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Proteomic profiling of tumor-initiating cells in HT-29 human colorectal cancer cells

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ABSTRACT

Recent reports have suggested that tumors are organized in heterogeneous populations. Within these populations, a small subpopulation of cells is more capable of initiating malignancy; these are called cancer stem cells. In this study, HT-29 cells were sorted according to the presence or absence of the cancer stem cell marker CD133. We confirmed that CD133+ cells possessed higher clonogenicity compared to CD133− cells. Furthermore, proteomic analysis identified 10 proteins, including actin-related protein 2/3 complex subunit 5-like and profilin 2. In conclusion, our data demonstrated that the expression of specific proteins associated with metastasis and invasion in CD133+ cells contributed to the stemness and tumorigenic properties of these cells.

1. Introduction

Colorectal cancer, also called large bowel cancer, is the third most common form of cancer and the second leading cause of cancer-related death in the Western world [1]. Statistics from the American Cancer Society revealed that almost 150,000 new cases of colorectal cancer were diagnosed and approximately 50,000 people died from this disease in 2008.

Recent studies have supported the theory that tumors are composed of heterogeneous populations of cells differing in morphology, tumorigenic potential, and proliferation ability. In traditional models of carcinogenesis, this heterogeneity can be explained by stochastic genetic events. In contrast, according to the cancer stem cell (CSC) hypothesis, only a small fraction of cells within a tumor possesses cancer-initiating potential and is responsible for tumor growth and metastasis [2,3].

Previous studies have described the protein profiles of tumorigenic and nontumorigenic colon cancer cells. Enrichment of tumor-initiating cells has been reported after isolation of CD133+ colon cancer cells [4]. CD133, also called AC133 and prominin-1, is a 5-transmembrane, 120-kDa glycoprotein containing 865 amino acids. Additionally, these cells have been found on tumor-initiating or cancer stem cells in brain tumors, hepatocellular carcinoma, leukemia, retinoblastoma, teratocarcinoma, and colon cancer [5–12].

In this study, the proteome of colorectal cancer stem cells was analyzed. The human colorectal cancer cell line HT-29 was used, and cells were sorted by magnetic-activated cell sorting (MACS), which yielded CD133+ cells and CD133− cells. Subsequently, the sorted cells were confirmed by flow cytometry, reverse transcription-PCR (RT-PCR), and Western blot analysis. Highly sensitive two-dimensional gel electrophoresis (2-DE) was used for the identification of proteins differentially expressed in the 2 cell subpopulations. We hypothesized that the identification of specific proteins in colorectal cancer stem cells would provide the basis for early diagnosis and detection of colorectal cancer and promote our understanding of the molecular mechanisms that govern cancer progression.

2. Materials and methods

2.1. Cell culture and treatment

The human cancer cell line HT-29 was purchased from the Korean cell line bank (Seoul, Korea). Upon arrival, cells were transferred to a T-25 flask and cultured in RPMI-1640 (GIBCO, CA, USA) containing 2.05 mM glucose, 25 mM HEPES, 10% fetal bovine serum (FBS; GIBCO), 100 μg/mL penicillin, and 100 μg/mL streptomycin [13].

2.2. Magnetic-activated cell sorting (MACS)

Cells were labeled with anti-human CD133/1-PE antibodies (AC133; Miltenyi Biotec, CA, USA) and anti-PE microbeads (Miltenyi Biotec) and then isolated on a MACS LS column (Miltenyi Biotec). All procedures were performed according to the manufacturer's instructions. The purity of sorted cells was evaluated by flow cytometry and Western blotting [14].

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2.3. Colony formation assay

Sorted cells were plated at a density of 5000 cells per well in 6-well plates (Nunc, NY, USA) in RPMI 1640 containing 10% FBS. After 28 days, cultured colonies were stained with 0.05% crystal violet (Sigma, MO, USA) [15].

2.4. Soft agar colony formation assay

Sorted cells were plated in triplicate in soft agar at a density of 5000 cells per well in 6-wells plates (Nunc). For the base layer, a 1.2% agar stock solution (Sigma) was melted in a microwave oven, cooled to 40 °C in a water bath, and then mixed with culture medium to obtain a solution of 0.6% agar in RPMI 1640 containing 10% FBS. For the top layer, the agar stock solution was diluted with culture medium to obtain a solution of 0.3% agar in RPMI 1640 containing 10% FBS (0.5 mL/well), the solution was gently mixed, and aliquots were added to each well (total of 1 mL/well). Colonies were stained with 0.05% crystal violet and counted using an inverted light microscope after 28 days [14].

2.5. Colonosphere formation assay

Sorted cells were plated at a density of 5000 cells/mL in sphere culture medium. The stem cell medium contained RPMI 1640 supplemented with several factors that favor stem cell growth, including 10 ng/mL basic fibroblast growth factor (bFGF) and 20 ng/mL epidermal growth factor (EGF). FBS was excluded for immature tumor cells. Furthermore, nonmalignant or differentiated cells were negatively selected and died through anoikis. Fresh aliquots of EGF and bFGF were added every other day. After culturing for 48–72 h, spheres were visible by inverted phase-contrast microscopy [14].

2.6. Sample preparation

Sorted HT-29 cells were extracted in 400 μL of RIPA buffer (Sigma) for 5 min at 4 °C. Whole cell lysates were harvested and dissociated with an ultrasonicator. Acetone precipitation was performed to remove salts and lipids that may have disrupted separation by 2-DE. Then, sample buffer containing 7 M urea, 2 M thiourea, 0.5 M Tris (pH 8.5), 4% CHAPS, 65 mM DTT, and 2% protease inhibitor was added to equal amounts of protein [16].

2.7. Isoelectric focusing (IEF)

DryStrips were rehydrated with the samples (50 μg protein) in 450 μL of solubilization solution containing 8 M urea, 13 mM DTT, 2% CHAPS, 2% IPG buffer (pH 3–10 NL), and a trace of bromophenol blue for 6 h without current and 15 h with a current of 8000 V. IEF was carried out using the IPGphor IEF system (AP Biotech, Sweden) at 200 V for 30 min, 500 V for 1 min, 8000 V for 1 h, and a constant 8000 V until approximately 120,000 V h was reached [17].

2.8. SDS–PAGE

Gel strips were equilibrated in 2 steps for 15 min each with gentle shaking. The DTT equilibration solution contained 50 mM Tris–HCl buffer (pH 8.8) with 6 M urea, 1% DTT, 2% SDS, and 20% glycerol. In the IAA equilibration solution, DTT was replaced with 2.5% IAA. After equilibration, IPG strips were gently rinsed with distilled water, blotted to remove excess equilibration buffer, applied onto 12.5% SDS–PAGE gels (26×20 cm^2), and overlaid with a solution of 0.5% agarose that contained a trace of bromophenol blue. Two-dimensional SDS–PAGE was performed at 55 V for 1 h, 160 V for 1 h, and 330 V for 4 h using the Ettan DALT II system (Amersham Biosciences) [18].

2.9. Image analysis

Computer analysis of the 2-DE image was performed using Image Master 2D Platinum Software (AP Biotech). Protein expression was determined by calculating the volume of each spot divided by the total volume of all of the spots on the gel (Total Spot Volume Normalization). For each spot, the relative volume intensity was averaged and expressed as mean ± standard error of the mean (SEM). Differentially expressed spots, with a statistical difference according to Student's *t*-test analysis ($P < 0.05$), were selected and identified by LC MS/MS [19].

2.10. LC MS/MS

MS/MS of peptides generated by in-gel digestion was analyzed by nano-ESI on a Q-TOF2 mass spectrometer (Micromass, Manchester, UK). The source temperature was 80 °C. A potential of 1 kV was applied to precoated borosilicate nanoelectrospray needles (EconoTip, New Objective, USA) in the ion source, combined with a nitrogen back-pressure of 0–5 psi to produce a stable flow rate (10–30 nL/min). The cone voltage was 40 V. A quadrupole analyzer was used to select precursor ions for fragmentation in the hexapole collision cell. The collision energy was 20–30 V, and the collision gas used was Ar, at a pressure of $6–7 \times 10^{-5}$ mbar. Product ions were analyzed using an orthogonal TOF analyzer fitted with a reflector, a micro-channel plate detector, and a time-to-digital converter. Data were processed using a Mass Lynx Windows NT PC system [20].

2.11. Protein identification and sequence processing

For protein identification, all MS/MS spectra recorded on tryptic peptides derived from spots were searched against protein sequences from NCBI databases using the MASCOT search program (http://www.matrixscience.com) [21].

2.12. Reverse-transcription PCR

Total RNA was extracted using TRIzol (Invitrogen, CA, USA), and cDNA was synthesized with 1 μg total RNA using the Superscript II system (Invitrogen) in accordance with the manufacturer's instructions. After synthesis, cDNA products were used for the PCR reaction. The primers used for RT-PCR are listed in Supplementary Table 1 [14].

2.13. Western blotting

For Western blot analysis, proteins from sorted HT-29 cells were separated by SDS–PAGE on 12% (w/v) gels, transferred to nitrocellulose membranes, and then immunoblotted with mouse anti-CD133 (Abcam), mouse anti-ARPC5L (Abcam), or chicken anti-profilin 2 (Abcam) antibodies. Values are expressed as means ± SEMs. The statistical significance was evaluated by unpaired least significant difference (LSD) test [13].

2.14. Statistical analysis

The Mann–Whitney *U*-test was used to determine the statistical significance of the results and to compare the means of the two groups. Data are expressed as means ± standard deviations, unless otherwise indicated. A *P*-value of less than 0.05 was accepted as statistically significant [18].

3. Results

3.1. HT-29 human colorectal cancer cells contained both cancer stem cells and noncancer stem cells

To isolate tumor-initiating cells from colorectal cancer cells, CD133+ cells and CD133− cells were sorted from HT-29 cells using MACS and analyzed by flow cytometry (Fig. 1A). Immunoblotting with specific antibodies revealed expression of CD133 protein in the sorted tumorigenic and nontumorigenic cells, indicating that CD133 was expressed more than 1.9-fold higher in CD133+ cells than in CD133− cells (Fig. 1B).

Many scientists believe that cancer stem cells can resist anticancer treatment and apoptosis. Therefore, the mRNA expression of stemness genes *SOX2*, *OCT-3/4*, and *β-catenin* in CD133+ cells was compared to expression of the same genes in CD133− cells by RT-PCR (Fig. 1C). In our results, relative expression of *SOX2*, *OCT-3/4*, and *β-catenin* was upregulated in CD133+ cells, suggesting that this set of genes may play roles in the regulation of HT-29 cancer stem cells. The same method was used to measure the expression of differentiation markers to evaluate presence of undifferentiated colorectal cancer stem cells. Our data showed that the marker CK20 was expressed only at very low levels, or even undetectable, in CD133+ cells. Moreover, CD133+ cells were populated with blast cells, including cytokeratins, based on the absence of differentiation markers. Taken together, these data indicated that CD133+ subpopulations of HT-29 cells, isolated using the CD133 surface marker, acted as tumor-initiating cells and possessed stemness characteristics.

3.2. CD133+ HT-29 cells were highly tumorigenic and clonogenic

Cancer stem cells have the ability to form colonies in the absence of serum and without attaching to culture plates. Accordingly, to evaluate the clonogenic ability of CD133+ and CD133− HT-29 cells, we examined whether these cells could form spheres under serum-free conditions. As shown in Fig. 2A, 1.79-fold more colonies were formed in CD133+ cells than in CD133− cells. The enhanced ability of CD133+ cells to form colonies was observed by means of soft agar assays. As shown in Fig. 2B, colonies were formed more efficiently in CD133+ cells, with 2.86-fold more colonies formed than in CD133− cells. Furthermore, colon cancer, glioblastoma, and melanoma cells, which were able to form sphere clusters, were found to be highly tumorigenic, able to propagate, and reconstitute the original tumor shape when injected into secondary recipients [22]. Our results also indicated that colonosphere clusters were clearly observed after 28 days in CD133+ HT-29 cells, whereas CD133− cells did not form spheres (Fig. 2C). Consequently, our data suggested that CD133+ HT-29 populations were able to form clones and had the potential to grow under anchorage-independent conditions.

3.3. 2-DE analysis of differences in protein expression between CD133+ and CD133− cells

Proteomics is a powerful platform for investigating protein expression profiles and protein modifications in response to specific physiological conditions in biological systems. In the present study, 2-DE and LC/MS/MS were conducted to investigate differences in protein expression between CD133+ and CD133− cells. As shown in Fig. 3A, ImageMaster 2D Platinum 6.0 was used to identify differentially expressed proteins. After gel analysis, relative intensities of spots from CD133- HT-29 cell lysates after 2-DE analysis were measured and compared to those of CD133+ HT-29 cell lysates. A comparative analysis of the normalized volume of each spot showed that 27 spots were upregulated in CD133+ cells by more than 100% (over 2-fold, $p < 0.01$) compared with CD133− cells, and 31 spots were highly upregulated in

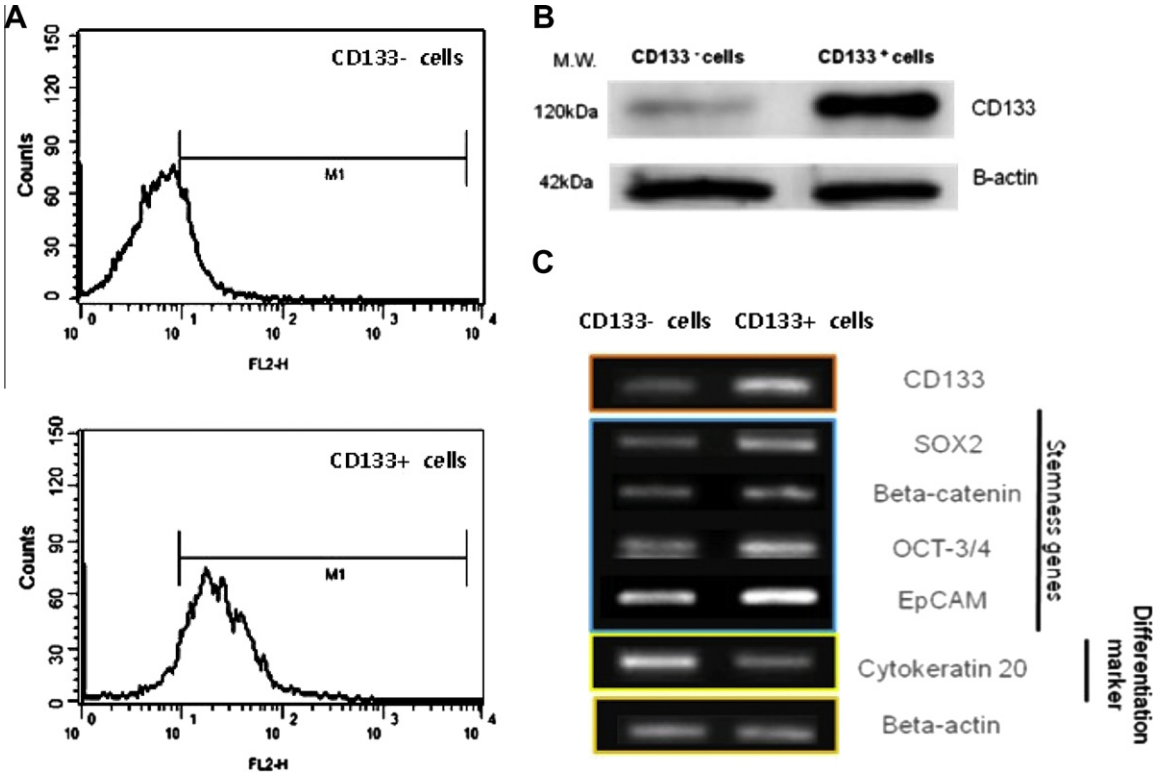

Fig. 1. CD133 expression in HT-29 human colorectal cancer cells was identified by Western blotting and RT-PCR. (A) Sorted HT-29 cells were analyzed by flow cytometry after magnetic-associated cell sorting. (B) Western blotting was used to demonstrate protein levels of the colon cancer stem cell marker CD133. (C) RT-PCR was conducted to determine the mRNA levels of CD133 in CD133− (left) and CD133+ (right) HT-29 cells.

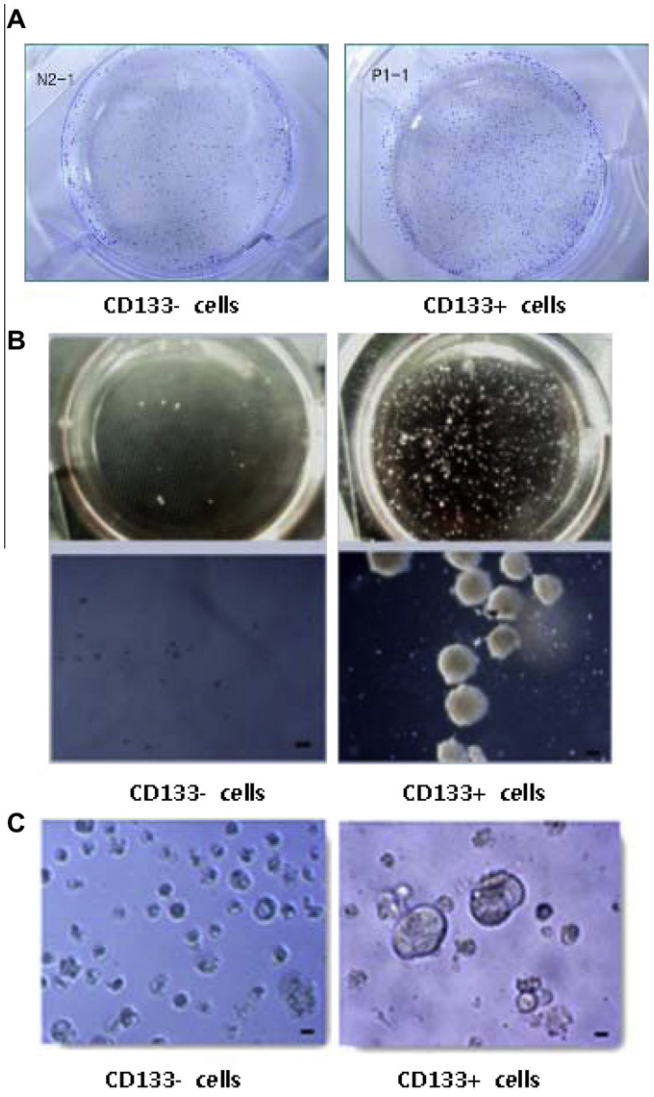

Fig. 2. Colony, soft agar, and colonosphere formation assays confirmed the tumor-initiating characteristics of CD133+ cells. (A) Colony formation was determined after 14 days of incubation, using 5000 sorted HT-29 cells. (B) To examine anchorage-independent growth, cells were incubated in soft agar. CD133− and CD133+ colonies were formed after 14 days of incubation, using 5000 sorted cells. Scale bar, 50 μm (original magnification, 100×). (C) After 14 days, CD133− cells did not form spheres in serum-free medium (original magnification, 200×); colonosphere clusters were formed by CD133+ cells after 14 days (original magnification, 200×).

CD133− cells by more than 100% (over 2-fold, $p < 0.01$) compared with CD133+ cells (Fig. 3).

To analyze differentially expressed proteins, the protein spots were excised from the gels. These excised proteins were then identified by mass spectrometry and by searching databases. Subsequently, an LC MS/MS analyzer was used to obtain more accurate protein identification data. Table 1 shows confirmed proteins that were up- or downregulated in CD133+ and CD133− cells. Upregulated spots were identified as actin-related protein 2/3 complex subunit 5-like protein (ARP2/3), prefoldin subunit 5, histidine triad nucleotide-binding protein 1, putative uncharacterized protein ENSP00000382160, and profilin 2. Downregulated spots were identified as glutathione S-transferase P, actin-aortic smooth muscle, RPSAP12 similar to 40S ribosomal protein SA (p40), eukaryotic translation initiation factor 3 subunit I, and fructose-bisphosphate aldolase A.

3.4. ARP2/3 and profilin 2 were highly expressed in CD133+ HT-29 cells

The identified proteins were subjected to Western blotting analyses to confirm the upregulation of ARP2/3 (also known as ARPC5L) and profilin 2 in CD133+ HT-29 cells. Both of these upregulated proteins play roles in actin polymerization. ARP2/3 regulates actin filament polymerization by binding to actin [23]. Together with a nucleation-promoting factor, such as the Wiskott-Aldrich syndrome protein (WASP), ARP2/3 mediates the formation of branched actin networks [24]. Another upregulated protein, profilin 2, also functions as an actin-binding protein and is known to affect the structure of the cytoskeleton. Profilin 2, expressed primarily in neurons, has been shown to be associated with the WAVE complex, which is involved in actin dynamics [25]. To confirm the differential expression level of these proteins between CD133+ and CD133− cells, Western blotting analyses were performed. Compared to their expressions in CD133− cells, both ARP2/3 and profilin 2 were upregulated in CD133+ cells (Fig. 4).

4. Discussion

Previous studies have shown that small populations of cancer cells could be isolated and characterized using cell culture methods, dye elimination, and cell surface markers, such as CD24, CD44, and CD133 [26–30]. Of these markers, CD133 has been used to isolate various types of cancer cells and cancer stem cells, and some previous reports have characterized these CD133+ cells as tumor-initiating cells [4,8,31,32]. Other reports on colon cancer have shown that the high-density CD133+ population has highly tumorigenic and proliferative capacities. In addition, these cells were found to be able to maintain themselves, differentiate, and regenerate tumor heterogeneity even after transplantation [8,9]. In this study, CD133+ clusters displayed both tumorigenic and stemness capacities. Proteomic analysis, consisting of 2-DE and LC MS/MS, revealed 10 proteins that were differentially expressed between CD133+ and CD133− cells; and 5 upregulated and 5 downregulated proteins were analyzed, including ARP2/3 and profilin 2.

In previous studies, the ARP2/3 complex, which consists of 7 subunits, including ARP2 and ARP3, has been shown to bind to profilin [33]. The ARP2/3 complex is also a central player in the regulation of both the initiation and organization of actin polymerization through interaction with WASP family proteins [34,35]. The formation and disassembly of actin filaments play an important role in cell movement, which is essential for embryogenesis, organogenesis, cancer invasion, and metastasis. ARP2/3 expression is also known to be involved in cancer invasion following carcinogenesis; thus, this supports the principle that cancer stem cells are involved in the migration and metastasis of malignant tumors [13,36,37]. In the present study, our results showed the increased expression of ARP2/3 in CD133+ cells, as compared to CD133− cells, also supporting the aforementioned principle of cancer stem cells. Together with previous studies, our analysis of cancer and noncancer stem cells in colorectal cancer indicated that tumor-initiating cells were more frequently positive for ARP2/3 and that the frequency of ARP2/3-positivity increased with the invasion index.

Profilins are small actin-associated proteins that are required, in all organisms, for the regulation of actin cytoskeleton dynamics; they function by preventing the spontaneous addition of actin monomers to barbed actin-filament ends [38]. In addition to profilin 2, 3 additional profilin isoforms have been identified, including profilin 1, profilin 3, and profilin 4. Among these, profilin 2 is distributed mainly in the central nervous system, but its specific function is unknown [39]. Previous studies have shown that the

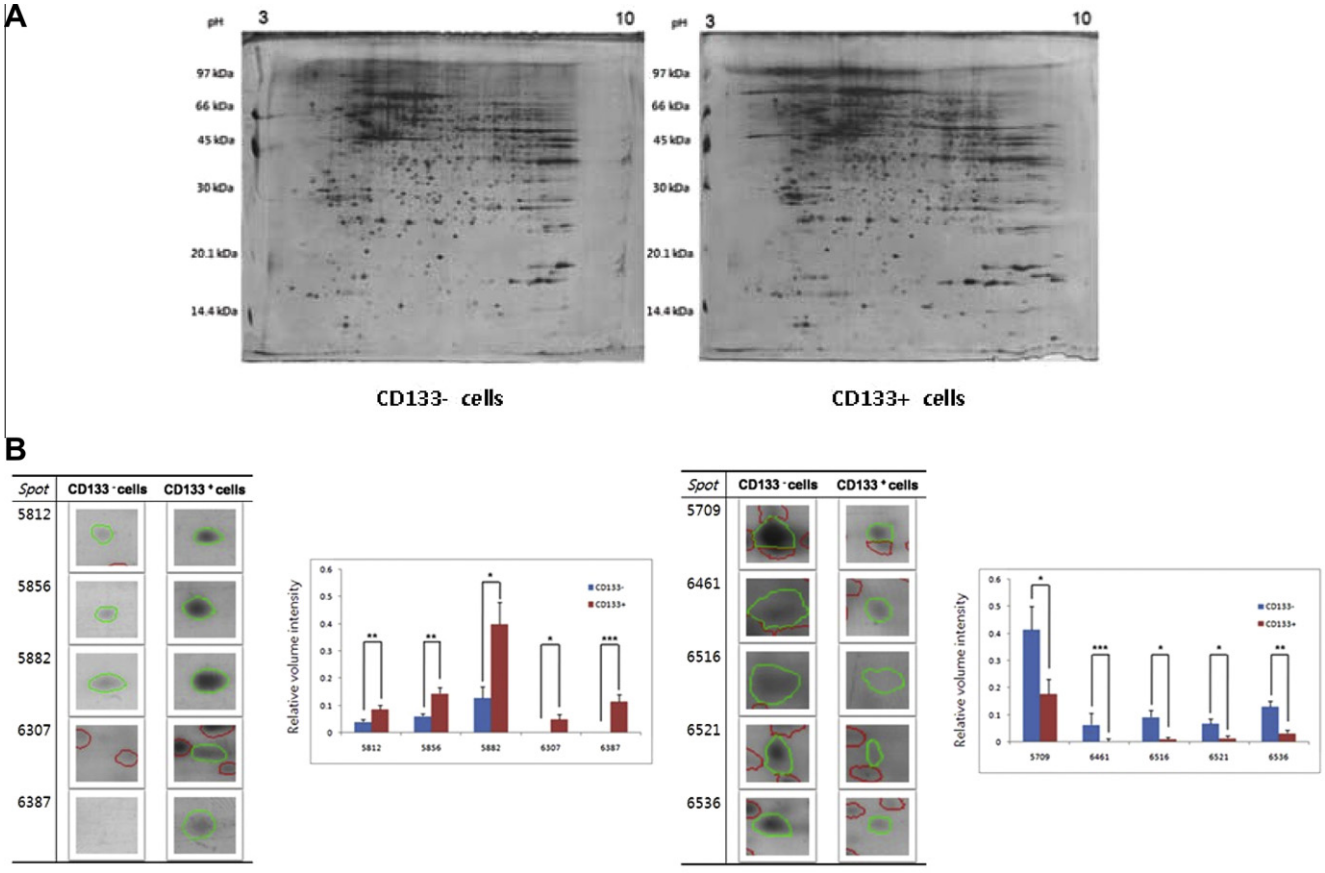

Fig. 3. Proteomic analysis of CD133− and CD133+ HT-29 cells was conducted by two-dimensional gel electrophoresis (2-DE). (A) 2-DE was performed with 50 μg protein using 24-cm pH 3–10 NL IPG strips and 12.5% SDS–PAGE. The gels were visualized by silver staining and analyzed with Image Master Software. (B) The protein spot figures (left) and relative volume intensities (right) were analyzed by Image Master 2D platinum 6.0. Each bar represents the mean ± SEM for each spot. * Significantly different from the control by Student's *t*-test (∗, $P < 0.01$). ** Significantly different from the control by Student's *t*-test (∗∗, $P < 0.005$). *** Significantly different from the control by Student's *t*-test (∗∗∗, $P < 0.001$).

Table 1
Identification of proteins that are differently expressed in CD133− and CD133+ cells using LC/MS/MS.

Spot no.	Classification	Protein	Accession no.	M.W. (kDa)	Score
5709	Down-regulated	Glutathione S-transferase P (GSTP1)	CAG29357	23.3410	20.27
5812	Up-regulated	Actin-related protein 2/3 complex subunit 5-like protein (ARPC5L)	NP_112240	16.9308	10.17
5856	Up-regulated	Prefoldin subunit 5 (PFDN5)	NP_005331	17.3170	10.28
5882	Up-regulated	Histidine triad nucleotide-binding protein 1 (HINT1)	NP_005331	13.7931	10.20
6307	Up-regulated	Putative uncharacterized protein ENSP00000382160	IPI00180956.6	48.4880	20.15
6387	Up-regulated	Profilin (PFN2)	AAH43646	20.8262	10.22
6461	Down-regulated	Actin, aortic smooth muscle (ACTA2)	CAG38756	41.9818	30.13
6516	Down-regulated	Similar to 40S ribosomal protein SA (p40) (RPSAP12)	NG_010141	32.7231	10.18
6521	Down-regulated	Eukaryotic translation initiation factor 3 subunit I (EIF3I)	CAI22322	36.4786	10.17
6536	Down-regulated	Fructose-bisphosphate aldolase A (ALDOA)	NP_908932	39.3953	10.16

Score is $-10 * \log(P)$, where P is the probability that the observed match is a random event; it is based on the NCBInr database using the MASCOT search program with our MS/MS data.

function of profilin 2 is related to dynamin 1 ligands, which are involved in signal transduction, vesicle recycling, endocytosis, and membrane trafficking [40]. In this study, profilin 2 was found to be expressed at high levels in tumor-initiating cells. Additionally, the protein is thought to play a key role in the control of cell motility. Therefore, it is possible that profilin 2 is another regulator of the metastatic activity of cancer stem cells.

In addition to ARP2/3 and profilin 2, other proteins were identified and found to be differentially expressed between CD133+ and CD133− HT-29 cells during 2-DE analysis (Table 1). Among them, the cell motility-related protein prefoldin 5, which is also called genes involved in microtubule biogenesis protein 5 (Gim-5) and c-Myc-binding protein Mm-1 or Myc modulator 1 (MM-1), has also been shown to be overexpressed in cancer stem cells and is known to affect actin processing within human chaperones [41]. Additionally, Fujioka et al. have suggested that prefoldin 5 represses the transcriptional activity of the proto-oncogene c-Myc and that this activity is associated with tumor suppression [42,43]. Furthermore, prefoldin (PFDN), a heterohexameric co-chaperone, has been shown to be required for proper folding and correct synthesis of nascent proteins. In particular, PFDN plays an essential role in actin- and tubulin-mediated functions in cell division, motility, and cytoskeletal stability [41]. In the present study, prefoldin 5, which regulates synthesis of actin and tubulin, showed

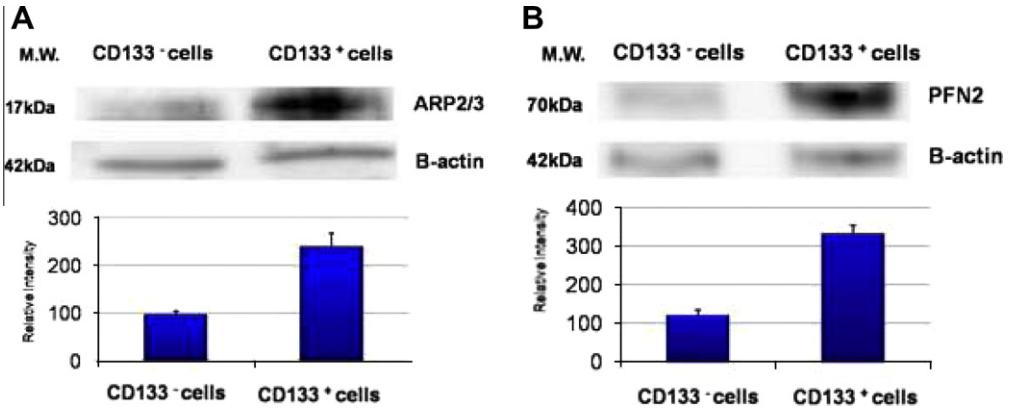

Fig. 4. Actin-related protein 2/3 complex subunit 5 (A) and profilin 2 (B) protein expression was demonstrated by western blotting in CD133− and CD133+ cells.

high expression levels in CD133+ cells. Although further studies are needed, we believe that the high expression of prefoldin 5 is partly attributable to the properties of cancer stem cells.

The therapeutic targeting of cancer stem cells is a promising research focus in the fields of diagnostics and therapeutic development in cancer. The identified upregulated proteins, ARP2/3 and profilin 2, are central players in actin polymerization and cell migration. Thus, it is plausible that these proteins, which are overexpressed in tumor-initiating cells, contribute to the tumorigenic and clonogenic phenotypes of these cells. Although further investigation of the biological functions of these proteins is necessary to confirm their involvement in the cancer stem cell phenotype, our current results imply that these differentially expressed proteins could be possible targets for the development of novel therapeutic agents.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.036.

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