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Gene expression profiling of cancer stem cells in the Du145 prostate cancer cell line

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Abstract. Cancer stem cells (CSCs) that exhibit tumor-initiating properties have been identified in primary prostate cancer and prostate cancer cell lines. CSCs are the root of tumor metastasis and recurrence even in the cases where they constitute a minority of the tumor mass. In this study, putative CSCs were isolated from the Du145 cell line and an Affymetrix microarray was used to investigate their gene expression profile. The results were validated by real-time polymerase chain reaction (PCR). The results of the microarray indicated that 138 genes were upregulated and 93 genes were downregulated in the CSCs. Certain genes that may be significant in the regulation of CSCs from the Du145 cell line were identified. These results may aid the studying of the mechanisms through which CSCs acquire their distinctive properties.

Introduction

Cancer stem cells (CSCs) are found in small populations in tumors. They have the properties of self-renewal, extensive differentiation and unlimited proliferation (1-3). The survival of CSCs was thought to be the ultimate cause of the metastasis and recurrence of a tumor (4). Prostatic CSCs have been identified in primary tumors and prostate-metastasized cancer (5).

We previously isolated and identified CSCs from the androgen-independent prostate carcinoma Du145 cell line (6). The CSCs express CD44, integrinα2β1 and CD133. The cells have distinctive signal transfer pathways and gene expression patterns that are different from the rest of the cell population. Tsai (7) found that certain pathways associated with somatic stem cells, including wnt, notch and β-catenin, are also found in CSCs. Previously, we showed that a number of genes that are associated with the differentiation of somatic stem cells and cell apoptosis, including β-catenin, bax and myc, are differentially expressed in CSCs (6). However, the different levels of expression of certain genes do not explain the mechanism of the unique nature of CSCs. To elucidate this mechanism, it is necessary to screen all the differentially expressed genes and describe the gene regulatory network in CSCs. Therefore, an oligonucleotide microarray was used to profile gene expression in $CD44^+integrin\alpha2\beta1^+C133^+$ CSCs and the unsorted cells from the Du145 cell line.

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Key words: prostate cancer, cancer stem cells, microarray, gene expression profile

Materials and methods

MACS cell sorting for $CD44^+integrin\alpha2\beta1^+CD133^+$ cells. An anti-PE MultiSort kit (anti-PE MACS microbeads, microbead release reagent) and a MACS separator (Miltenyi Biotech Ltd.) were used to indirectly isolate $CD44^+$ cells. As reported previously (6), cells were labeled with anti-CD44-PE (eBioscience) for 5 min and then incubated with MACS goat anti-PE IgG microbeads for 15 min at 4˚C. The cell suspension was rinsed with PBS containing 0.5% bovine serum albumin and 2 mM EDTA (PBE), centrifuged, resuspended in 1,000 μl PBE and applied to a MACS column. Labeled $CD44^+$ basal cells were eluted and resuspended in 1 ml PBE. Release reagent (20 μl) was added to the cell suspension, which was then incubated for 10 min at 4˚C. The reaction was stopped with 30 μl MultiSort stop reagent per 10^7 cells. The cells passed through a MACS column, with the free microbeads removed from the cells. $CD44^+$ basal cells were plated onto dishes coated with type I collagen (100 μg/ml) for 5 min. Following this time interval, the adherent cells were found to be $CD44^+integrin\alpha2\beta1^+$ cells. The cells were then rinsed with PBS and harvested with Accutase™ (Chemicon International, Inc., Temecula, CA, USA). As we have previously reported (6), the $CD44^+integrin\alpha2\beta1^+$ cells were applied to the direct MACS cell sorting for $CD133^+$ cells.

Flow cytometric analysis. Aliquots of sorted cells were suspended in PBE in a volume of 100 μl for antibody labeling. Anti-CD133-FITC (10 μl) and 1:200 anti-CD44 antibody-PE (Novus Biologicals, LLC, Littleton, CO, USA) monoclonal antibodies were added to the cell suspension. The reaction was incubated in the dark for 10 min at 4˚C. The cells were washed and centrifuged in triplicate and resuspended in 500 μl PBE. Flow analysis was performed with a FACScan (FACSCalibur, Becton-Dickinson) with a 488-nm laser. A control with no antibody was analyzed to delineate the unstained and autofluorescent population, with the percentage of positive events determined.

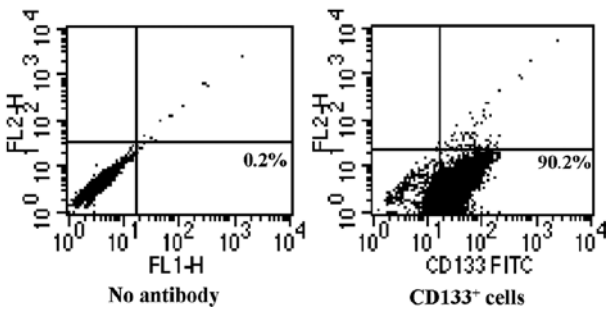

Figure 1. Results of flow cytometric analysis revealed that the purity of the CD133$^+$ cells was 90.2%.

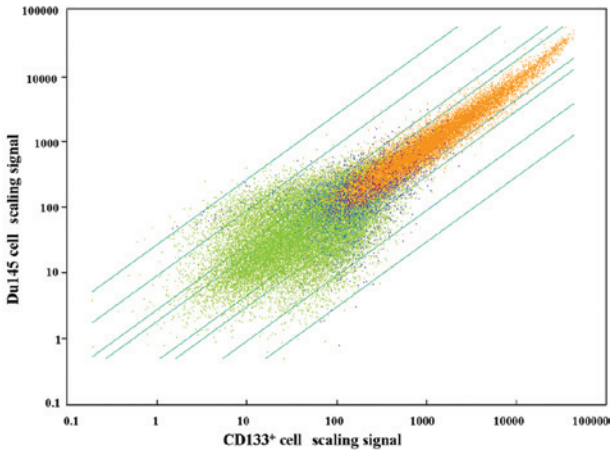

Figure 2. Gene expression scaling signal scatters in the unsorted cells and CD44$^+$integrin α2β1$^+$C133$^+$ cells from the Du145 cell line. Orange, gene expression signal detected in the two cell groups; blue, gene expression signal detected only in one of the cell groups; green, gene expression signal detected in neither of the cell groups.

Isolation of total RNA. An RNeasy Mini kit (Qiagen, Hilden, Germany) was used to extract total RNA from the cell samples according to the manufacturer's instructions. The RNA in the aqueous phase was moved to a fresh RNA-free tube and mixed with 1/10 volume of 3 M NaOAc (Sigma-Aldrich, St. Louis, MO, USA) and 2.5 volumes of ethanol. The samples were incubated at -20˚C for at least 1 h and centrifuged at 12,000 x g for 20 min at 4˚C. The supernatant was removed and the RNA pellet was washed twice with 80% ethanol, redissolved in diethylpyrocarbonate-treated water and stored at -80˚C. The quality of the RNA was analyzed using a UV spectrophotometer (Affymetrix, Santa Clara, CA, USA). To determine the concentration and purity of the samples, the absorbance was checked at 260 and 280 nm. The 260:280 ratio of the RNA was 1.8:2.0.

Affymetrix microarray for gene expression. Affymetrix microarrays (Affymetrix U 133 plus 2.0, Affymetrix) were used to analyze the transcriptional profiles of the RNA samples. There were 54,000 probes on each slide. Biotin-labeled cRNA was generated and linearly amplified from total RNA using the MEGAscript® T7 kit (Ambion, Inc., USA) according to the manufacturer's instructions.

Array hybridization, chemiluminescence detection and image acquisition and analysis were performed using a chemiluminescence detection kit (Affymetrix) and a chemiluminescence scanner (Affymetrix® GeneChip® Scanner 3000, Affymetrix) according to the manufacturer's instructions. The experiments were performed in triplicate. Each microarray was first prehybridized at 45˚C for 10 min in a hybridization buffer with a blocking reagent. Labeled cRNA targets were first fragmented by incubation with a fragmentation buffer at 94˚C for 35 min and hybridized to each prehybridized microarray in a volume of 250 μg at 45˚C for 16 h. Following hybridization, the arrays were washed with a hybridization wash buffer and a chemiluminescence rinse buffer. Chemiluminescent signals were generated by first incubating the arrays with biotinylated antibody (anti-streptavidin antibody, Vector Laboratories, Burlingame, CA, USA) and adding chemiluminescence-enhancing solution and chemiluminescence substrate. Images were captured for each microarray using the chemiluminescence scanner and GCOS software (Affymetrix).

Data analysis. The GCOS software was used to extract assay signals and assay signal-to-noise ratios from the microarray images. The chemiluminescence intensity for each spot was calculated using local mean background subtraction. Normalization was performed as a two-step process, first using the internal standards present on the array (different genes allowing quantification/normalization and estimation of experimental variation) and second using a set of housekeeping genes. The variance of the normalized set of housekeeping genes was used to generate a confidence interval to test the significance of the gene expression ratios obtained (sorted cells versus control). Ratios outside the 95% confidence interval were considered to indicate statistically significant results. The ratios of assay signals of the genes were log-transformed and represented graphically using Microsoft Excel. Standard deviations were not calculated due to the use of a log scale. The genes with an expression ratio change >2-fold were selected and classified according to their functions (cell cycle, cell proliferation and cell apoptosis) with GCOS 1.2.

Real-time polymerase chain reaction (PCR). Real-time PCR was performed using the method we reported previously (6). RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA was quantified using UV spectrophotometry and was used to create cDNA with the SuperScript RT-PCR kit (Invitrogen Life Technologies). The PCR co-amplification of the genes was carried out with β-actin in the iCycler iQTM real-time PCR detection system (Bio-Rad, Richmond, CA, USA) using the reaction mixture in a total volume of 25 μl containing 12.5 μl SYBR-Green real-time PCR mix (Toyobo, Osaka, Japan), 11 μl ddH_2O, 0.5 μl (1 μM) sense and antisense primers and 0.5 μl cDNA. Real-time PCR was performed in triplicate. A reaction mixture containing β-actin primers but omitting cDNA was used as the negative control. The primers of bax, fas, c-myc, survivin, β-catenin and β-actin and the detailed methods used were as previously reported (6). At the end of each cycle, the fluorescence emitted by SYBR-Green was measured. The expression level of the target gene in CD44$^+$integrinα2β1$^+$C133$^+$ cells relative to that in unsorted cells was calculated using the formula: fold change=$2^{-\Delta\Delta Ct}$, where

Table I. Overexpressed genes in cancer stem cells.

Signal log ratio	Representative public ID	Gene title	Gene symbol
Apoptosis			
2.4	NM_000399	Early growth response 2 (Krox-20 homolog, *Drosophila*)	EGR2
1.7	M57731	Chemokine (C-X-C motif) ligand 2	CXCL2
1.5	NM_000948	Prolactin	PRL
1.3	AA005141	Met proto-oncogene (hepatocyte growth factor receptor)	MET
1.3	AF130085	Catenin (cadherin-associated protein), β 1, 88 kDa	CTNNB1
1.1	NM_001511	Chemokine (C-X-C motif) ligand 1 (melanoma growth-stimulating activity, α)	CXCL1
1	NM_002006	Fibroblast growth factor 2 (basic)	FGF2
1	NM_000620	Nitric oxide synthase 1 (neuronal)	NOS1
Cell binding			
4.8	BF977837	KIAA0527 protein	KIAA0527
4.6	NM_007136	Zinc finger protein 80 (pT17)	ZNF80
4.3	BC007908	TBC1 domain family, member 10	TBC1D10
4.1	AU148255	Eukaryotic translation initiation factor 4E-binding protein 3	ANKHD1
3.5	NM_006186	Nuclear receptor subfamily 4, group A, member 2	NR4A2
3	S77154	Nuclear receptor subfamily 4, group A, member 2	NR4A2
2.5	NM_002702	POU domain, class 6, transcription factor 1	POU6F1
2	NM_001674	Activating transcription factor 3	ATF3
Signaling			
3.5	NM_006186	Nuclear receptor subfamily 4, group A, member 2	NR4A2
3	S77154	Nuclear receptor subfamily 4, group A, member 2	NR4A2
2.1	AA295257	Neuropilin 2	NRP2
1.8	AV700298	CD44 antigen (homing function and Indian blood group system)	CD44
1.2	J05008	Endothelin 1	EDN1
1.1	NM_001511	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	CXCL1
1	NM_000584	Interleukin 8	IL8
1	NM_003877	Suppressor of cytokine signaling 2	SOCS2
Degeneration			
1.4	AI468201	Thrombospondin 3	THBS3
1.3	AF130085	Catenin (cadherin-associated protein), β 1, 88 kDa	CTNNB1
1	AA156873	Integrin, α 1	PELO
1	AL039870	Guanine nucleotide-binding protein (G protein), γ 7	GNG7
Metabolism			
2.5	U20489	NADH dehydrogenase 5	MTND5
2.2	AI651445	Hypothetical protein MGC4655	MGC4655
2.1	AA295257	Neuropilin 2	NRP2
1.9	BC003143	Dual specificity phosphatase 6	DUSP6
1.9	AK025352	Microtubule-associated serine/threonine kinase 2	MAST2
Cell cycle			
3	NM_006732	FBJ murine osteosarcoma viral oncogene homolog B	FOSB
1.9	AA994334	B-cell CLL/lymphoma 10	BCL10
1.9	BC003143	Dual specificity phosphatase 6	DUSP6
1.8	AK025101	Exostoses (multiple) 1	EXT1
1.8	BC003143	Dual specificity phosphatase 6	DUSP6

ΔΔCt=[Ct(target gene)-Ct(β-actin)] $CD44^+$integrinα2β1^+C133^+ cells - [Ct(target gene)-Ct(β-actin)] unsorted cells.

Results

Highly purified CSCs were sorted from Du145 cells. We obtained the CSCs using the multitarget MACS cell sorter. The purity of the $CD133^+$ cells was 90.2 % (Fig. 1).

Identification of genes differentially expressed in CSCs and Du145 cells. Using the Student's t-test examination and fold change methods (signal log ratio ≥1 or ≤-1), 454 transcripts and variants were found to be differentially expressed, including 231 genes whose titles and functions were determined. Of these, 138 genes were upregulated and 93 were downregulated in the CSCs. The signal scatters of the two groups of cells are shown in Fig. 2.

Table II. Underexpressed genes in cancer stem cells.

Signal log ratio	Representative public ID	Gene title	Gene symbol
Apoptosis			
-3.4	L22454	Nuclear respiratory factor 1	NRF1
-1.4	AI880383	Natural killer-tumor recognition sequence	NKTR
-1.1	BG612458	Heat shock 90 kDa protein 1, β	HSPCB
-1.1	NM_001729	Betacellulin	BTC
-1	NM_000978	Ribosomal protein L23	RPL23
-1	NM_015965	Cell death-regulatory protein GRIM19	GRIM19
Cell binding			
-1.3	M74447	Transporter 2, ATP-binding cassette, subfamily B	TAP2
-2		Zinc finger protein 555	ZNF555
-1	BG032366	Interleukin enhancer-binding factor 3, 90 kDa	ILF3
-1	BC020837	Zinc finger protein 505	ZNF505
Transcription regulation			
-3.4	L22454	Nuclear respiratory factor 1	NRF1
-2.8	AB055703	LIM homeobox 4	LHX4
-1.2	NM_004862	Lipopolysaccharide-induced TNF factor	LITAF
-1	U26455	Ataxia telangiectasia mutated	ATM
Signaling			
-3.7	AK027217	LIM protein	LIM
-1.7	AW504458	Guanine nucleotide-binding protein (G protein), β polypeptide 4	GNB4
-1.3	AF033111	CD27-binding (Siva) protein	SIVA
-1.3	M74447	Transporter 2, ATP-binding cassette, subfamily B	TAP2
-1.2	NM_004862	Lipopolysaccharide-induced TNF factor	LITAF
-1.1	NM_006579	Emopamil-binding protein (sterol isomerase)	EBP
-1.1	NM_013437	Low density lipoprotein-related protein 12	LRP12
-1	NM_000820	Growth arrest-specific 6	GAS6
Degeneration			
-1.7	AW504458	Guanine nucleotide-binding protein (G protein), β polypeptide 4	GNB4
-1.3	BF063156	RNA-binding motif protein 17	RBM17
-1.2	BE561798	HLA class II region expressed gene KE2	HKE2
-1	AW675725	Baculoviral IAP repeat-containing 4	BIRC4
Metabolism			
-4.2	AF153430	Cyclin-dependent kinase (CDC2-like) 10	CDK10
-2	AU156915	Methylcrotonoyl-Coenzyme A carboxylase 2 (β)	MCCC2
-1.8	AI142677	Glutamyl-prolyl-tRNA synthetase	EPRS
-1.8	NM_014502	PRP19/PSO4 homolog (*S. cerevisiae*)	PRP19
-1.7	AI809582	E1A-binding protein p400	EP400
-1.6	D84430	Phenylalanine-tRNA synthetase-like, β subunit	FARSLB
-1.5	U18197	ATP citrate lyase	ACLY
-1.5	BG252666	ATPase, class I, type 8B, member 1	ATP8B1
-1.5	BF127479	Ribonuclease H1	RNASEH1
-1.4	AI880383	Natural killer-tumor recognition sequence	NKTR
-1.4	S72422	Dihydrolipoamide S-succinyltransferase	DLST
-1.4	AB037769	Pyruvate dehydrogenase phosphatase isoenzyme 2	PDP2
-1.3	M74447	Transporter 2, ATP-binding cassette, subfamily B	TAP2
-1.3	AF281132	Exosome component 3	EXOSC3
-1.3	AV706522	Hypothetical protein DKFZp761G058	DKFZp761G058
-1.2	AA639797	ATPase, class VI, type 11B	ATP11B
-1.2	C18318	Ring finger and KH domain-containing 1	RKHD1
Cell cycle			
-4.2	AF153430	Cyclin-dependent kinase (CDC2-like) 10	CDK10
-1	NM_002312	Ligase IV, DNA, ATP-dependent	LIG4
-1	NM_003158	Serine/threonine kinase 6	STK6
-1.5	AK024690	Hypothetical protein LOC90110	FBXL20
-1.5	AA761259	Rap guanine nucleotide exchange factor (GEF) 1	RAPGEF1

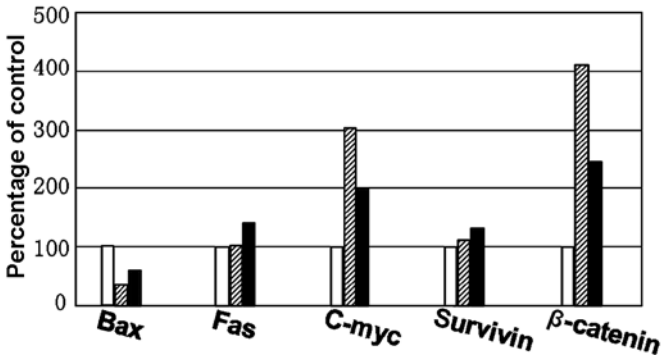

Figure 3. Results of the microarray compared with the results of real-time PCR. The results of the microarray were similar to those of real-time PCR. The gene expression level of the cells in the control group was set at 100%. White bar, control; striped bar, the gene expression level detected with real-time PCR; black bar, the gene expression level detected with microarray.

Classification of significantly expressed genes. Hierarchical cluster analysis was used to classify the differentially expressed genes according to their function by means of GCOS 1.2 software. The roles of the genes included tumorigenesis, tumor suppression, apoptosis, tumor angiogenesis and cell cycle regulation. The results are shown in Tables I and II.

Validation of significantly expressed genes by real-time PCR. To validate the microarray results, we selected several genes which we previously detected using real-time PCR (6). Therefore, the expression of bax, fas, c-myc, survivin and β-catenin was examined.

C-myc and β-catenin in CSCs were detected to be overexpressed and bax underexpressed. The expression levels of fas and survivin had no significant difference between the CSCs and unsorted cells, similar to the microarray results. The magnitude of expression changes detected by real-time PCR was comparable with the fold changes detected by the microarray platform (Fig. 3).

Discussion

The proportion of CSCs in a tumor is very small. CSCs have distinctive biological properties; their signal transfer pathways and patterns of expression of certain genes are different from those of other types of cells, but similar to those of stem cells (8,9). CSCs are able to survive in adverse environments and resist anticancer therapy through certain mechanisms (10,11). For example, leukemia stem cells are capable of resisting apoptosis by regulating bcl-2 and bax (12).

Less is known concerning the molecular biological nature of prostate CSCs. In this study, an Affymetrix microarray was used to detect the genes which were expressed at significantly different levels in CSCs from the Du145 cell line.

We found that 44 genes associated with apoptosis, tumor proliferation and differentiation were overexpressed (including b-FGF, EGR2, CXCL1, PRL, MET, IL-24, NOS1, CXCL2 and β-catenin), 22 were downregulated (including RPL23, HSPCB and NKTR), 31 signal transducer-associated genes were upregulated (including SOCS2, EDN1 and CXCL1) and 10 were downregulated (including GNB4, SIVA and LITAF) in CSCs. In addition, the expression levels of genes that are involved in metabolism, transcription regulation, enzyme regulation and the cell cycle were also altered. The underexpression of LITAF (lipopolysaccharide-induced TNF factor) and NKTR (natural killer-tumor recognition sequence) may protect prostate CSCs from destruction by the immune system or immunotherapy. The different expression levels of genes involved in the regulation of transcription and enzyme activity may affect the migration and invasion of cancer cells.

Gene profiling microarray is very efficient. It provides an accurate timepoint analysis of a sample (13,14), but its results are variable even in similar tissues or cells due to the changes in environment as well as the sample age. The treatment of samples (15) and the quality of the RNA and probes (16) also affect the microarray results. Therefore, the microarray results are thought to be difficult to repeat. In this study, in order to guarantee an identical environment, the generation and treatment of the samples were exactly the same. CSCs were immediately isolated from the Du145 cells prior to the extraction of total RNA. The standard Affymetrix microarray and processes were then used to examine the expression profile of the CSCs and unsorted cells. We compared the results of the microarray with that of real-time PCR. The results of the microarray were similar to those of the real-time PCR.

The CSCs acquire their distinctive properties through the regulation of a number of pathways and multiple genes. This study primarily selected the differentially expressed genes in CSCs from the Du145 cell line. It provides new insights into the study of the molecular biological nature of prostate CSCs.

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