이학박사 학위논문

Expression of CD133 and CD133-regulated Nucleophosmin linked to 5-Fluorouracil Susceptibility in Human Colon Cancer Cell Line SW620

인체 대장암 세포주 SW620 에서 5-Fluorouracil 감수성과 연관된 CD133 과 Nucleophosmin 의 발현

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협동과정 종양생물학 전공

김 경 희

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Expression of CD133 and CD133-regulated Nucleophosmin linked to 5-Fluorouracil Susceptibility in Human Colon Cancer Cell Line SW620

By

Kyung-Hee Kim

(Directed by Ja-Lok Ku, D.V.M., Ph.D)

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Abstract

Expression of CD133 and CD133-regulated Nucleophosmin linked to 5-Fluorouracil Susceptibility in Human Colon Cancer Cell Line SW620

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Cancer Biology Course

The Graduate School

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Cancer stem cells (CSCs) are known to be resistant to conventional chemotherapy and radiotherapy. Specific CSC targeting and eradication is therefore a therapeutically important challenge. CD133 is a colorectal CSC marker with unknown function(s). Assessing proteomic changes induced by CD133 expression may provide clues not only to new CD133 functions but also to the chemotherapy and radiation susceptibility of colon cancer cells. To

identify the proteins affected by CD133 expression, CD133-positive (CD133+) and CD133-negative (CD133–) human colon cancer cells were obtained by cell sorting. Whole proteomes were profiled from SW620/CD133+ and SW620/CD133– cells and analyzed by two-dimensional-based proteome analysis. Nucleophosmin (NPM1) was identified as a protein regulated by CD133. CD133 expression was not affected by NPM1, and an interaction between the two proteins was not observed. CD133 and NPM1 expression was positively correlated in 11 human colon cancer cell lines. The CD133+ subpopulation percentage or its value normalized against CD133 expression was only linked to intrinsic susceptibility of human colon cancer cells to 5-fluorouracil (5-FU). However, either suppression of CD133 or NPM1 increased 5-FU susceptibility of SW620. The present study suggests that CD133-regulated NPM1 expression may provide a clue to novel CD133 function(s) linked to human colon cancer cell susceptibility to chemotherapy.

Key Words: Colon Cancer; Cancer Stem Cells; CD133; Nucleophosmin; Drug-Resistance

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Introduction

Colon cancer is the third most prevalent cancer and the third leading cause of cancer deaths worldwide. It results in almost a half million deaths every year (Siegel *et al.,* 2012). Over the past 40 years, the standard chemotherapy treatment for advanced colon cancer was 5-Fluorouracil (5-FU)-based chemotherapy (Nordman *et al.,* 2006). 5-FU, the chemotherapy agent belongs to the class of drugs known as antimetabolites. It interferes with the growth of cancer cells. However, partial or complete response of colon cancer to 5-FU are generally followed by eventual tumor re-growth (Li *et al.,* 2009). Numerous studies have focused on identifying the mechanisms and key molecules involved in natural or acquired 5-FU resistance. However, conclusive and consistent results have not been demonstrated so far.

The discovery of cancer stem cells (CSCs) has changed our view of carcinogenesis and chemotherapy (Reya *et al.,* 2001). The differences in biology and clinical prognosis of solid tumors suggest that tumors might harbor different tumor entities arising from different cells of origin. CSCs are proposed to be the cancer initiating cells responsible for tumorigenesis and contribute to cancer resistance (Guo *et al.,* 2006). Although the CSCs concept

has captured great interest recently, the field of CSCs must seem very confusing (Jordan 2009). On one side there are individuals who contend the discovery of CSCs is huge step toward ultimately curing cancer, and that understanding such cells is tremendously important. Conversely, others actually question the existence of CSCs, and argue that the relative importance of the putative population is negligible (Jordan 2009, Takebe *et al.,* 2010). The CSCs hypothesis is supported by controversial findings, but it could offer new hope for winning the battle against cancer.

Considerable research efforts have been directed toward the identification of CSCs markers associated with the initiation and progression of colon cancer. Intestinal stem cells generate $>10^{10}$ new cells daily, which differentiate along a vertical axis within the human gut. In the colorectum, stem cells reside at the bottom of epithelial crypts, and dysregulation of the AKT/PKB, Wnt and/or bone morphogenic protein (BMP) signaling pathways disturbs intestinal stem cell self-renewal (Kosinski *et al.,* 2007). Musashi-1, a marker for neuronal stem cells, has been proposed as a potential stem cell marker in the gastrointestinal tract and colon epithelial crypt cells (Nishimura *et al.,* 2003). The identification of CSC-specific marker sets (Table 1) and the targeted therapeutic destruction of CSCs remain a challenge.

CD133 is one of five transmembrane glycoproteins described as surface antigens specific for human hematopoietic stem and progenitor cells (Miraglia *et al.,* 1997, Yin *et al.,* 1997). The tumorigenic potential of CD133-positive (CD133+) cells was confirmed in severe combined immunodeficient mice (O'Brien *et al.,* 2007, Ricci-Vitiani *et al.,* 2007). CD133+ cells are not only resistant to apoptosis (Todaro *et al.,* 2007), but also exhibit CSC-like characteristics in brain, prostate, breast, pancreatic, and colorectal cancers, as well as heterogeneity, or potential for multidirectional differentiation (Al-Hajj *et al.,* 2003, Singh *et al.,* 2004, Bao *et al.,* 2006, Dirks 2006, Li *et al.,* 2007, Gil *et al.,* 2008). CD133 has also been reported as a prognostic factor for colon cancer, but CD133 suppression in colon cancer cell lines has no effect on proliferation, migration, invasion, and colony formation (Horst *et al.,* 2009).

Nonetheless, much controversy exists as to whether CD133 is a valid CSCs marker in tumors, despite its use in isolating cells with cancer-initiating ability. Shmelkov *et al*. demonstrated that CD133 was neither a specific marker of organ-specific stem or progenitor cells nor a key molecule for metastasis (Shmelkov *et al.,* 2008). Furthermore, recent studies showed that CD133 could be differentially expressed depending on the stage of the cell

cycle, and demonstrated that resistance to chemo- or radiotherapy might be related to expression of anti-apoptotic molecules, such as survivin, but not CD133 (Jaksch *et al.,* 2008, Yi *et al.,* 2008). Since the CD133+ subpopulation contains stem cell-like epithelial-specific antigen^{high}/CD44+ cells, Dalerba *et al*. proposed that CD44 and CD166 may be markers of colon CSCs (Dalerba *et al.,* 2007).

CD133 function(s) is unclear, although roles in asymmetric division and self-renewal have been suggested. The role of CD133 in regulating proliferation is supported by its polarized localization and concentration in cell surface domains corresponding to the spindle pole region during metaphase (Bauer *et al.,* 2008). Recent studies have also indicated a role for CD133 in tumor angiogenesis. CD133+ glioma cells produce pro-angiogenic factors that can modify endothelial cell behavior (Bao *et al.,* 2006). Another study demonstrated that CD133+ cells can give rise to endothelial cells that promote vascularization and tumor growth (Bruno *et al.,* 2006). In the intestine, CD133+ cells tend to aberrantly activate Wnt signaling, and such an event may disrupt normal tissue maintenance, ultimately resulting in neoplastic transformation of the intestinal mucosa (Zhu *et al.,* 2009).

My study focused on differential protein expression in a CD133+

subpopulation of cells to investigate the role of CD133 in the response of human colon cancer cells to chemotherapy or radiation therapy. Proteomic assessment of CD133+ and CD133-negative (CD133–) subpopulations of SW620 cells led to the identification of nucleophosmin (NPM1) as a protein associated with CD133 expression. I herein discuss the expressional link between CD133 and NPM1, which may affect intrinsic or induced chemotherapy resistance of colon cancer cells.

Table 1. Markers frequently used to identify cancer stem cells

Tumor Type	Surface Marker	References
Acute myeloid leukemia	CD34+, CD38-	Costello *et al.,* 2000
Brain tumor	CD133+	Singh *et al.,* 2004
Colon cancer	CD133+	O'Brien *et al.,* 2007
Hepatocellular carcinoma	CD133+	Ma *et al.,* 2008
Metastatic melanoma	CD20+	Monzani *et al.,* 2007
Pancreatic cancer	CD24+, CD44+, CD326+	Immervoll *et al.,* 2008
Prostate cancer	CD44+, CD133+	Shepherd *et al.,* 2008
Renal cancer	CD133+	Bruno *et al.,* 2006

Materials and Methods

Cell culture

Eleven human colon cancer cell lines (SNU-81, SNU-407, SNU-C4, SNU-C5, DLD-1, NCI-H508, NCI-H747, SW620, Caco-2, HCT 116, and LoVo) were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). Four SNU-colorectal cancer cell lines were established and reported previously by this laboratory (Park *et al.,* 1987, Oh *et al.,* 1999). SNU-C4 and SNU-C5 with induced 5-FU resistance were established as described in previous study (Shin *et al.,* 2005). All the cell lines were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 0.1 mg/mL streptomycin. The cultures were maintained in humidified incubators at 37°C in a 5% CO_2 and 95% air atmosphere.

Flow cytometry analysis

Single-cell suspensions were obtained by trypsinizing adherent culture cells. Cell preparations were stained with antibody against human CD133 with allophycocyanin (APC)-conjugated anti-human CD133 antibody (Miltenyi

Biotec, Auburn, CA, USA). The cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Fluorescence-activated cell sorting

Cultured SW620 cells were trypsinized and resuspended in 1% FBS in phosphate-buffered saline (PBS). Mouse antihuman CD133-APC (Miltenyi Biotec) monoclonal antibody was then added (1:11 dilution) and incubated for 15 min at 4°C in the dark. After two washes, labeled cells were isolated using a FACSAria cell sorter (BD Biosciences). Dead cells, cell debris, doublets and aggregates were excluded from the analysis using forward and side scatter and pulse-width gating. Matched isotype primary antibodies were used as controls.

Two-dimensional gel electrophoresis (2-DE)

SW620/CD133+ and SW620/CD133– cells were homogenized in lysis buffer and incubated for 45 min at room temperature. After centrifugation at 12,000 rpm for 15 min, 150 μg of protein was dissolved in rehydration buffer. Immobilized pH gradient (IPG) strips were rehydrated using 250 μL of each paired preparation. Once isoelectric focusing (IEF) was completed, the strips were equilibrated in equilibration buffer for 10 min. The second dimension

was performed using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V constant current per gel. The gels were stained using a colloidal blue staining kit (Invitrogen, Carlsbad, CA, USA).

Mass analysis and data alignment

2-DE gels containing proteins of interest were excised, destained, and dried in a SpeedVac evaporator. Dried gel pieces were rehydrated with 30 μL of 25 mM sodium bicarbonate containing 50 ng trypsin (Promega, Madison, WI, USA) at 37^{o}C overnight. Alpha-cyano-4-hydroxycinnamic acid (10 mg; AB Sciex, Foster City, CA, USA) was dissolved in 1 mL 50% acetonitrile in 0.1% trifluoroacetic acid (TFA), and 1 μL of the matrix solution was mixed with an equivalent volume of sample. Analysis was performed using a 4700 Proteomics Analyzer TOF/TOF system (AB Sciex). The TOF/TOF system was operated in positive ion reflect mode. Mass spectra were first calibrated in the closed external mode using the 4700 proteomics analyzer calibration mixture (AB Sciex), and analyzed with GPS Explorer software, version 3.5 (AB Sciex). The acquired tandem mass spectrometry spectra were searched against Swiss-Prot and NCBI databases using an in-house version of MASCOT.

Western blot analysis

Western blot analysis was carried out as described previously (Shin *et al.,* 2005). Briefly, 4,000 × *g* supernatant fractions of cell homogenates containing equivalent amounts of protein were subjected to SDS-PAGE. Following electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA), which were blocked by incubation for 2 h at 4°C in 1% Tween 20 and tris-buffered saline (TBS) containing 1.5% nonfat dry milk (Bio-Rad, Richmond, CA, USA) and 1 mmol/L $MgCl_2$. Membranes were incubated for 2 h at room temperature with primary antibodies against either CD133 (1:1,000; Abcam, Cambridge, MA, USA), NPM1 (1:1,000; Abcam), p53 (1:1,000; Cell Signaling Technology, Beverly, MA, USA), p21 (1:1,000; Cell Signaling Technology), NF-κB (1:1,000; Cell Signaling Technology), p-mTOR (1:1,000; Cell Signaling Technology), p-ERK (1:1,000; Cell Signaling Technology), p-PI3K (1:1,000; Cell Signaling Technology), p-AKT (1:1,000; Cell Signaling Technology), β-catenin (1:1,000; Abcam), or Actin (1:2,000; Sigma-Aldrich, St. Louis, MO, USA). Membranes were washed three times for 15 min each with blocking solution and incubated with diluted horseradish peroxidase (HRP)-conjugated secondary antibody (Southern Biotech, Birmingham, AL, USA) for 1 h at

room temperature. Membranes were rewashed three times for 15 min with blocking solution, incubated with WEST-ZOL plus chemiluminescence reagent (Intron Biotechnology, Gyeonggi, Korea) for 1 min, and exposed to film (Kodak Blue XB-1; Kodak, Rochester, NY, USA).

Immunofluorescence and confocal microscopy

SW620/CD133+ and SW620/CD133– cells were seeded on each glass chamber slides, fixed for 15 min with 4% paraformaldehyde, and incubated 30 min with PBS containing 1% bovine serum albumin (BSA) at room temperature. The cells were incubated in the diluted CD133 or NPM1 antibodies (Abcam) overnight at 4°C. Cells were washed three times for 5 min each with PBS and incubated with diluted green-fluorescent Alexa Fluor 488, or orange-red-fluorescent Alexa Fluor 568 secondary antibody (Invitrogen) for 1 h at room temperature in dark. Cell nuclei were stained by incubating with DAPI (1 μg/mL) for 1 min. The stained cells were examined using LSM 510 META inverted confocal microscope (Carl Zeiss, Oberkochen, Germany) with 40X water immersion objective lens (Carl Zeiss) coupled with three optical zoom.

Knockdown of CD133 and NPM1 expression

The following target sequences were used to generate siRNA (Qiagen, Chatsworth, CA, USA): 5`-GGUAAGAACCCGGATCAAA-3` for CD133, 5`-AAAGGTGGTTCTCTTCCCAAA-3` for NPM1, and 5`-AATTCTCCGA ACGTGTCACGT-3` for the non-silencing control (NC). Transfection of siRNA was performed using HiferFect transfection reagent (Qiagen). CD133 siRNA solution (6 μL, 2 μM) and 12 μL of the transfection reagent were incubated in 100 μL of serum-free RPMI-1640 medium for 10 min to facilitate complex formation. The resulting mixture (final concentration of 5 nM) was added to 3×10^5 SW620 cells and incubated in a 6-well plate with 2 mL of RPMI-1640 medium.

Immunoprecipitation

All procedures were performed at 4°C unless otherwise specified. Approximately 1×10^7 cells in 1 mL of cold 1× RIPA buffer containing protease inhibitors (Roche Diagnostics, Basel, Switzerland) were incubated on ice for 30 min with occasional mixing. Cell lysates were centrifuged at 12,000 × *g* for 10 min, and the supernatant was carefully collected without disturbing the pellet. The supernatant was mixed with a primary antibody against CD133

(Abcam) and incubated for 2 h on a rocking platform. Prepared protein G Sepharose beads (100 μL; GE Healthcare Life Sciences, Piscataway, NJ, USA) were added, followed by further incubation on ice for 1 h on a rocking platform. The mixture was centrifuged at 10,000 × *g* for 30 s, and the supernatant was completely removed. Protein G Sepharose beads were washed five times with 1 mL of cold 1× RIPA to minimize background. Next, 100 μL of 2× SDS sample buffer was added to the bead pellet and heated to 100°C for 10 min. After boiling, immunoprecipitates were centrifuged at 10,000 × *g* for 5 min, and the supernatant was collected for Western blot analysis.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

A colorimetric assay using the tetrazolium salt MTT was used to assess cell proliferation. MTT assays were performed as described in a previous report (Shin *et al.,* 2005). Briefly, equal numbers of cells were incubated in each well in 0.2 mL of culture medium. At 24 to 96 h of culture after either 5-FU or radiation treatment, 0.1 mg of MTT was added to each well, and cells were incubated at 37°C for a further 4 h. Plates were centrifuged at 450 × *g* for 5

min at room temperature and the medium was removed. Dimethyl sulfoxide (0.15 mL) was added to each well to solubilize the crystals, and plates were immediately read at 540 nm with a scanning multiwell spectrometer (Bio-Tek Instruments Inc., Winooski, VT, USA). All experiments were performed three times, and the values are presented as means ± standard deviation (SD).

Statistical analysis

Within-group correlations were calculated using the Spearman rank coefficient. Significance was set at $P < 0.05$.

Results

Differential CD133+ cell subpopulations in human colon cancer cell lines

CD133+ cell subpopulations were determined in 11 human colon cancer cell lines (SNU-81, SNU-407, SNU-C4, SNU-C5, DLD-1, NCI-H508, NCI-H747, SW620, Caco-2, HCT 116, and LoVo; Figure 1A). All 11 cell lines had a differential distribution of CD133+ cell subpopulations. The CD133+ subpopulation percentage was very low in SNU-81, SNU-C4, and Caco-2 cells, but accounted for the majority of the total population of SNU-407, SNU-C5, and HCT 116 cells (Figure 1A).

Subpopulation changes in SW620 cells after CD133+ and CD133– cell sorting

Among the cell lines tested, CD133+ cells in the SW620 and LoVo cell lines accounted for about half of the total population (Figure 1A). SW620 cells were sorted using a CD133-APC monoclonal antibody (Figure 1B), and the subpopulation change was monitored (Figure 1C). From 3 days after sorting, CD133+ cells increased in the SW620/CD133– subpopulation, but no

significant subpopulation change was noted in the SW620/CD133+ subpopulation (Figure 1C).

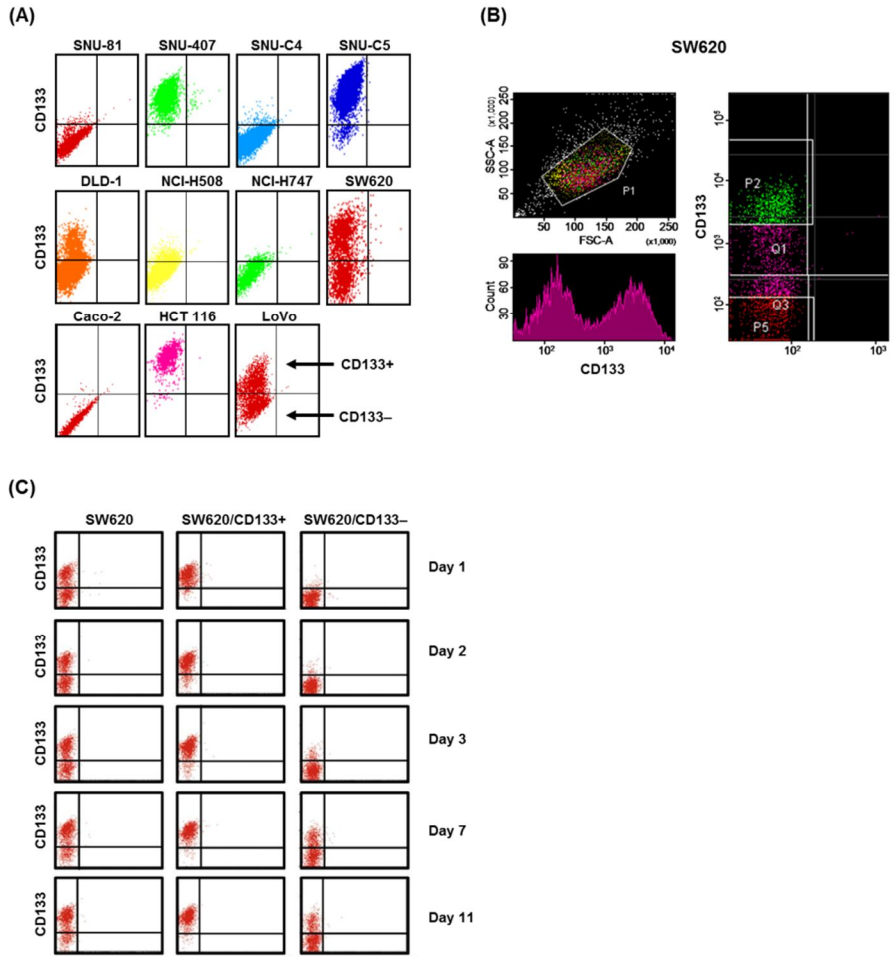


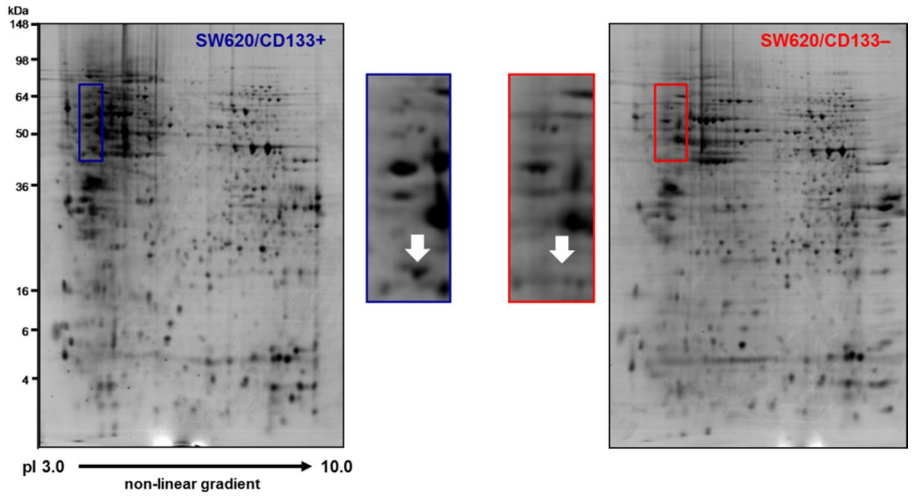
Figure 1. Preparation of SW620/CD133+ and SW620/CD133– cells for proteomic analysis. (A) Differential CD133+ subpopulations of human colon cancer cell lines. All 11 human colon cancer cell lines utilized in the present study had a different amount of CD133+ subpopulations; however, CD133+ and CD133– subpopulations in DLD-1, SW620, and LoVo were similar. (B) Fluorescence-activated sorting of SW620/CD133+ and SW620/CD133– cells. SW620 colorectal cancer cells were incubated with a CD133-APC

monoclonal antibody. SW620/CD133+ (P2) and SW620/CD133– (P5) populations were isolated using a cell sorter. (C) Time-dependent change in SW620/CD133+ and SW620/CD133– cell subpopulations. From 3 days after cell sorting, the amount of CD133+ cells increased in the CD133– subpopulation, but no significant changes were observed in the CD133+ subpopulation. SW620/CD133+ and SW620/CD133– cells were sorted and used for the next proteome analysis before subpopulation change.

Upregulated NPM1 in SW620/CD133+ cells

SW620/CD133+ and SW620/CD133– cells were sorted and lysed to prepare a proteome source for identifying proteins linked to CD133 expression. 2-DE gels were constructed using whole proteins from SW620/CD133+ and SW620/CD133– cells, and an upregulated protein with an acidic *p*I and an approximate 40 kDa molecular mass was selected by image analysis of 2-DE gels (Figure 2A). The selected protein spot was analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and was identified as NPM1 (Figure 2B, Table 2). Increased NPM1 expression in the SW620/CD133+ subpopulation was confirmed by Western blotting and confocal analyses (Figure 2C and 2D).

(A)



(B)

Protein Name	Accession No.	Protein Score	Protein Score C.I.%	Total Ion Score	Total Ion C.I.%	Sequence Coverage
Nucleophosmin OS=Homo Sapiens GN=NPM1	P06748	80	99.977	65	99.965	33%

Peptide Information								
Calc. Mass	Obsrv. Mass	± Da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C.I.%
599.3875	599.3743	-0.0132	-22	151	155	VPQKK		
1023.4894	1023.4744	-0.015	-15	25	32	ADKDYHFK		
1303.6616	1303.6300	-0.0316	-24	268	277	FINYVKNCFR		
1568.7300	1568.6631	-0.0669	-43	33	45	VDNDENEHQLSLR	16	0
1568.7300	1568.6631	-0.0669	-43	33	45	VDNDENEHQLSLR		
1819.8433	1819.7635	-0.0798	-44	278	291	MTDQEAIQDLWQWR		
1819.8433	1819.7635	-0.0798	-44	278	291	MTDQEAIQDLWQWR	25	0
1835.8381	1835.7401	-0.0980	-53	278	291	MTDQEAIQDLWQWR		
1835.8381	1819.7401	-0.0980	-53	278	291	MTDQEAIQDLWQWR		
2224.9832	2225.0808	0.0976	44	55	73	DELHIVEAEAMNYEGSPIK		
2227.2156	2227.0903	-0.1253	-56	81	101	MSVQPTVSLGGFEITPPVVLR	24	0
2227.2156	2227.0903	-0.1253	-56	81	101	MSVQPTVSLGGFEITPPVVLR		
2243.2104	2243.0881	-0.1223	-55	81	101	MSVQPTVSLGGFEITPPVVLR	11	0
2243.2104	2243.0881	-0.1223	-55	81	101	MSVQPTVSLGGFEITPPVVLR		

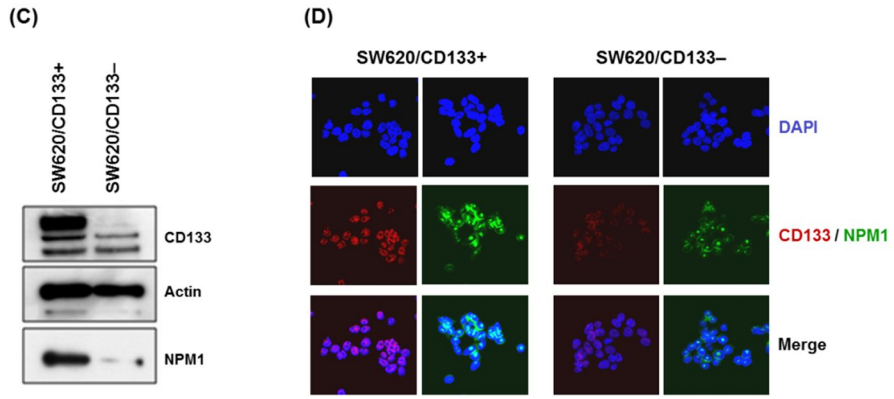


Figure 2. Identification of highly expressed nucleophosmin (NPM1) in SW620/CD133+ cells. (A) 2-DE gel images showing differential protein expression between SW620/CD133+ and SW620/CD133– cells. Enlarged 2-DE gel images demonstrate that the protein indicated by a white arrow was significantly upregulated in SW620/CD133+ cells. (B) Identification of NPM1 by MALDI-TOF analysis. The protein indicated in figure 2A was unambiguously identified as NPM1. Mass analysis data are as shown in the table. Western blot (C) and confocal (D) analyses to confirm NPM1 overexpression in SW620/CD133+ cells.

Table 2. Proteins with expressional change in SW620 cells sorted as CD133– cells

No.	Protein Name	Accession No.	Protein Score	Nominal mass	Calculated *p*I value	Sequence Coverage	Regulation
1	Methylosome subunit pICln	P54105	62	26199	3.97	45%	Down
2	Reticulocalbin-1	Q15293	65	38866	4.86	37%	Down
3	Nucleophosmin	P06748	80	32555	4.64	33%	Down
4	hnRNP C1/C2	P07910	151	33650	4.95	28%	Down
5	Proliferating cell nuclear antigen	P12004	187	28750	4.57	61%	Down
6	Elongation factor 1-beta	P24534	59	24748	4.5	12%	Down
7	Inorganic pyrophosphatase	Q15181	135	32639	5.54	63%	Up
8	Prohibitin	P35232	73	29786	5.57	47%	Up

9	Glutathione S-transferase P	P09211	293	23341	5.43	66%	Down
10	Rho GDP-dissociation inhibitor 1	P52565	131	23193	5.02	48%	Down
11	Histone H2B type 1-M	Q99879	62	13882	10.31	35%	Down
12	Cofilin-1	P23528	96	18491	8.22	56%	Down
13	Profilin-1	P07737	358	15045	8.44	78%	Down
14	Macrophage migration inhibitory factor	P14174	95	12468	7.74	49%	Down

NPM1 expression regulated by CD133 in SW620 cells

To determine whether artificial CD133 suppression cause same effect of cell sorting on NPM1 expression, Western blot analysis was performed using the total lysate of SW620 cells transfected with CD133 siRNA. Like CD133, NPM1 expression was significantly downregulated in both CD133– cells and SW620 cells transfected with CD133 siRNA (Figure 3A). Expression levels of p-AKT and NF-κB also decreased in both CD133– and CD133-suppressed cells (Figure 3A). However, NPM1 suppression itself did not affect CD133 expression (Figure 3B), and protein interaction between CD133 and NPM1 was not found (Figure 3C).

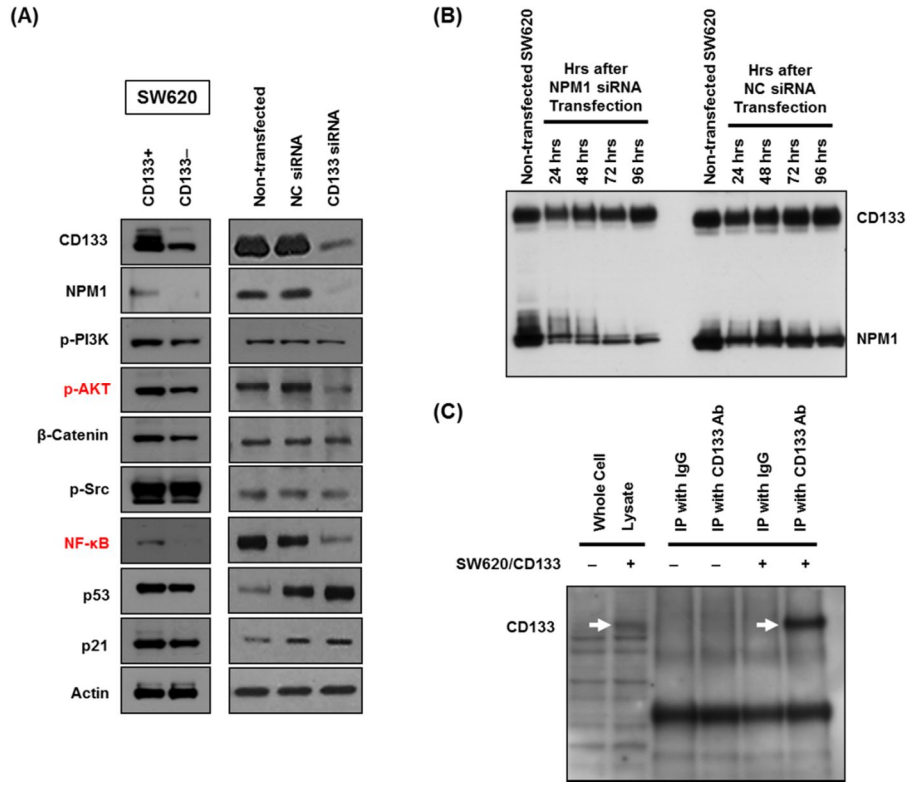


Figure 3. CD133-regulated NPM1 expression in SW620 cells. (A) NPM1 expression changes after either CD133+ sorting or CD133-specific siRNA transfection. NPM1 expression was significantly lower in SW620/CD133– and CD133-suppressed SW620 cells as compared to SW620/CD133+ and non-silenced SW620 control cells. NPM1 expression was regulated by CD133 expression status. Expression change of major signaling molecules and transcription factors in SW620 cells transfected with CD133 siRNA was also monitored in SW620/CD133– cells. Like NPM1, p-AKT and NF-κB were significantly downregulated in SW620/CD133– and CD133-suppressed

SW620 cells. (B) No change in CD133 expression was observed after NPM1 suppression. NPM1 expression was successfully suppressed until 96 h after specific siRNA transfection. However, CD133 expression was not affected by NPM1 suppression. (C) No interaction was observed between CD133 and NPM1. Western blot analysis of the immunoprecipitate (IP) using a CD133 antibody demonstrated that CD133 did not interact with NPM1.

Positive expressional correlation between NPM1 and CD133 in human colon cancer cell lines

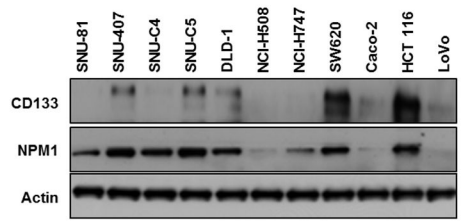
The intracellular expression of CD133 and NPM1 was determined by Western blot analysis in 11 human colon cancer cell lines (Figure 4A). Although the Spearman correlation (P = 0.0759) did not reveal statistical significance, a positive correlation between CD133 and NPM1 was found in human colon cancer cell lines (Figure 4B). In most cell lines tested, NPM1 expression followed CD133 expression. However, SNU-81, SNU-C4 and NCI-H747 did not express CD133, but moderately expressed NPM1 (Figure 4A).

Positive correlation between the CD133+ subpopulation percentage and susceptibility of human colon cancer cell lines to 5-FU

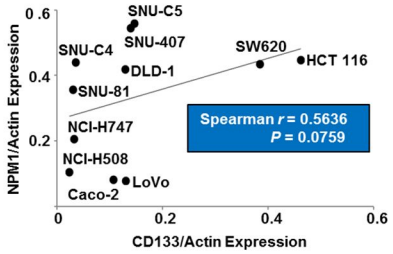
CD133 or NPM1 expression was not correlated with the susceptibility of human colon cancer cell lines to chemotherapy or radiation treatment (Figure 4C). Spearman correlation analysis revealed that proliferating human colon cancer cell lines with either a higher CD133+ subpopulation or lower CD133 expression when normalized to the CD133+ subpopulation percentage were more resistant to 5-FU treatment (Figure 4C). However, no factors tested in

Figure 4C were linked to radiation susceptibility.

(A)



(B)



(C)

Spearman Coefficient *r*, *P*		% Cell Proliferation after Treatment					
		96 Hrs	96 Hrs	48 Hrs	96 Hrs	48 Hrs	96 Hrs
		5-FU (1 μg/ml)	5-FU (10 μg/ml)	4 Gy	4 Gy	8 Gy	8 Gy
% of CD133+ Subpopulation		0.5727, 0.0706	0.6727, 0.0281	0.1182, 0.7343	0.2818, 0.4021	0.1727, 0.6144	0.0545, 0.8815
CD133 Expression / % of CD133+ Subpopulation		-0.6091, 0.0519	-0.7364, 0.0134	-0.0545, 0.8815	-0.2182, 0.5209	-0.2455, 0.4682	-0.0364, 0.9244
Expression	CD133	0.4182, 0.2031	0.3091, 0.3560	0.3273, 0.3270	0.1182, 0.7343	-0.3091, 0.3560	-0.0727, 0.8388
	NPM1	0.2091, 0.5391	0.3818, 0.2484	-0.0545, 0.8815	-0.1455, 0.6734	-0.4636, 0.1543	-0.2364, 0.4855

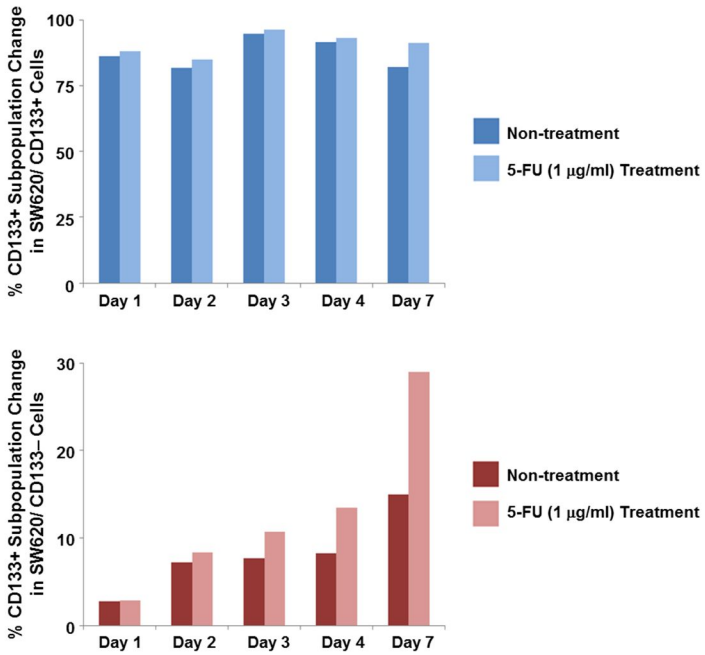
Figure 4. Correlation between CD133 and NPM1 expression and its relevance to 5-FU susceptibility in human colon cancer cell lines. (A) Variable expression of CD133 and NPM1 in human colon cancer cell lines. Western blot analysis showed CD133 and NPM1 in 11 human colon cancer cell lines. (B) Expressional correlation of NPM1 with CD133 in human colon cancer cell lines. CD133 and NPM1 expression was calculated by determining the optical density of Western blots and normalized against actin. Spearman correlation coefficient, r = 0.5636; P = 0.0759. (C) CD133+ subpopulation percentage in human colon cancer cell lines linked to 5-FU treatment response in human colon cancer cells. Spearman correlation analysis was performed to

reveal the link between the CD133 subpopulation percentage and CD133 and NPM1 expression to chemotherapy and radiation susceptibility of 11 human colon cancer cell lines. Increased CD133+ subpopulation percentage (or decreased CD133 expression/% of CD133+ subpopulation) was positively correlated with % cell proliferation rate at 96 h after 1 or 10 μg/mL 5-FU treatment.

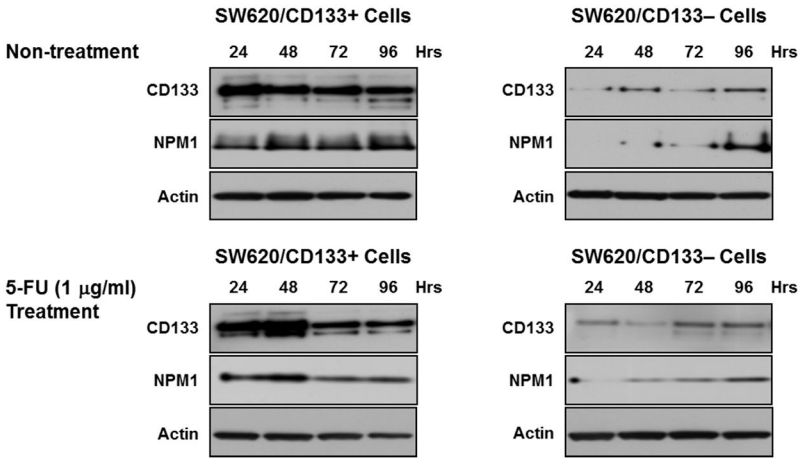
Differential 5-FU susceptibility between SW620/CD133+ and SW620/CD133– cells

After sorting of SW620/CD133+ and SW620/CD133– cells, CD133+ subpopulation did not show any significant changes in SW620/CD133+ cells, regardless of 5-FU treatment (Figure 5A, upper panel). However, much less portion of CD133+ subpopulation (about 2-3%), not sorted completely and mixed in SW620/CD133– cells was gradually increased in up to about 15% in 7 days after sorting of cells (Figure 5A, lower panel). Such increasing CD133+ subpopulation in SW620/CD133– cells was augmented by 5-FU treatment (Figure 5A, lower panel), and also accompanied by upregulated expression of CD133 and NPM1 (Figure 5B, right two panels). However, altered expression of CD133 and NPM1 in SW620/CD133+ cells was not found (Figure 5B, left two panels). When investigated differential 5-FU susceptibility between SW620/CD133+ and SW620/CD133– cells, SW620/CD133+ cells showed slightly less susceptible to 5-FU compared to SW620/CD133– cells, but in the absence of 5-FU proliferating rates of SW620/CD133+ and SW620/CD133– cells were not different (Figure 5C).

(A)


% CD133+ Subpopulation Change in SW620/ CD133+ Cells
100
75
50
25
0
Day 1
Day 2
Day 3
Day 4
Day 7
Non-treatment
5-FU (1 μg/ml) Treatment
% CD133+ Subpopulation Change in SW620/ CD133– Cells
30
20
10
0
Day 1
Day 2
Day 3
Day 4
Day 7
Non-treatment
5-FU (1 μg/ml) Treatment

(B)


SW620/CD133+ Cells
SW620/CD133– Cells
Non-treatment
24 48 72 96 Hrs
CD133
NPM1
Actin
5-FU (1 μg/ml) Treatment

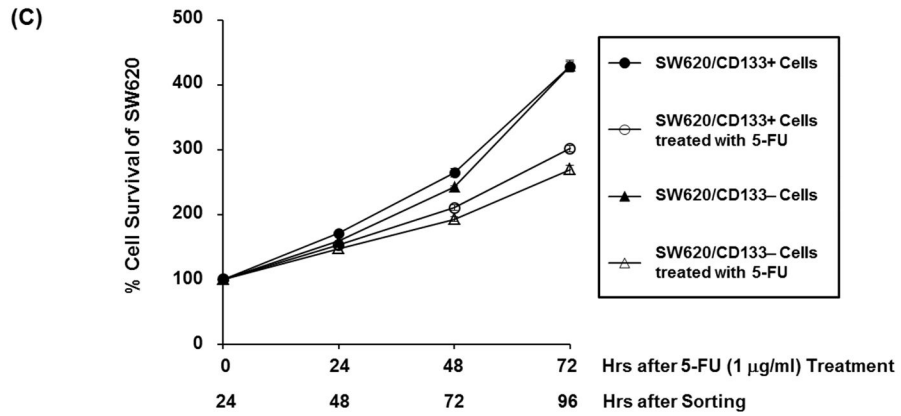


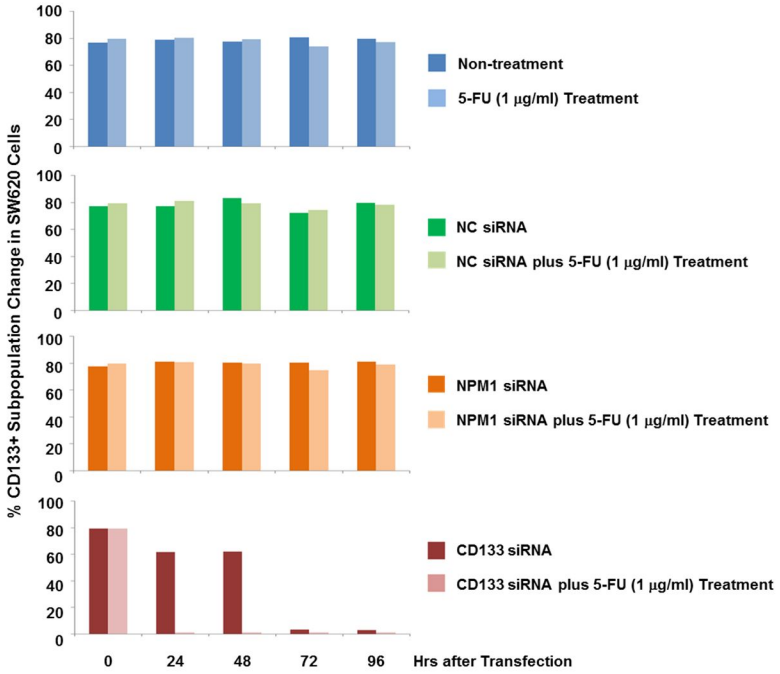
Figure 5. Differential 5-FU susceptibility between SW620/CD133+ and SW620/CD133– cells. (A) Increased CD133+ subpopulation in SW620/CD133– cells. CD133+ subpopulation was maintained in in both non-treated and 5-FU treated SW620/CD133+ cells (upper panel). But minor portion of CD133+ subpopulation (about 2-3%) not separated completely in SW620/CD133– cells was slowly increased up to about 15% in 7 days (lower panel). 5-FU treatment accelerated increase of CD133+ subpopulation in SW620/CD133– cells (lower panel). (B) CD133 and NPM1 expression in sorted cells. Minor expression of CD133 and NPM1 in both non-treated and 5-FU treated SW620/CD133– cells became increased depending on increasing of CD133+ subpopulation (right two panels). However, expression pattern of CD133 and NPM1 in SW620/CD133+ cells was not changed (left two panels). (C) 5-FU susceptibility difference between SW620/CD133+ and

SW620/CD133– cells. SW620/CD133+ cells were less susceptible to 5-FU compared to SW620/CD133– cells. Data are means ± SD from three independent experiments.

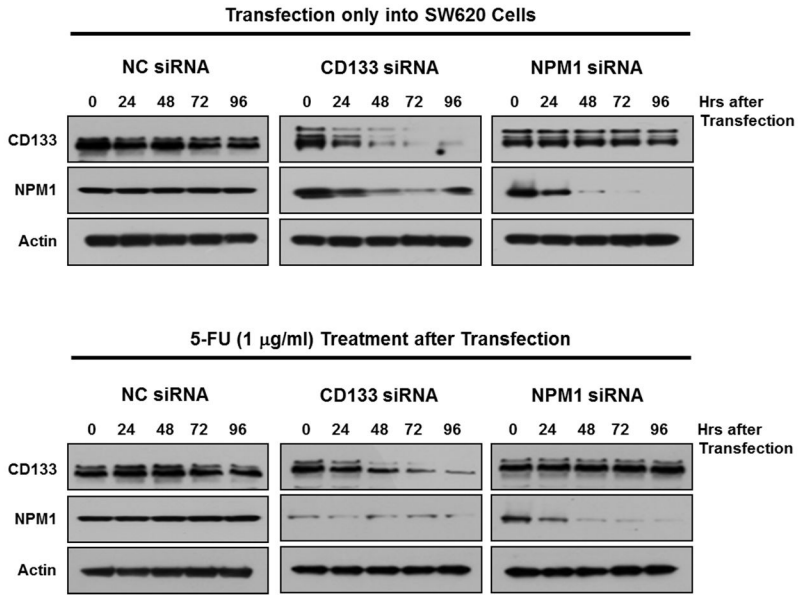
Significant increased 5-FU susceptibility in SW620 cells after suppression of CD133 and NPM1

Artificial suppression of CD133 by transfection of specific siRNA eliminated CD133+ subpopulation (Figure 6A), and also induced downregulation of NPM1 (Figure 6B). However, NPM1 suppression and 5-FU treatment did affect neither CD133+ subpopulation nor CD133 expression (Figure 6A and 6B). Unlike in CD133+ cell sorting, suppression of CD133 expression significantly increased 5-FU susceptibility, and interestingly, such induced susceptibility was also monitored after NPM1 suppression (Figure 6C).

(A)


% CD133+ Subpopulation Change in SW620 Cells
100
80
60
40
20
0
Non-treatment
5-FU (1 μg/ml) Treatment
NC siRNA
NC siRNA plus 5-FU (1 μg/ml) Treatment
NPM1 siRNA
NPM1 siRNA plus 5-FU (1 μg/ml) Treatment
CD133 siRNA
CD133 siRNA plus 5-FU (1 μg/ml) Treatment
0
24
48
72
96
Hrs after Transfection

(B)


Transfection only into SW620 Cells
NC siRNA
CD133 siRNA
NPM1 siRNA
0 24 48 72 96
Hrs after Transfection
CD133
NPM1
Actin
5-FU (1 μg/ml) Treatment after Transfection
NC siRNA
CD133 siRNA
NPM1 siRNA
0 24 48 72 96
Hrs after Transfection
CD133
NPM1
Actin

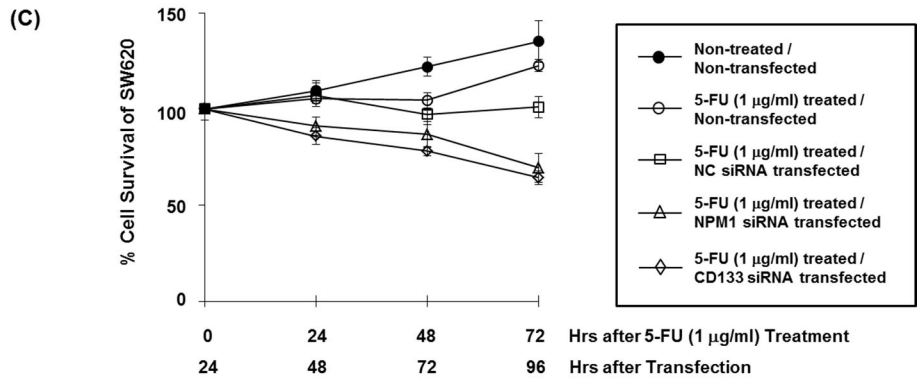
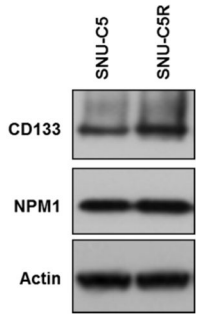


Figure 6. Increase of 5-FU susceptibility in SW620 cells by suppression of CD133 and NPM1. (A) Elimination of CD133+ subpopulation by transfection of specific siRNA to CD133. CD133+ subpopulation was almost disappeared after CD133 siRNA transfection. (B) Artificial suppression of CD133 leading to downregulation of CD133 and NPM1. NPM1 suppression did not affect CD133 expression. (C) Increased 5-FU susceptibility after CD133 suppression. Increased 5-FU susceptibility was also found in NPM1 suppressed cells. Data are means ± SD from three independent experiments.

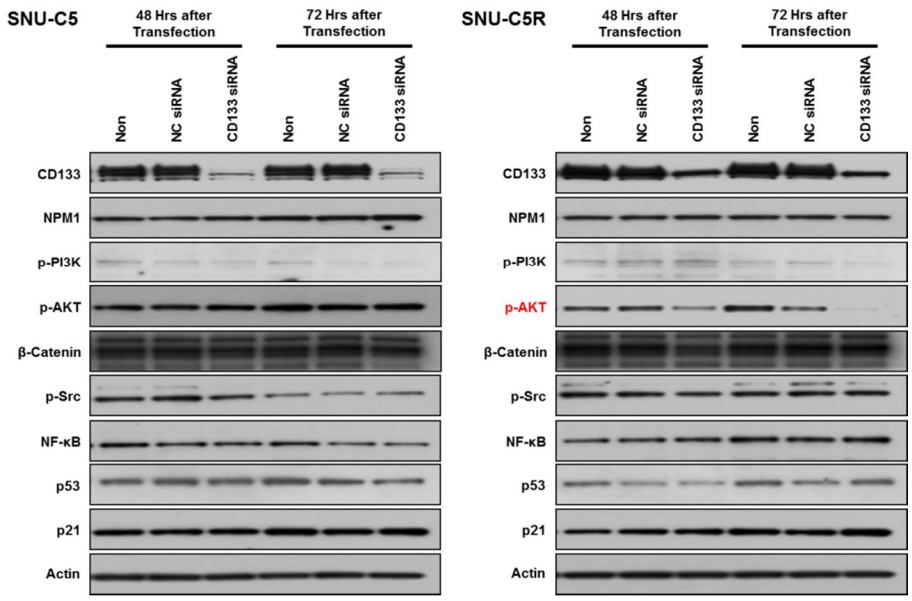
Effects of CD133 and NPM1 suppression on 5-FU susceptibility in human colon cancer cell line SNU-C5R with induced 5-FU resistance

To clarify whether CD133 and NPM1 suppression also leads to increase of 5-FU susceptibility in other human colon cancer cell lines, SNU-C5R with induced 5-FU resistance and its parent cell line SNU-C5 were employed. CD133 expression was slightly upregulated in SNU-C5R compared to its parent cell line SNU-C5, while NPM1 did not show any expressional difference in both cell lines (Figure 7A). Transfection of siRNA specific to CD133 significantly decreased level of p-AKT (Figure 7B). However, unlike in SW620, expression of NF-κB was not dysregulated after CD133 suppression (Figure 7B), and NPM1 expression was not regulated by CD133 (Figure 7C and 7D). Although CD133-regulated NPM1 expression was not found, increased 5-FU susceptibility after CD133 or NPM1 suppression was unambiguously monitored in both SNU-C5 and SNU-C5R cells (Figure 7E).

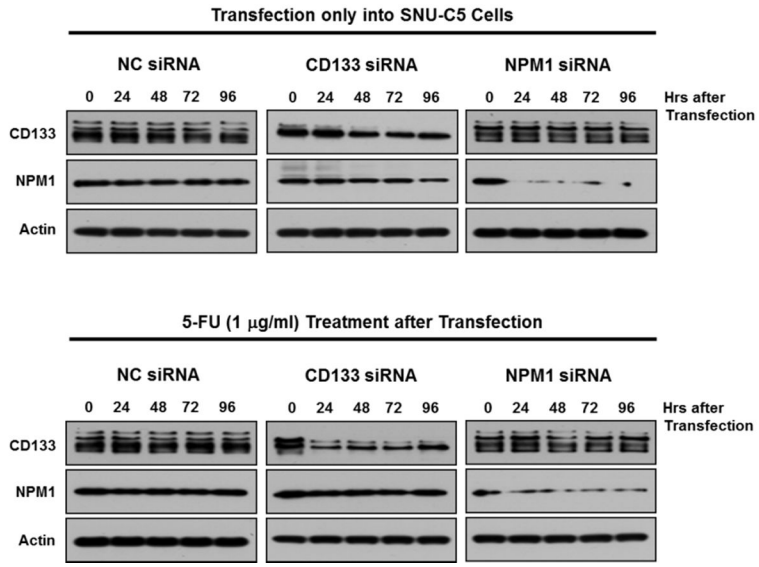
(A)


SNU-C5
SNU-C5R
CD133
NPM1
Actin

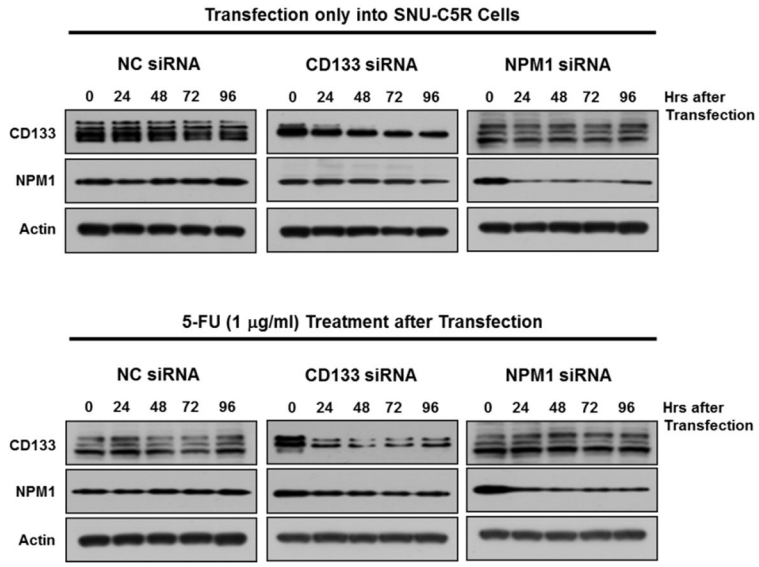
(B)


SNU-C5
48 Hrs after Transfection
72 Hrs after Transfection
Non
NC siRNA
CD133 siRNA
CD133
NPM1
p-PI3K
p-AKT
β-Catenin
p-Src
NF-κB
p53
p21
Actin
SNU-C5R
48 Hrs after Transfection
72 Hrs after Transfection
Non
NC siRNA
CD133 siRNA
CD133
NPM1
p-PI3K
p-AKT
β-Catenin
p-Src
NF-κB
p53
p21
Actin

(C)


Transfection only into SNU-C5 Cells
NC siRNA
CD133 siRNA
NPM1 siRNA
0 24 48 72 96
Hrs after Transfection
CD133
NPM1
Actin
5-FU (1 μg/ml) Treatment after Transfection
NC siRNA
CD133 siRNA
NPM1 siRNA
0 24 48 72 96
Hrs after Transfection
CD133
NPM1
Actin

(D)


Transfection only into SNU-C5R Cells
NC siRNA
CD133 siRNA
NPM1 siRNA
0 24 48 72 96
Hrs after Transfection
CD133
NPM1
Actin
5-FU (1 μg/ml) Treatment after Transfection
NC siRNA
CD133 siRNA
NPM1 siRNA
0 24 48 72 96
Hrs after Transfection
CD133
NPM1
Actin

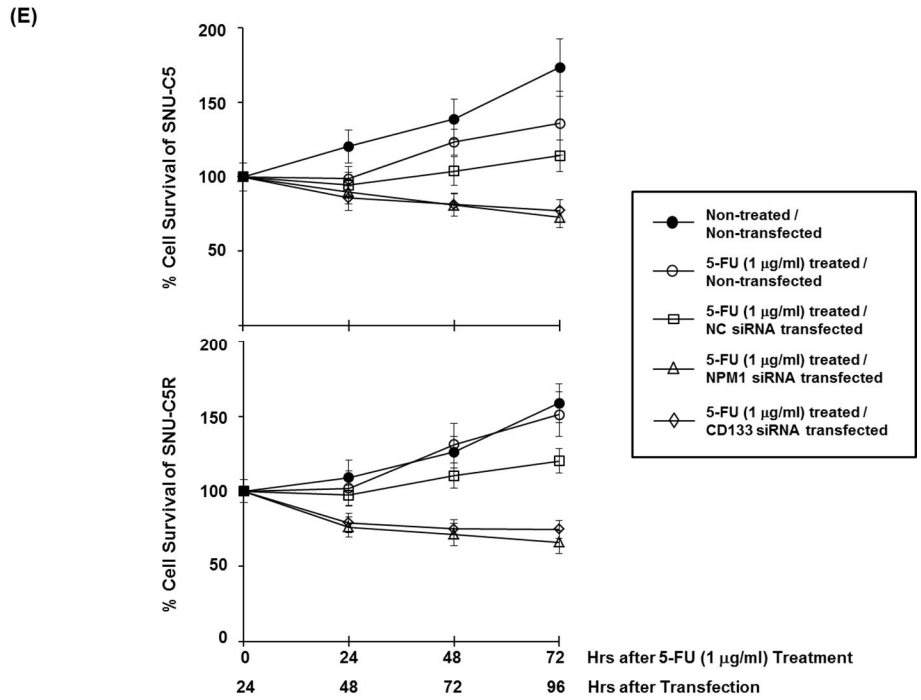


Figure 7. Increase of 5-FU susceptibility in SNU-C5R cells with induced 5-FU resistance by suppression of CD133 and NPM1. (A) CD133 and NPM1 expression in SNU-C5R and its parent SNU-C5 cells. CD133 expression was upregulated in SNU-C5R, but NPM1 did not show any expressional difference in both cell lines. (B) Expressional change of intracellular molecules after CD133 suppression. Among the molecules tested, p-AKT only showed downregulation after CD133 siRNA transfection. Successful suppression of CD133 and NPM1 in SNU-C5 (C) and SNU-C5R (D) cells. Unlike in SW620, expression of NPM1 was not modulated by

CD133 in both cell lines. (E) Increase of 5-FU susceptibility in SNU-C5R and its parent SNU-C5 cells by suppression of CD133 and NPM1. 5-FU susceptibility after CD133 or NPM1 suppression was unambiguously increased in both SNU-C5 and SNU-C5R cells. Data are means ± SD from three independent experiments.

Discussion

CD133+ and CD133– cancer cells are often reported to have different protein expression profiles that are proposed to affect their ability to self-renew and their level of resistance to drugs (Chen *et al.,* 2008). I therefore performed proteomic analysis using CD133+ and CD133– human colon cancer cells to find a protein with differential expression depending on CD133 expression status. CD133 was expressed differently in the 11 colon cancer cell lines tested (Figures 1A and 4A), but SW620 cells contained the most dispersed subpopulations between CD133+ and CD133– cells (Figure 1A and 1B). Therefore, I utilized SW620 cells for the present proteomic study. After 11 days of culturing the two groups of the sorted SW620 cells separately, I observed a gradual shift from a CD133– to CD133+ subpopulation in SW620/CD133– cells as well as in the unsorted SW620 control group (Figure 1C). After sorting, SW620/CD133+ and SW620/CD133– cells were used immediately for the next proteome analysis.

From 2-DE-based proteome assessment of sorted SW620 cells, increased NPM1 was found in SW620/CD133+ cells (Figure 2). The correlation between NPM1 and CD133 was confirmed by Western blotting, as NPM1 was

significantly downregulated in both SW620/CD133– and CD133-suppressed SW620 cells (Figure 3A). Although statistical significance was not observed (P = 0.0759), this correlation pattern was also found in the 11 human colon cancer cell lines (Figure 4). NPM1 promotes ribosome biogenesis and protein synthesis, acts on DNA polymerase-alpha to increase DNA synthesis, and facilitates cell cycle progression (Grisendi *et al.,* 2006). Moreover, NPM1 inhibits p53, which promotes cell survival and suppresses differentiation, apoptosis, and DNA fragmentation (Grisendi *et al.,* 2006). Therefore, NPM1 might play an important role in giving CD133+ cells their ability to self-renew and resist drugs. My data clearly demonstrate that NPM1 expression can be regulated by CD133 (Figure 3A).

When compared the effects of CD133 suppression and CD133 sorting, the changes observed in major signaling molecules and transcription factors were similar (Figure 3A). In either CD133-suppressed or SW620/CD133– cells, downregulation of p-AKT and NF-κB was found as compared to the non-suppressed or SW620/CD133+ cells, respectively (Figure 3A). Conversely, CD133 siRNA transfected cells showed significantly increased p53 compared to the non-silenced cells (Figure 3A). AKT is a key signaling protein in multiple cellular processes. AKT activates NF-κB by regulating IκB kinase

and enhances the ubiquitination-promoting function of Mdm2, which results in reduced p53 protein (Ogawara *et al.,* 2002). The CD133 siRNA transfected cells also had relatively low NPM1, which is involved in the regulation of cell growth, proliferation, and transformation (Grisendi *et al.,* 2006). NPM1 is a natural p53 repressor and may also contribute to the dampening of p53 function (Maiguel *et al.,* 2004). Therefore, my findings suggest that CD133 suppression induces inactivation of the AKT signaling cascade in SW620 cells and that the CD133 protein may be involved in AKT-mediated cell proliferation and survival.

I also investigated the link between CD133 and NPM1 expression with 5-FU and radiation in human colon cancer cells. Although CD133 has been implicated in chemotherapy and radiation therapy responses (Liu *et al.,* 2006, Kojima *et al.,* 2010), my data showed that CD133 expression itself and CD133-regulated NPM1 expression were not correlated with intrinsic susceptibility to 5-FU or radiation treatment (Figure 4C). Nevertheless, the CD133+ subpopulation percentage or CD133 expression normalized to the percentage of the CD133+ subpopulation was significantly correlated with intrinsic 5-FU susceptibility of human colon cancer cells (Figure 4C). Increased CD133+ subpopulation percentage was positively correlated with 5-

FU resistance, but this correlation was negative when CD133 expression was normalized with the CD133+ subpopulation percentage (Figure 4C).

CD133+ cells not completely separated from SW620/CD133– subpopulation, continuously proliferated and gradually increased its cell number in SW620/CD133– subpopulation (Figure 5A). Such increasing of number of CD133+ cells was promoted by the treatment of 5-FU (Figure 5A). These findings demonstrate that CD133+ cells could be less susceptible to 5-FU compared to CD133– cells. Actually, survival of CD133+ cells was better than that of CD133– cells (Figure 5C). Furthermore, suppression of CD133 increased 5-FU susceptibility not only in SW620 (Figure 6C), but also SNU-C5 and SNU-C5R (Figure 7E). Especially, 5-FU resistance of SNU-C5R was effectively decreased by CD133 suppression (Figure 7E, lower panel).

The CD133– cell subpopulation is more migratory and invasive; CD44+/CD133– cells are proposed to be the CSCs population, as they display greater proliferation and less spontaneous apoptosis and were more resistant to drug-induced cell death in SW620 cells (Wang *et al.,* 2012). On the other hand, Tentes *et al.* reported that SW620 Cells with acquired 5-FU resistance adopted a CD44–/CD133+ phenotype (Tentes *et al.,* 2010). Also, other findings support the claim that CD133 is a valid marker for CSCs (Wang *et al.,*

2012). The CD133+ subpopulation was observed to have higher take rates and shorter doubling time in tumor growth than the unsorted cell line or CD133– cells (Schneider *et al.,* 2012). Moreover, Kawamoto *et al*. showed that CD133+ SW620 cells were more proliferative and chemoradiation therapy-resistant than CD133– cells (Kawamoto *et al.,* 2010).

Interestingly, CD133-regulated NPM1 expression was only found in SW620 (Figure 3A), but not in both SNU-C5 and SNU-C5R (Figure 7B). As well as, downregulated NF-κB caused by CD133 suppression in SW620 (Figure 3A) was not observed in both SNU-C5 and SNU-C5R (Figure 7B). Although CD133-regulated NPM1 expression was limited in SW620, NPM1 suppression itself was enough to increase 5-FU susceptibility of all cell lines tested, SW620, SNU-C5 and SNU-C5R (Figures 6C and 7E). These findings suggest that like CD133, expression of NPM1 may not only play an important role for cellular response to 5-FU, but also be a predictive factor for chemotherapy response prediction.

In conclusion, although the role of CD133 in chemotherapy and radiation therapy responses is still unclear, my present study suggests that CD133-regulated NPM1 expression may provide a clue to possible CD133 function(s) that can be linked to susceptibility of human colon cancer cells to

chemotherapy.

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국문 초록

대장암은 서구에서 흔한 고형암 중의 하나로 최근 국내에서도 발생 빈도가 높아지고 있는 암이다. 대장암 치료에 있어서 5-fluorouracil(5-FU)은 가장 널리 사용되고 있는 항암제이나, 진행된 대장암의 경우 약 25% 미만의 환자만이 5-FU 에 반응을 보이며 5-FU 에 대한 내성으로 인한 암의 재발로 인하여 효과적인 치료에 어려움을 겪고 있다. 따라서, 대장암의 항암치료에 있어서의 가장 큰 어려움은 항암제에 대한 내성이다. 최근 연구에 의하면 줄기세포(stem cell)의 특성을 가진 세포들이 암 세포의 통합적인 발달과 지속에 관여하고 있다는 이론이 부각되고 있으며 종양의 종양줄기세포(cancer stem cell)를 제거하는 것이 암의 재발을 막고 결과적으로 항암치료에 효과적인 역할을 할 것으로 논의되고 있다. *In vivo* 실험에서 면역시스템이 결핍된 쥐에게 인간의 종양세포를 CD133 양성인 종양세포와 CD133 음성인 종양세포로 나누어 각각 주입한 결과 CD133 양성인 종양세포를 주입한 실험 군에서만 종양이 형성되는 것이 보고되면서 CD133 은 종양줄기세포 표지자로 논의되기 시작하였다. 대장암에서도 CD133 은 종양줄기세포 표지자로 보고된 바가 있으나, 현재까지 CD133 의 생체 내 기능에 대한 명확한

연구결과는 밝혀진 바가 없다. 이에 본 연구팀에서는 CD133 의 생체 내 기능에 대한 연구를 진행하고자 하였다. 11 개의 대장암 세포주에서 CD133 단백질의 발현을 확인한 후 CD133 양성 세포와 CD133 음성 세포가 고르게 분포하는 SW620 대장암 세포를 분석 대상 세포로 선정하였다. 유세포 분석방법을 이용하여 SW620 대장암 세포를 CD133 양성 세포와 CD133 음성 세포로 각각 분리한 후 비교단백질체 분석법인 2-DE 방법을 이용하여 CD133 단백질의 발현에 따라 다르게 발현되는 단백질을 관찰하였으며 MALDI-TOF 질량분석기를 이용하여 CD133 양성 세포에서 높게 발현되는 단백질로 nucleophosmin 단백질을 동정하였다. Western blot 방법과 면역형광법을 사용하여 CD133 양성 세포는 nucleophosmin 단백질의 발현이 높고, CD133 음성 세포는 nucleophosmin 단백질의 발현이 낮음을 확인하였다. SW620 대장암 세포주에 siRNA transfection 방법을 사용하여 인위적으로 CD133 의 발현을 저해하였을 때에도 nucleophosmin 단백질의 발현이 함께 감소하는 것을 확인하였으나, 인위적으로 nucleophosmin 단백질의 발현을 억제하였을 때에는 CD133 단백질의 발현에 변화가 없는 것을 확인하였다. 이를 통해 SW620 대장암 세포에서 CD133 에 의해 nucleophomin 의 발현이 조절되는 것을 확인할 수 있었다. 또한 CD133 과 nucleophomin 의

발현과 5-FU 에 대한 감수성과의 관계를 알아보기 위하여 SW620 대장암 세포를 유세포분석방법 또는 siRNA transfection 방법을 통해 CD133 발현을 다르게 한 후 세포 배양을 하며 5-FU 를 처리하여 5-FU 에 대한 감수성을 조사하였다. 유세포 분석방법을 통해 CD133 의 발현 정도를 다르게 한 후 세포 독성 조사(MTT assay)를 하여 CD133 음성 세포가 CD133 양성 세포에 비해 5-FU 에 대한 감수성이 높은 것을 확인할 수 있었다. 또한, siRNA transfection 방법을 통해 nucleophosmin 의 발현을 인위적으로 억제하였을 때에도 5-FU 에 대한 감수성이 높아지는 것을 확인하였다. SW620 대장암 세포 외에 다른 대장암 세포에서도 CD133 과 nucleophosmin 의 발현과 5-FU 에 대한 감수성의 연관성을 확인하기 위해 CD133 이 과발현되고 있는 SNU-C5 대장암 세포와 5-FU 에 대한 내성을 가지고 있는 SNU-C5R 세포를 대상으로 추가 실험을 진행하였다. 비록 CD133 에 의한 nucleophosmin 의 발현 조절은 SW620 대장암 세포에서만 확인되었지만, CD133 과 nucleophosmin 의 발현과 연관된 5-FU 에 대한 감수성은 SW620, SNU-C5, SNU-C5R 대장암 세포에서 모두 관찰되었다.

본 연구는 종양줄기세포인자로 알려진 CD133 단백질의 발현과 연관된 단백질을 동정하고 대장암 세포주에서 CD133 과 항암제

내성과의 연관성을 알아보고 이를 통해 CD133 의 기능을 확인하는데 그 의의가 있다.

주요어: Colorectal cancer; Cancer Stem Cell; CD133; Nucleophosmin; Drug-Resistance

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