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Chemoresistance of CD133$^+$ colon cancer may be related with increased survivin expression

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ABSTRACT

CD133, putative cancer stem cell marker, deemed to aid chemoresistance. However, this claim has been challenged recently and we previously reported that patients with CD133$^+$ colon cancer have benefit from 5-fluorouracil (5-FU) chemotherapy incontrast to no benefit in patients with CD133$^-$ cancer. To elucidate the role of CD133 expression in chemoresistance, we silenced the CD133 expression in a colon cancer cell line and determined its effect on the biological characteristics downstream. We comparatively analyzed the sequential changes of MDR1, ABCG2, AKT1 and survivin expression and the result of proliferation assay (WST-1 assay) with 5-FU treatment in CD133$^+$ and siRNA-induced CD133$^-$ cells, derived from Caco-2 colon cancer cell line. 5-FU treatment induced significantly increase of the mRNA expression of MDR1, ABCG2 and AKT1genes, but not protein level. CD133 had little to no effect on the mRNA and protein expression of these genes. However, survivin expression at mRNA and protein level were significantly increased in CD133$^+$ cells compared with siRNA-induced CD133-cells and Mock (not sorted CD133$^+$ cells) at 96 h after siRNA transfection. The cytotoxicity assay demonstrated notable increase of chemoresistance to 5-FU treatment (10 μM) in CD133$^+$ cells at 96 h after siRNA transfection. From this study, we conclude that CD133$^+$ cells may have chemoresistance to 5-FU through the mechanism which is related with survivin expression, instead of MDR1, ABCG2 and AKT1 expression. Therefore a survivin inhibitor can be a new target for effective treatment of CD133$^+$ colon cancer.

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1. Introduction

The cancer stem cell (CSC) theory postulates that a small subset of tumor cells have the capacity of tumor initiation, progression [1] and chemoresistance and can also establish distant metastasis [2]. The conventional therapies fail to respond clinically, due to strategies solely based on tackling only the highly proliferating cells and therefore spare the small population of slowly growing but resilient CSCs in the tumor [3]. Nevertheless, efforts to foster and explore the effective scope of CSC-based target therapy are on the verge.

Thus far, a number of different CSC markers have been proposed. One such marker is CD133, a trans-membrane glycoprotein which was initially classified as a marker of primitive hematopoietic [4] and neural stem cells [5]. There are accumulating pieces of evidence highlighting the role of CD133 as a reliable marker for cancer stem cells (CSCs) in several human tumors [6]. The collective studies suggest that CD133 expression confers poor prognosis [7] and mediates cancer relapse after chemotherapy in colorectal cancers [8,9]. Numerous reports from in vivo and/or in vitro studies have previously shown that CD133$^+$ cells are more resistant to chemotherapy as compared to CD133$^-$ cells [2,10–15]. Notwithstanding, these claims have been challenged recently [16,17]. In our previous report, we analyzed the outcome of the colorectal cancer patients after 5-FU chemotherapy and demonstrated that CD133 expression was not an independent prognostic factor. We demonstrated that the group of patients with CD133$^+$ colon cancer showed better overall survival (OS) if they received adjuvant therapy compared to patients without adjuvant therapy by multivariate analysis ($p < 0.0001$, HR 0.125, 95% CI 0.052–0.299). On the other hand, the patients with CD133$^-$ tumors did not show any difference in OS between the two groups ($P = 0.055$, HR 0.500, 95% CI 0.247–1.015), suggesting that CD133$^+$ tumor cells may have effect on chemotherapeutic response [16]. Noteworthy, this finding asks for further elucidation of the functional role of CD133.

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CSCs escape various toxic stimuli and apoptosis caused by cancer therapies [18] and there are numerous speculations on the specific signaling pathways utilized by CSCs to evade such effects. Several possible etiological mechanisms have been delineated so far, which include elevated expression of ATP binding cassette (ABC) transporters such as MDR1 [19] and ABCG2 [20], high expression of aldehyde dehydrogenase [21], expression of anti-apoptotic proteins such as survivin [22]. Since the discovery of AKT as a target of phosphoinositide 3-kinase almost two decades ago and the subsequent evidence indicating the increased cell survival upon the upregulation of AKT, there has been large interests to inhibit this pathway to treat cancer [23]. The activation of AKT survival pathway was shown to confer chemoresistance to $CD133^+$ CSCs in hepatocellular carcinoma and neuroblastoma [24,25]. The chemoresistance of $CD133^+$ cells was previously attributed to the high expression of specific ATP-binding cassette drug transporters such as ABCG2 and MDR1 [26,27]. Importantly, it has been recently verified that AKT activation upregulates the expression of these drug efflux pumps [28,29]. Survivin, a member of the inhibitor of apoptosis proteins (IAP) has been described as a biomarker for chemoresistance in various cancers. The recent studies explain the role of survivin in drug resistance of cancer and propose targeted therapy for survivin for effective chemotherapy [30]. However, the relation of survivin expression to CD133 in colon cancer is not clear yet.

To validate the role of CD133 as a stem cell marker and to find the relation with chemoresistance of colorectal cancer, we comparatively evaluated the its effect on the downstream and biological characteristics of $CD133^+$ cells and siRNA-induced $CD133^-$ cells, derived from a single colon cancer cell line.

2. Materials and methods

2.1. Cell lines and culture

The human colon adenocarcinoma cell lines were obtained from Korean Cell Line Bank (Seoul, South Korea). Caco-2 cells were cultured in MEM/EBSS (Hyclone, Logan, Utah), other cell lines (SW1116, SW480, SNU-C4, DLD-1, HCT-15, HT-29) were cultured in RPMI-1640 (Hyclone) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), Penicillin 100 U/ml and Streptomycin 100 ug/ml (Gibco) in humidified atmosphere at 37 °C and 5% CO_2.

2.2. Flow cytometry analysis

Trypsinized cells were centrifuged and resuspended in FACS buffer (PBS containing 0.5% FBS and 2 mM EDTA). IgG-PE (Isotype control), CD133/1-PE and CD133/2-PE (Miltenyi Biotec, Bergisch Gladbach, Germany) were added to each conditions with FcR blocking reagent (Miltenyi Biotec) and incubated in dark for 10 min at 4 °C. After washing, cells were resuspended in FACS buffer (PBS (pH7.2)-containing 0.5% FBS and 2 mM EDTA) and analyzed by BD FACSAria III (BD Bioscience, San Jose, CA, USA).

2.3. Inhibition of CD133 expression by siRNA transfection and cell purification

SiRNAs (QIAGEN, Hilden, Germany) targeted against CD133 gene sequence were mixed with 200 μl PBS and 4 μl G-fectin (Genolution, Seoul, Korea) and incubated for 10 min at room temperature. Approximately 8×10^4 Caco-2 cells were plated in six-cluster plates followed by treating them with the transfection mixture as describe above. Cells were harvested at 48, 72 and 96 h post-transfection. The knockdown of CD133 expression was confirmed by real-time PCR, western blot and flow cytometry.

$CD133^+$ and siRNA-induced $CD133^-$ cells were sorted using CD133 Microbead kit (Miltenyi Biotec). CD133 siRNA transfected-Caco-2 cells were labeled with CD133/1 Microbead for 30 min. Subsequently, the cells were stained with CD133/2-PE antibody (Miltenyi Biotec) for 5 min and then magnetically separated with MS column and MACS separator (Miltenyi Biotec) by following the manufacturer's instructions. Isolated cells were analyzed by flow cytometry.

2.4. Colony formation assay

The cells were transfected with control siRNA or CD133 siRNA for 48 h. Trypsinized cells were sorted by CD133 MACS sorter and re-plated into new 6-well plates (1000 cells/well) and media was changed every 3 days. After 10 days, colonies were fixed by methanol for 5 min and visualized by using 1% methylene blue.

2.5. Quantitative RT-PCR for CD133, ABCG2, AKT1, MDR1 and survivin

Total RNA was extracted from $CD133^+$ and siRNA-induced $CD133^-$ cells, separately, using RNeasy plus Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol and QuantiTect Reverse Transcription Kit (QIAGEN) was used for cDNA synthesis from 1 μg of total RNA. Real-time RT-PCR was performed as described elsewhere [17] in 384 well PCR plates containing the Fast SYBR Green Master Mix (Applied Biosystems, California, USA), cDNA template, primers in a final volume of 10 pmole each. Each primer/cDNA set was set up in triplicate. Real-time PCR reactions in a 7900HT Fast Real-Time PCR System (Applied Biosystems) were initiated by heating to 50 °C for 2 min and then to 95 °C for 10 min, followed by 40 cycles of 95 °C (15 s), and 60 °C (60 s). The relative quantification of gene expression was performed via the ΔCt method.

2.6. Western blot for CD133 and survivin

Total proteins were extracted from $CD133^+$ and siRNA-induced $CD133^-$ cells, separately, after lysis in PRO-PREP (iNTRON Biotechnology, Daejeon, South Korea). Sodium dodecyl suphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Equal amount of proteins of each sample was subjected to electrophoresis then transferred to 0.45 um PVDF membranes. After blocking in 5% fat-free milk for an hour, membranes were incubated with anti-CD133/1 (AC133, Miltenyi Biotech, Bergisch Gladbach, Germany) and anti-survivin (Abcam, Cambridge, MA, USA) at 4 °C overnight. Anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as loading control. After incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the immune-complexes were visualized by Luminata Forte western HRP substrate (Millipore, Billerica, MA, USA). Images were obtained using the Biospectrum Imaging System (UVP, Upland, CA, USA) and densitometry was done with Image j 1.45 software (Image Processing and Analysis in Java).

2.7. Cytotoxicity assay

The Caco-2 cells were seeded at a concentration of 8×10^4 cells per well into 6-well plates. Cells were transfected with siRNA against CD133 and incubated for 48 h. Trypsinized cells were sorted by CD133 MACS sorter and re-plated into 96-well microplates. The old media were replaced with new media containing 5-FU

(Sigma–Aldrich, St. Louis, USA) at various concentrations and incubated for 72 h. After 72 h, 10 ul of WST-1 reagent (Roche, Indianapolis, IN, USA) was added to each well and the plates were incubated for 1 h at 37 °C and 5% CO_2. The absorbance of the samples was measured using ELISA reader at a 450 nm wavelength.

3. Results

3.1. CD133 expression in various colon cancer cell lines

To determine the most suitable colon cancer cell line for CD133 functional analysis, we evaluated CD133 expression level in 7 primary colon cancer cell lines (Caco-2, HT-29, DLD-1, HCT-15, SNU-C4, SW1116, SW480) using RT-PCR, western blot and flow cytometry. CD133 mRNA and protein were only expressed in Caco-2 and HT-29 cell lines, and not in DLD-1, HCT-15, SNU-C4, SW1116 and SW480 (Fig. 1A and B). Especially, Caco-2 cell line was preferred for CD133 knock-down using siRNA due to robust CD133 expression in Caco-2 than HT-29 cell line by flow cytometry analysis (Fig. 1C).

3.2. CD133 knockdown induces increase of survivin expression but does not alter ABCG2, AKT1 and MDR1 expression in colon cancer cells

We achieved successful inhibition of CD133 expression in Caco-2 cells using four different CD133 siRNA target sequences. Forty eight hours after siRNA transfection, the expression of CD133 mRNA was quantified by QRT-PCR. All four siRNA sequences resulted in significant inhibition of CD133 expression (Fig. 2), but only one of them was used for further transfection analysis. The mRNA and protein expression level of CD133 drastically dropped at 48 and 72 h after CD133 siRNA transfection compared to control siRNA group. In colony formation assay, $CD133^-$ cells showed much fewer numbers of colonies than that of $CD133^+$ cells, suggesting that CD133 may have a role in the stemness of colon cancer cells (data not shown).

To determine the effect of CD133 knockdown on the expression of genes involved in the acquisition of chemoresistance, such as ABCG2, AKT1 and MDR1, CD133 siRNA were transfected for 48 h and then MACS was used to sort $CD133^+$ cells and siRNA-induced $CD133^-$ cells. The result of flow cytometry analysis after sorting was presented in Fig. 3A. SiRNA-induced $CD133^-$ group composed of 77.2% of $CD133^-$ cell but still contained 22.8% of $CD133^+$ cells while CD133 + group composed of 72.6% of $CD133^+$ cells and 27.4% of $CD133^-$ cells. The sequential changes in the level of transcribed mRNA of ABCG2, AKT1 and MDR1 were not epoch-making in relation with CD133 (Fig. 3B). After 5-FU treatment, both of $CD133^+$ and siRNA-induced $CD133^-$ cells induced notable increase of mRNA expression of these genes, but western blot failed to demonstrate the increased protein expression of all these genes (Data not shown). However, the expression of survivin at mRNA and protein level were significantly increased in $CD133^+$ cells at 96 h after siRNA transfection (Fig. 3B and C).

As seen in Fig. 4, WST-1 assay showed 5-FU treatment significantly reduced tumor cells regardless of CD133 48 h after siRNA transfection (57.42% for mock, 55.86% for ctrl siRNA, 59.2% for siRNA-induced $CD133^-$ cells, 61.25% for $CD133^+$ cells). However, 96 h after siRNA transfection, 5-FU reduced tumor cells 47.85% for mock, 48.63% for ctrl siRNA, 71.25% for siRNA-induced $CD133^-$ cells, 64.37% for $CD133^+$ cells. It indicates $CD133^+$ cells have more chemoresistance than siRNA-induced $CD133^-$ cells. Observing the result of expression analysis, the chemoresistance of $CD133^+$ cells may be related with survivin expression.

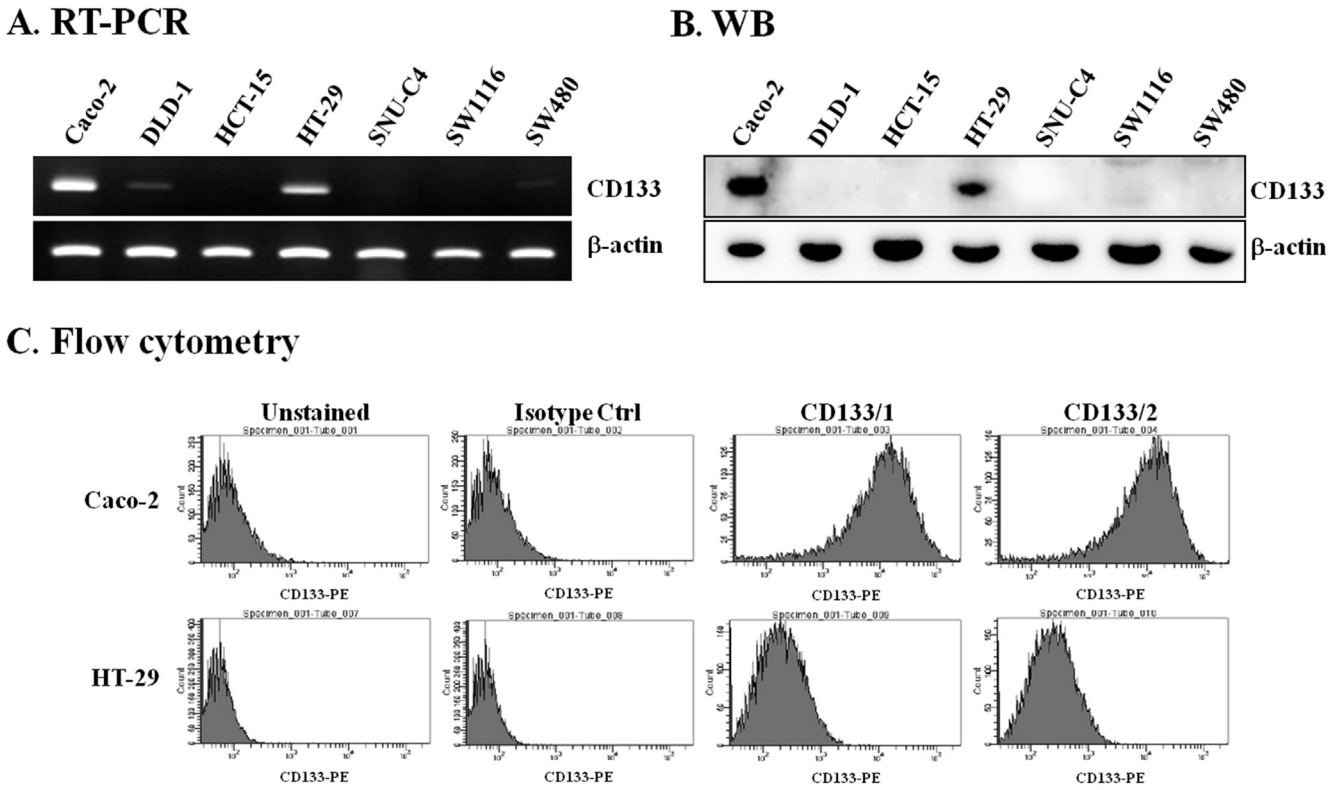

Fig. 1. CD133 expression was detected in Caco-2 and HT29 colon cancer cell lines at both mRNA (A) and protein level (B) among 7 colon cancer cell lines. DLD-1 cell line only demonstrates weak mRNA expression without detectable protein expression. Flow cytometry analysis demonstrated that CD133 expression was much more and stronger in Caco-2 cell line compared to HT-29 cell line (C).

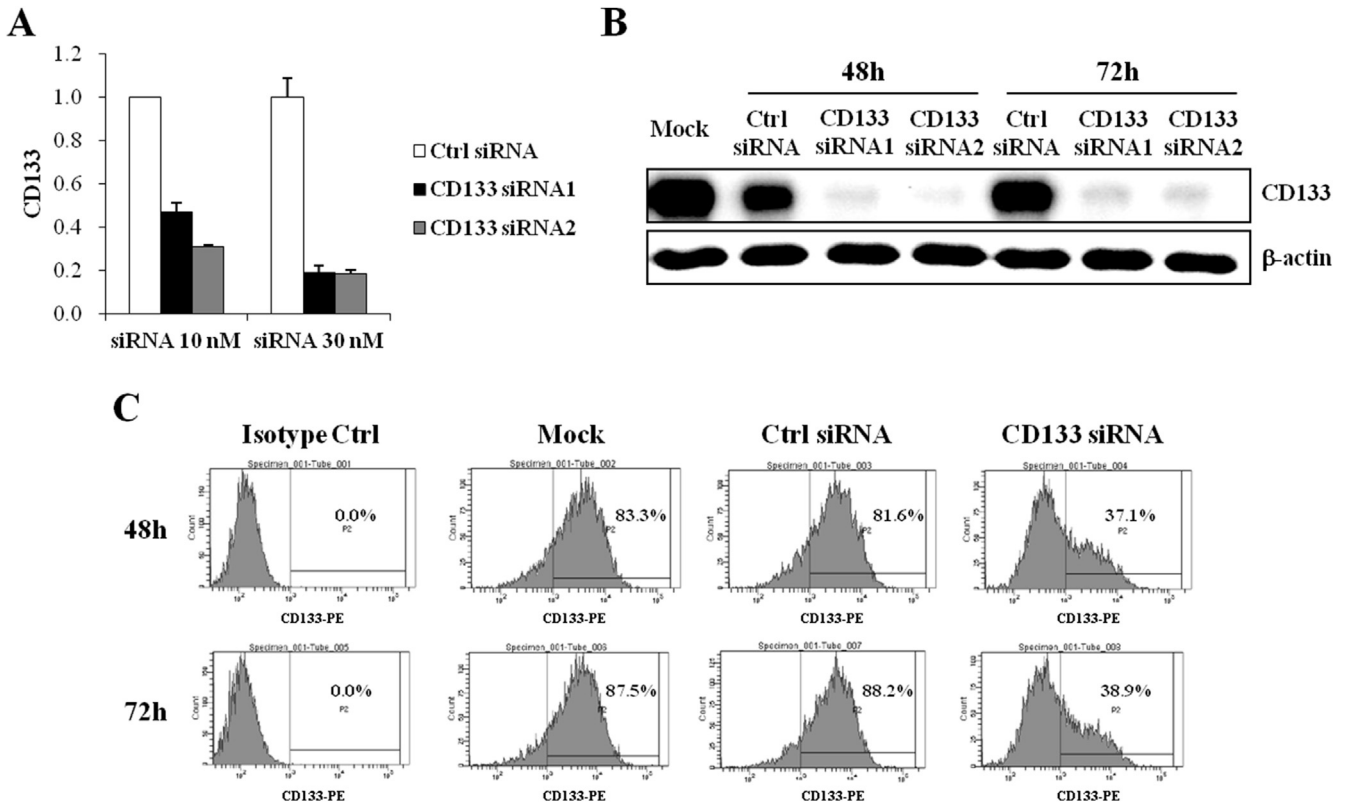

Fig. 2. QRT-PCR revealed that 30 nM of siRNA was optimum for inhibition of CD133 mRNA expression in Caco-2 cells (A). CD133 siRNA-transfected Caco-2 cells were collected at the 48 and 72 h and then the loss of CD133 expression at protein level were analyzed by western blot (B). The flow cytometry analysis demonstrated the changes of $CD133^+$ population after siRNA transfection at the 48 and 72 h (C).

4. Discussion

CSCs expand their colonies through asymmetric cell division, the result of which is two daughter cell populations, one being similar to the mother cells, retaining stem cell properties, while the other one is committed to undergo a specified differentiation. These characteristics of CSC are responsible for tumor heterogeneity which makes the expression of CD133 varies widely within cancer cell lines or a single tumor. Therefore, to reduce the bias of tumor heterogeneity, we designed siRNA transfection in a single cell line and then comparatively analyzed the biologic characteristics of $CD133^+$ and siRNA-induced $CD133^-$ cells. Hongo et al. reported that $CD133^+$ cells can produce $CD133^-$ but not vice versa, which indicates that CD133 is responsible for stemness of cells. In this study, colony formation assay revealed the difference of colony forming ability between sorted siRNA-induced $CD133^-$ cells and $CD133^+$ cells. The result of WST-1 assay without 5-FU treatment which showed higher proliferation activity of $CD133^+$ cells compared with siRNA-induced $CD133^-$ cells (Fig. 4) concordant with the result of colony formation assay. They may support the role of CD133 as a stem cell marker, considering its effect on self-renewal capability. As seen in Figs. 2 and 3, most of $CD133^+$ cells were converted to $CD133^-$ after siRNA transfection, yet some cells still remained to be $CD133^+$, which would explain why there were still many colonies in the siRNA-induced $CD133^-$ cells even after a marked decrease in CD133 expression. CD133 expression was found in Caco-2 and HT-29 among 7 primary colon cancer cell lines examined in this study, however, the remaining $CD133^-$ cell lines might possess stemness and may express a different stem cell marker which supports the report claiming the insufficiency of using only CD133 as a cancer stem cell marker. Aldehyde dehydrogenase 1 (ALDH1) which oxidizes intracellular aldehydes has been described as another potential colon cancer stem cell marker. Lin et al. reported that using both ALDH1 and CD133 is better than using ALDH or CD133 alone to detect colon cancer stem cell [31]. To acquire more comprehensive understanding regarding the role of cancer stem cell marker, we will use both ALDH and CD133 in our future studies.

Hongo et al. also described that $CD133^-$ colon cancer cells from Lovo cell line were more resistant to 5-FU than $CD133^+$ cells [16]. We found 5-FU chemotherapy induce increase in the level of mRNA expression of MDR1, ABCG2 and AKT1 in both siRNA-induced $CD133^-$ cells and $CD133^+$ cells. Multidrug resistance protein 1 (MDR1) is known to play a crucial role in drug disposition as well as distribution. Also MDR1 was reported to have a connection to ABC transporter gene expression. Last but not least, the evidence of cross-reactivity of CD133 and MDR1 in glioblastoma has been illustrated [19]. In this study, we found a marked increase in MDR1 protein expression at 48 h after 5-FU treatment both in $CD133^+$ cells and siRNA-induced $CD133^-$ cells which indicates the expression of MDR1 is not related with CD133. ABCG2 protein, also referred as breast cancer resistance protein (BCRP), has been contended to be associated with chemoresistance through its role in the cellular efflux of biological molecules [32]. In vitro studies indicate that ABCG2 could potentially cause multidrug resistance in several malignancies via actively exporting various drugs across the plasma membrane. Moreover, it was also shown that elevated expression and the activity of ABCG2 are linked to cancer stem-like phenotypes [33]. However, the increase of ABCG2 expression at protein level after 5-FU treatment was not found in this study. The serine/threonine kinase AKT was also described to positively regulate ABCG2, thereby holds a critical position in multidrug resistance. In this study, sequential changes of mRNA expression suggests their expressions are related with each other. However, 5-FU treatment did not induce increase of protein expression of all three genes regardless of CD133.

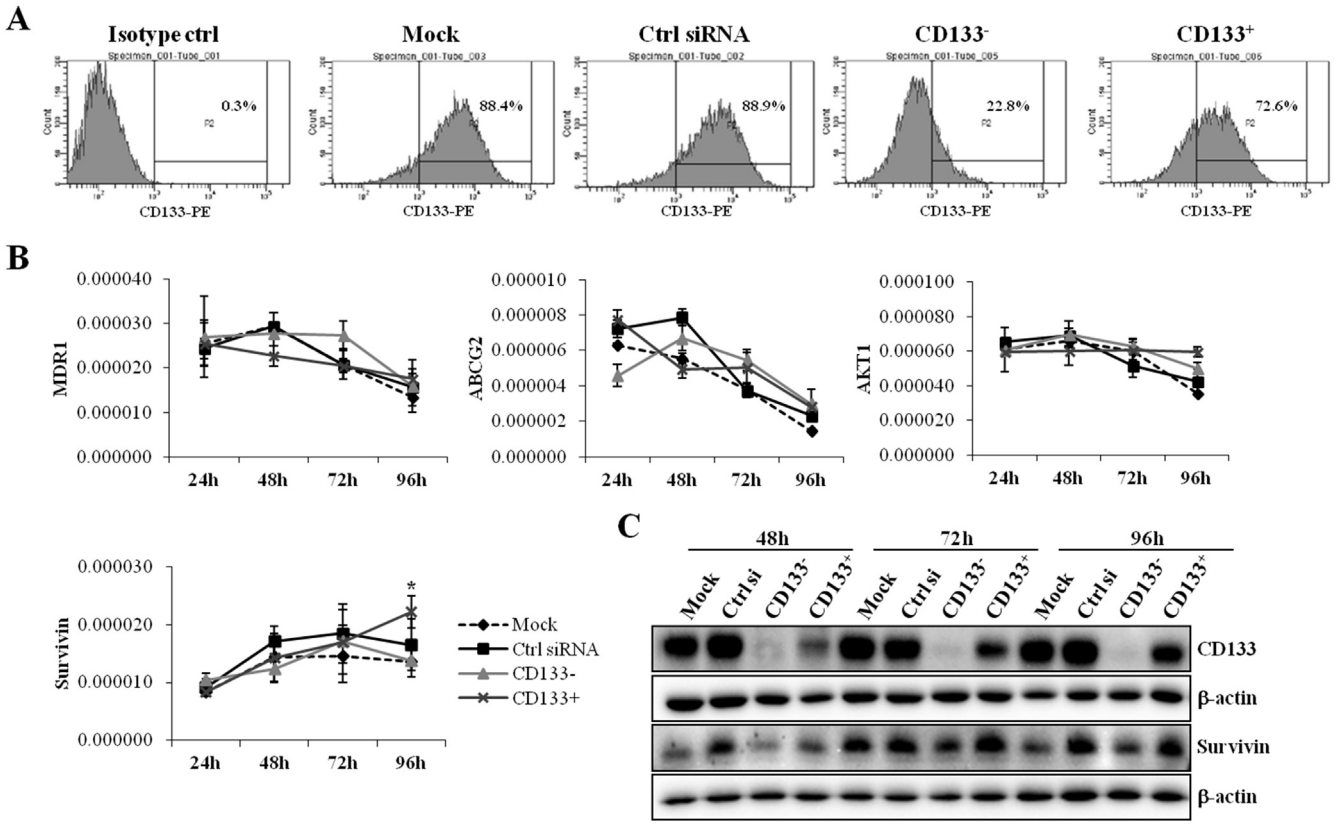

Fig. 3. Flow cytometry analysis was performed to evaluate the population of $CD133^-$ and $CD133^+$ cells in each group sorted by MACS separator. The siRNA-induced $CD133^-$ group composed of 22.8% of $CD133^+$ cells incontrast to 72.6% in $CD133^+$ group (A). Sequencial changes of the level of mRNA transcription by QRT-PCR revealed no significance difference in β-catenin, survivin, ABCG2, AKT1 and MDR1 inregard to the CD133 expression. However, the survivin expression is significantly increased in $CD133^+$ cells at 96 h ($p = 0.0206$) (B). The western blot also demonstrated the increase of survivin expression in $CD133^+$ cells (C).

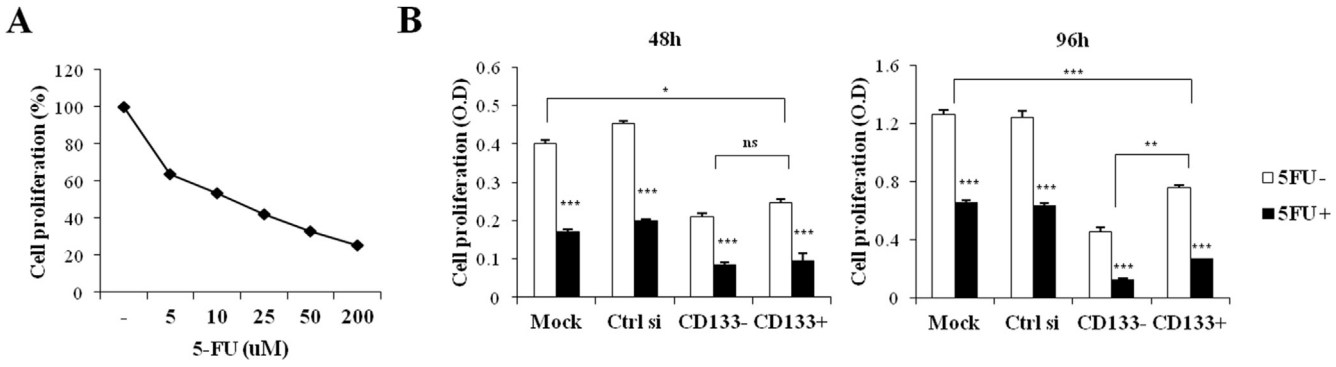

Fig. 4. In WST-1 assay, Caco-2 colon cancer cells were treated with 5-FU in a dose-dependent manner (0–200 uM) for 72 h, and the approximate IC50 value was found to be 10 uM (A). The chemoresponse of $CD133^+$ cell to 5-FU treatment was not different from that of siRNA-induced $CD133^-$ at 48 h after siRNA transfection, but 96 h after siRNA transfection, it showed significant difference in the chemoresistance to 5-FU between $CD133^+$ cells and siRNA-induced $CD133^-$ cells (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$) (B).

On the other hand, both mRNA and protein expression level of survivin were significantly higher in $CD133^+$ cells at 48 h and 96 h compared to siRNA-induced $CD133^-$ in this study. Survivin is widely expressed in the tumor and promotes cell survival by suppression of apoptosis and regulation of cell division. Interestingly, $CD133^+$ cells exhibited noticeably higher cell survival at 96 h after siRNA transfection in WST-1 assay, which may be related with the increase of survivin expression. We found that the increase of survivin expression in Mock and Ctrl siRNA group was a little bit less than sorted $CD133^+$ cells. It may be related with sorting which can have an additional effect on survivin expression of $CD133^+$ cells.

In conclusion, we deduce that CD133 is a putative stem cell marker and may have an effect on chemoresistance to 5-FU in colon cancer through the mechanism related with survivin expression, instead of MDR1, ABCG2 and AKT1. The effect of survivin inhibitor on cancer stem cell biology will be the next candidate for future study.

Conflict of interest

None.

Acknowledgments

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Transparency document

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