (34.6% ± 3.5% to 91% ± 2.2% of the selected population), and negative for CD133, CXCR4, and CD90 markers (Q4 quadrant; Figs. 5A–G). In the P2 population, the cells that were positive for CD44 and CXCR4 coexpressed CD133 and CD90 (Q_2 quadrant; Figs. 4C–E, 5). These cells were present in low percentage (Table 6). P3 population showed (2.7%–35.1% of total cells) higher percentage of cells expressing the combina-

TABLE 6. Differential Expression of Markers in the FSC^{lo}/SSC^{lo} Population in Different Samples

Case No. and Selected Scatter Gate Cells (%)	$CD44^+$ $CXCR4^+$ Cells (%)	$CD44^+$ $CXCR4^+$ and $CD90^+$ Cells (%)	$CD44^+$ $CXCR4^+$ and $CD133^+$ Cells (%)
Case 1 (1.7)	17.9 ± 3	17.5	17.1
Case 2 (4.6)	30.7 ± 11.1	29.6	30.5
Case 3 (4.6)	60.7 ± 6.2	58.57	58.2
Case 4 (2.5)	4.7 ± 5.3	2.8	4.7
Case 5 (2.1)	0.9 ± 0.9	14.3	79.2
Case 6 (12)	2.85 ± 1.6	40	89.8
Case 7 (17.7)	14.7 ± 4.2	84.2	73.9
Case No. and Selected Scatter Gate Cells (%)	**$CD44^+$ and $CXCR4^-$ Cells (%)**	**$CD44^+$ and $CXCR4^-$ Cells That Are $CD90^+$ Cells (%)**	**$CD44^+$ and $CXCR4^-$ Cells That Are $CD133^+$ Cells (%)**
Case 1 (1.7)	54.2 ± 15.6	3.7	0
Case 2 (4.6)	39.9 ± 7.3	4.5	1.8
Case 3 (4.6)	34.6 ± 3.5	4.1	0.3
Case 4 (2.5)	66.5 ± 12.9	2.5	1.8
Case 5 (2.1)	51.5 ± 1.8	0.9	1.2
Case 6 (12)	91 ± 2.2	2.5	0.5
Case 7 (17.7)	84.6 ± 6.4	5.2	3.8

TABLE 7. Differential Expression of Markers in FSC^{hi}/SSC^{lo} Population in Different Samples

Case No. and Selected Scatter Gate Cells (%)	$CD44^+$ $CXCR4^+$ Cells (%)	$CD44^+$ $CXCR4^+$ and $CD90^+$ Cells (%)	$CD44^+$ $CXCR4^+$ and $CD133^+$ Cells (%)
Case 1 (2.7)	92.7 ± 3.4	91.8	91.5
Case 2 (12.2)	95.4 ± 5.5	93.5	94.9
Case 3 (16.9)	99 ± 0.5	98.8	98.7
Case 4 (3.7)	52.5 ± 16.1	52.5	52.5
Case 5 (32.2)	0.16 ± 0.1	66.7	99.6
Case 6 (15.9)	38.6 ± 17.1	96.9	88.2
Case 7 (35.1)	51.7 ± 6.5	81.7	57.5

Case No. and Selected Scatter Gate Cells (%)	$CD44^+$ and $CXCR4^-$ Cells (%)	$CD44^+$ and $CXCR4^-$ Cells That Are $CD90^+$ Cells (%)	$CD44^+$ and $CXCR4^-$ Cells That Are $CD133^+$ Cells (%)
Case 1 (2.7)	4.5 ± 3.1	2.56	0.21
Case 2 (12.2)	1.8 ± 2	0.99	0.2
Case 3 (16.9)	0.6 ± 0.4	2.9	0.81
Case 4 (3.7)	4.8 ± 3.8	4.3	1.4
Case 5 (32.2)	2.7 ± 0.11	33.6	32.5
Case 6 (15.9)	70.8 ± 22.4	61	35
Case 7 (35.1)	37.6 ± 6.6	27.3	6.5

tion of CD44, CD133, CXCR4, and CD90 markers when compared to CD44 alone (0.16% ± 0.1% to 99% ± 0.5%). Since a consistency was observed across seven samples, the cells of case 6 were sorted for RTPCR analysis (Tables 6, 7).

Gene Expression Profile of the FSClo/SSClo (P2) Versus FSChi/SSClo (P3) Subpopulation

The two subpopulations (P2 and P3) were sorted based on the expression of CD44 and CXCR4 with a sorting efficiency of 97% and 61%, respectively (Figs. 6A–C). The gene expression profile of the P2 population showed a strong expression of retinal progenitor cell markers PROX1 and Syntaxin1A. In contrast, the P3 population showed low expression of retinal progenitor cell markers (Figs. 6A: Q4, Q2 quadrant). Differentiated cell markers CD133 and NSE were expressed in both the groups.

DISCUSSION

The study of Rb, the most common ocular tumor, has revolutionized our understanding of tumor biology: primarily genetics, mode of inheritance, and the means to improve survival. The identification of cells with stem cell markers such as multidrug resistant (MDR) related P-glyco protein (P-gp) and lung resistance protein has provided further insight into Rb.[16] Immunoreactivity to P-gp is frequently observed in well-differentiated Rb, especially those tumors treated by chemotherapy before enucleation.[16–18] In a retrospective study of 20 noninvasive Rb tumors, ABCG2 was expressed in 12 and MCM2 in 14. Furthermore, in 19 invasive Rb tumors, 15 tumors were positive for ABCG2 and 16 for MCM2.[11] Most of the tumor cells in Rb are dead, necrotic, or apoptotic. Analyzing the expression of markers in the heterogeneous tumor constituting many dead and necrotic cells is inappropriate. To overcome this anomaly, we attempted to show the expression of markers in the freshly isolated tumor cells by flow cytometry.

Our observation regarding the expression of ABCG2, CD133, and CD90 are in accordance with earlier studies.[11–13] This is the first study to use flow cytometry to document the percentage of cells expressing CD44 and CXCR4 in primary Rb. Previous studies have shown CD44 to be the hallmark of cancer stem cells in leukemia, pancreatic, and breast cancers.[19–24] Nevertheless, its expression is repressed in certain tumors of the neuroectoderm like neuroblastoma, small-cell lung carcinoma and gastrinoma.[24,25] The overexpression of CD44 (hyaluronate receptor) in Rb may be attributed to the microenvironment of vitreous fluid as it contains hyaluronic acid.

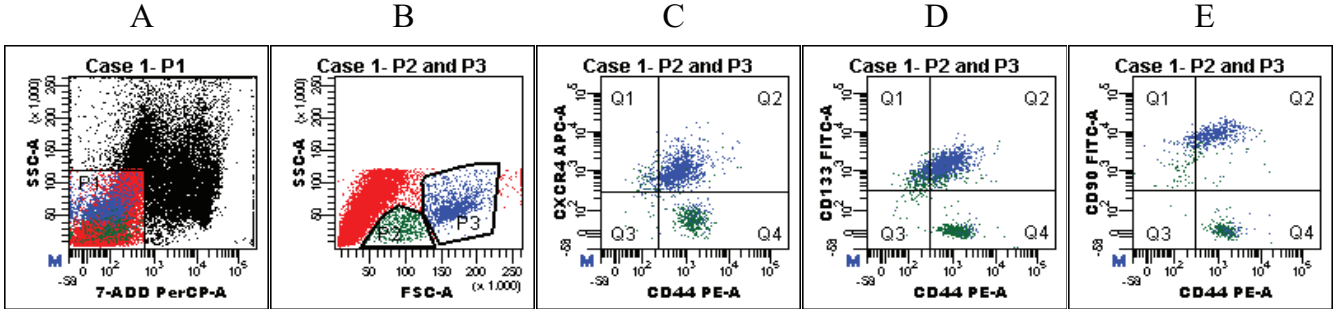

FIGURE 4. Differential expression of stem and differentiated cell markers in Rb cells. (**A**) The P1 gate in this plot shows cells that were negative for 7-AAD stain. (**B**) The P1 selected cells in FSC versus SSC plot. P2 gate showed the FSC^{lo}/SSC^{lo} subpopulation (*green*), and P3 gate showed the subpopulation FSC^{hi}/SSC^{lo} (*blue*). The population in *red* was eliminated from analysis. (**C–E**) The population of cells that were CD44-positive and -negative for CXCR4, CD133, and CD90 (represented in the Q4 quadrant).

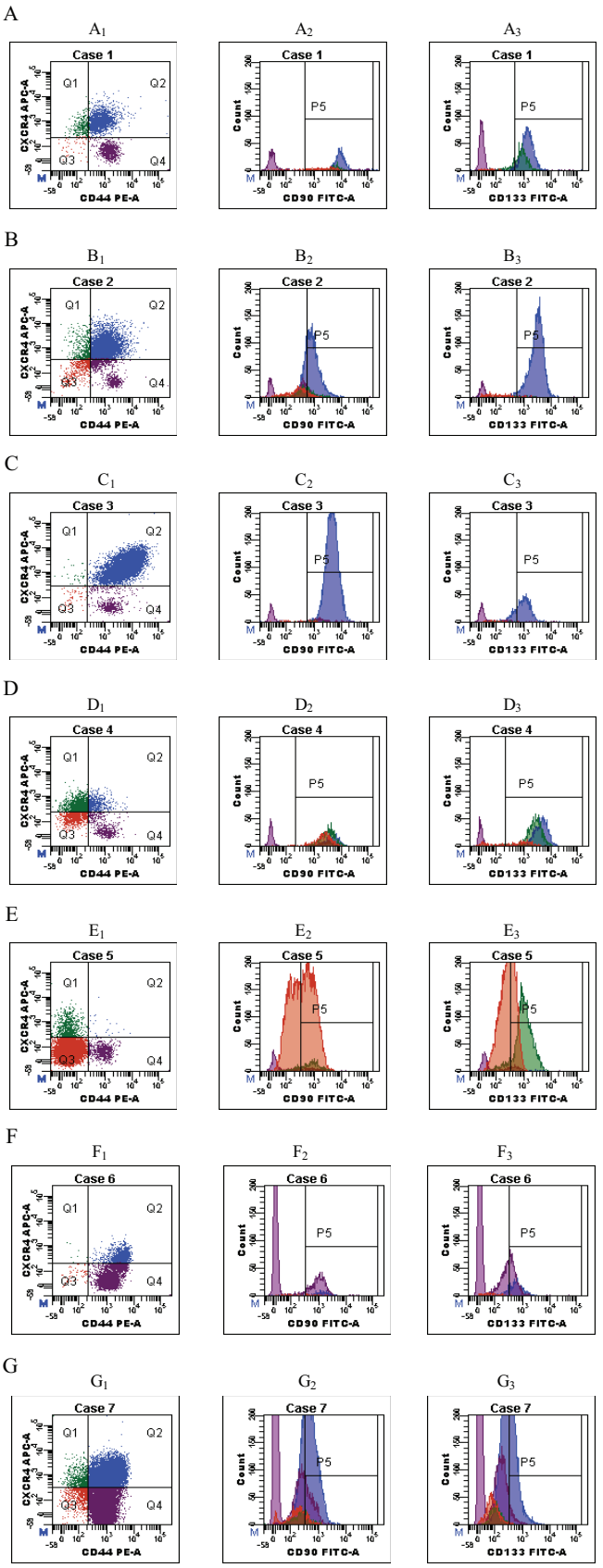

FIGURE 5. Flow cytometric analysis of stem and differentiated cell markers by tumor cells. (**A–G**) The expression profile of a combination of markers (CXCR4+CD44+CD90 and CXCR4+CD44+CD133) expressed in cases 1 to 7. (**A_1–G_1**) The population of cells expressing CXCR4 alone (Q1), CD44 alone (Q4), CD44 and CXCR4 (Q2), and negative for both the markers (Q3). (**A_2–G_2**) The populations from various quadrants of (**A_1**) to (**G_1**) that showed expression of CD90. (**A_3–G_3**) Populations from various quadrants of (**A_1**) to (**G_1**) that showed the expression of CD133.

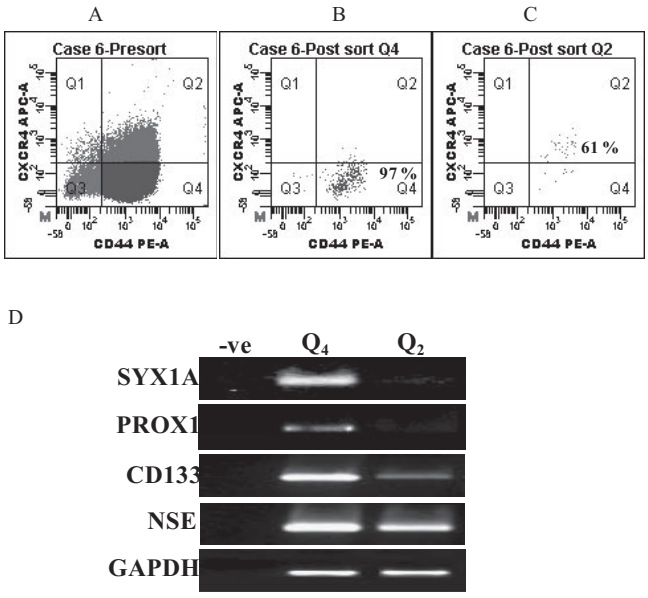

FIGURE 6. RT-PCR analysis of sorted cells. Total RNA was extracted from the cell populations in the Q2 and Q4 quadrants after flow cytometry sorting of Rb cells from the tumor of case 6. (**A**) The presort analysis of CD44 and CXCR4 expression. (**B**) The postsort efficiency of Q4 (97%). (**C**) The postsort efficiency of Q2 (61%). (**D**) The RT-PCR analysis for human Syntaxin1A, PROX1, CD133, NSE, and GAPDH.

We identified two subpopulations in Rb tumors based on surface marker expression and light-scatter properties. This finding prompted us to hypothesize that the cells in the P2 population (mean, 6.4% of total cells) which were positive for CD44 and negative for other markers are primitive, when compared with the P3 group (mean, 16.9% of total cells). We corroborated our hypothesis by analyzing for the expression of retinal progenitor cell markers (PROX1 and Syntaxin1A) in the sorted population using RT-PCR. Results have shown strong expression of retinal progenitor cell markers in the P2 population when compared with the P3 group (Fig. 6D). In addition, the expression of differentiated cell markers of retina CD90 (retinal ganglion cell marker), CD133 (photoreceptor cell marker) was more in the P3 group.[13,26] Although our sample size was limited, we noted that the proportion of CD44-positive cells was high in case 6, which was primitive and poorly differentiated. It is difficult to correlate the histopathologic risk factors with markers, because there was variability of the expression from sample to sample.

In conclusion, we have shown that Rb, a malignant round-cell tumor, harbors putative stem-like cells. Among these cells, there appeared to be two distinct subpopulations of cells as identified by flow cytometric analysis. The FSC^{lo}/SSC^{lo} cells showed high expression of retinal progenitor markers (Syntaxin 1A and PROX1), and a low percentage of differentiated markers (CD133 and CD90) compared with the FSC^{hi}/SSC^{lo} subpopulation, thus prompting us to postulate a hierarchy of stem cells within the tumor population. Further studies using clonal assays, in vivo experiments, and gene expression signatures are warranted to

improve our understanding of the basic biology, thus paving the way for potential targeted therapy in future.

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