

CD44-shRNA recombinant adenovirus inhibits cell proliferation, invasion, and migration, and promotes apoptosis in HCT116 colon cancer cells

SOO YOUNG LEE¹, KYUNG AE KIM¹, CHANG HYUN KIM¹,
YOUNG JIN KIM¹, JAE-HYUK LEE² and HYEONG ROK KIM¹

¹Division of Colorectal Surgery, Department of Surgery and ²Department of Pathology,
Chonnam National University Medical School and Hwasun Hospital,
Hwasun-gun, Jeonnam 58128, Republic of Korea

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Abstract. The cell-surface glycoprotein CD44 is closely associated with cell proliferation, tumor invasion, and metastasis. Previous studies have reported that knockdown of CD44 with short hairpin RNA (shRNA) reduced cell proliferation and migration, and induced apoptosis. However, more efficient means of delivering small interference RNA are still necessary. We developed an *in vitro* model of CD44-shRNA recombinant adenovirus (Ad-CD44-shRNA) and evaluated its ability to alter tumor invasion, migration, and apoptosis in human colon cancer cells. An shRNA against CD44 was used for knockdown of CD44 expression, and recombinant adenovirus was constructed using AD293 cells. The Ad-CD44-shRNA-treated HCT116 colon cancer cells showed a significant decrease in cell proliferation, migration, and invasion, while apoptosis was increased. The Ad-CD44-shRNA also decreased the phosphorylation of Akt and GSK-3 β . The levels of Bcl-2 and Bcl-xL expression were downregulated, whereas the expression levels of Bax, cleaved caspase-3 and -9, and PARP were increased in Ad-CD44-shRNA-treated colon cancer cells. These results support the feasibility of an adenovirus-mediated RNA interference therapy targeting human colon cancer via the CD44 as a potential future therapeutic intervention.

Introduction

Colorectal cancer is one of the most common malignancies causing mortality in the world (1). However, current knowledge of molecular carcinogenesis in the development of colorectal

cancer is still limited. Although significant advances have been achieved, more effective therapeutic options for advanced colorectal cancer are still needed, and many efforts have been made to develop novel treatments for targeting tumor-specific genes.

Emerging evidence suggests that specific sub-populations of cancer cells with stem cell characteristics within the bulk of tumors are implicated in the pathogenesis of heterogeneous malignant tumors (2-4). To study the behavior of cancer stem cells (CSCs), markers for prospective isolation of CSCs are crucial. CD44 has been proposed as one of the CSC markers of colorectal cancer (2,4,5). CD44 is a transmembrane glycoprotein acting as a cell adhesion molecule through the binding to hyaluronic acid, and plays a key role in remodeling and degradation of hyaluronic acid (4). Furthermore, CD44 is involved in fundamental aspects of cancer cell biology such as tumor stem cell phenotype, cell adhesion, invasion, and metastasis (6). Several studies have shown that CD44 expression was associated with tumor progression, metastasis, and poor prognosis (7-10).

Recent studies have reported that knockdown of CD44 resulted in the inhibition of tumor growth and metastasis (11-13). In our previous studies, we have shown that CD44 enhanced the epithelial-mesenchymal transition, which is associated with colon cancer invasion (14), and that knockdown of CD44 expression using inducible short hairpin RNA (shRNA) significantly reduced cell proliferation, invasion, and migration (13). The therapeutic effect of RNA interference depends on the stability and tissue specificity of small interference RNA (siRNA) and the efficiency of siRNA transduction. We have previously used plasmids to suppress CD44 expression (13); however, the efficiency of plasmid delivery remains poor. Therefore, more efficient means of delivering therapeutic siRNA are necessary. One promising therapeutic modality is the use of oncolytic viruses, which have cancer specificity and also act as a vector for stable introduction of siRNA (15,16).

Here, we developed an *in vitro* model using CD44-shRNA recombinant adenovirus, and evaluated the impact of CD44 knockdown adenovirus on proliferation, invasion, migration, and apoptosis of colon cancer cells.

Correspondence to: Dr Hyeong Rok Kim, Department of Surgery, Chonnam National University Medical School and Hwasun Hospital, 322 Seoyang-ro, Hwasun-eup, Hwasun-gun, Jeonnam 58128, Republic of Korea
E-mail: drkhr@chonnam.ac.kr

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Materials and methods

Cell culture. The HCT116 human colon cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were routinely maintained in complete medium (DMEM; Lonza, Walkersville, MN, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaille, France), 50 U/ml penicillin, and 50 µg/ml streptomycin (Lonza) at 37°C in a humidified incubator with 5% CO₂.

Construction of shRNA-CD44 plasmid. The shRNA with vector was purchased from OriGene (OriGene Technologies, Inc., Rockville, MD, USA). The shRNA-CD44 (sense: GACAG AAAGCCAAGTGGACTCAACGGAGA) and pGFP-V-RS vectors, the latter of which contained an ineffective shRNA cassette against GFP, were used for knockdown of CD44 expression.

Construction of recombinant adenovirus. The human shRNA targeting the CD44 sequence and a negative control scrambled sequence were each amplified by polymerase chain reaction (PCR) from plasmids containing the 29-mer shRNA construct using primers containing *KpnI* and *XbaI* restriction sites (Enzymomics, Daejeon, Korea). Purified (Qiagen, Valencia, CA, USA) PCR products and adenovirus shuttle plasmids (Agilent Technologies, Palo Alto, CA, USA) were digested with *KpnI* and *XbaI*, ligated with T4 DNA ligase (Promega, Madison, WI, USA), and then transformed into DH5a chemically competent *E. coli*. Miniprep DNA of the different clones was analyzed on a 0.8% agarose gel and the correct clones were confirmed by DNA sequencing.

Clones carrying the correct target sequences were selected, linearized with *PmeI*, subcloned into the pAdEasy-1 backbone, and transformed into BJ5183 bacteria. Recombinant adenoviral plasmids were selected against kanamycin and screened by diagnostic digestions. These plasmids were then digested by *PacI*, and the larger fragments were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) into AD293 cells (Stratagene, La Jolla, CA, USA). After 11-13 days, the recombinant adenoviruses were collected after freeze-thaw lysis of the AD293 cells. The primary viral stock was used to infect new AD293 cells for 2-3 days to produce a bulk viral stock. Infected cells exhibiting cytopathic effects were lysed for collection of the virus.

Viral particles were purified and concentrated using the Vivapure AdenoPACK™ 20 RT (Sartorius Stedim Biotech, Göttingen, Germany) kit. The viral particle concentration was determined by measuring absorbance at 260 nm, and a standard TCID₅₀ (50% tissue culture infective dose) assay was performed on AD293 cells to determine the infectious virus titer (17). Purified viral particles were stored at -70°C until use.

Adenoviral infection. The HCT116 colon cancer cells were seeded onto a 6-well plate at a density of 0.1x10⁶ cells/ml, cultured overnight, and infected with serially diluted concentrations of recombinant adenovirus. After a 24-h incubation, the previous growth medium was removed and fresh complete growth medium was added, and treated with adenoviral aliquots with or without shRNA-CD44.

Table I. Primers for RT-PCR.

Protein	Primers	Sequences
CD44	Forward	5'-GAA TAT AAC CTG CCG CTT TG-3'
	Reverse	5'-CTG AAG TGC TGC TCC TTT CAC-3'
GAPDH	Forward	5'-ACC ACA GTC CAT GCC ATC AC-3'
	Reverse	5'-TCC ACC ACC CTG TTG CTG TA-3'

Reverse transcription PCR (RT-PCR) analysis. Total RNA was isolated from cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The quantity and purity of total RNA were determined by measuring absorbance at 260 and 280 nm using the Nanodrop ND-1000 spectrophotometer (BCM, Houston, TX, USA). Next, cDNA was synthesized from 3 µg total RNA using Oligo(dT) (Promega) and reverse transcriptase (Beams Biotech, Seongnam, Korea). PCR amplification of cDNA was performed using gene-specific primers (Table I) and nTaq DNA polymerase (Enzymomics, Daejeon, Korea). PCR products were separated on a 1% agarose gel, visualized and photographed under UV light.

Western blotting. Total cell extracts were lysed in cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) with a protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentrations were determined by a BCA protein assay (Thermo Fisher Scientific, Rockford, IL, USA). The protein was separated by 10% SDS-PAGE and transferred onto a PVDF (polyvinylidene fluoride) membrane (Millipore, Billerica, MA, USA). The membranes were incubated for 1 h in blocking solution [5% skim milk in TBS with 0.1% Tween-20 (TBST)] and sequentially blotted with the following primary antibodies: anti-CD44 (R&D Systems, Minneapolis, MN, USA), anti-GAPDH (Aviva Systems Biology, San Diego, CA, USA), anti-AKT, anti-phospho-AKT (Ser/Thr), anti-phospho-GSK-3β, anti-β-catenin, anti-Bax, anti-Bcl-2, anti-Bcl-xL, anti-caspase-3, anti-phospho-caspase-3, anti-cleaved-caspase-3, anti-caspase-9, anti-phospho-caspase-9, anti-cleaved-caspase-9, anti-PARP poly(ADP-ribose) polymerase, and anti-cleaved-PARP (Cell Signaling Technology) at 4°C overnight. After rinsing in TBST (0.1%), membranes were incubated with horseradish peroxidase-labeled anti-rabbit (Thermo Fisher Scientific) or anti-mouse IgG secondary antibodies (Cell Signaling Technology) at room temperature for 1 h. The blot was detected by ECL (enhanced chemiluminescence) of an HRP substrate (Millipore) on an image reader (Ras4000, Fujifilm, Tokyo, Japan).

Cell viability assay. The viability of treated cells was measured using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Cells were plated into 96-well plate at 5,000 cells/well one day prior to the viral transduction. Then, cells were infected with the recombinant adenoviruses for 1 day, followed by medium replacement. Cell growth and viability were assayed 4-5 days post-infection. For cell viability assay, the cells are incubated with reagents from the CCK-8 kit for 1 h and the absorbance was measured at 450 nm in a microplate reader.

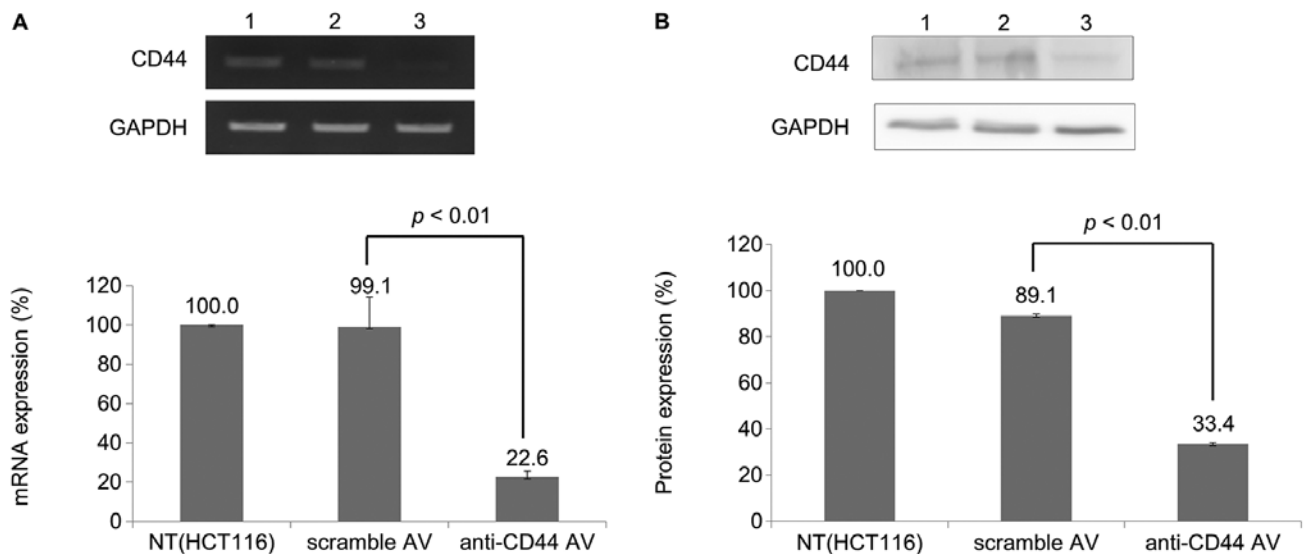


Figure 1. CD44 expression detected by (A) RT-PCR and (B) western blotting, with GAPDH as an internal control. Lane 1, non-treated WT cells (HCT116). Lane 2, cells infected with scramble Ad. Lane 3, cells infected with Ad-CD44-shRNA. Bands were analyzed and quantified by densitometry and the CD44/GAPDH ratio was evaluated.

(BioTek, Winooski, VT, USA). Each sample was assayed in triplicate, and each experiment was repeated at least twice.

Flow cytometry analysis. Apoptosis was quantified using flow cytometry after being stained with APC (allophycocyanin)-labeled Annexin V and 7-amino-dactinomycin (BD Biosciences, San Diego, CA, USA). We analyzed for intact cells (Annexin V/7AAD-double-negative), early apoptotic cells (Annexin V-positive), and late apoptotic cells or necrotic cells (Annexin V/7AAD-double-positive). The cells were plated in 6-well plates at of 200,000 cells/well prior to infected with the recombinant adenoviruses for 4-5 days. Both uninfected and infected HCT116 cells were trypsinized, washed twice with cold PBS, and resuspended in 1X binding buffer (BD Biosciences). Analysis of 400 μ l of this cell resuspension was performed on a fluorescence active cell sorting (FACS) Calibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) using the CellQuest version 3.3 software (Becton-Dickinson).

Cell migration assay. Cells were cultured in 6-well plates and infected with the recombinant adenoviruses for 24-48 h. The infected HCT116 cells were then seeded in culture-inserts (2x0.22 cm²; IBIDI GmbH, Martinsried, Germany) at 5x10⁴ cells/well. To create a cell-free gap, Culture-Inserts were gently removed using sterile tweezers after a 24-h incubation. The progress of cell migration into the cell-free gap was photographed at 0, 20, and 40 h using an inverted microscope. The distance between gaps was measured using the Focus Lite Ver 2.90 (Focus, Daejeon, Korea) software after three random sites were photographed.

Cell invasion assay. Cell invasion assays were carried out using 24-well Transwell filters with 8- μ M pores (Corning Inc., NY, USA). Transwell filters were coated with 500 μ g Matrigel/DMEM for 3-4 h and unbound material was aspirated at room temperature. Cells infected with the recombinant adenoviruses were resuspended at a density of 2.5x10⁵ cells in 120 μ l

0.2% BSA medium and then seeded into the upper chamber. Then 400 μ l of 0.2% BSA medium containing 50 μ g/ml human plasma fibronectin (Calbiochem, La Jolla, CA, USA) as a chemoattractant was loaded into the lower chamber. After a 24-h incubation, invaded cells on the bottom surface of the Transwell were stained with Diff-Quick solution (Sysmex, Kobe, Japan) and quantified in five selected fields (1 mm² each) using a hemacytometer under a light microscope.

Soft-agar colony formation assay. Soft agar assays were constructed in 6-well plates. The foundation layer of each well consisted of 1.5 ml of 0.6% agar solution in 1X media. The HCT116 cells were transduced (1.5x10⁴ cells/well) for 1 day, and then mixed with 0.6% soft agar (1:1) and seeded onto the bottom. An additional 3 ml of 1X media without agarose was poured on top of the growth layer. After a 2-week incubation, the colonies were stained with 0.05% crystal violet and photographed using an inverted microscope camera. The number of colonies was counted at x40 magnification.

Statistical analysis. All statistical analyses were performed using a t-test with SPSS 21.0 (IBM Inc., Armonk, NY, USA) software.

Results

Expression of CD44. The level of expression of CD44 mRNA was evaluated by RT-PCR; GAPDH served as an internal control. As shown in Fig. 1A, the level of expression of CD44 mRNA in cells infected with Ad-CD44-shRNA was significantly downregulated compared with parental (HCT116) and scramble-Ad-infected cells ($p < 0.01$). A significant reduction in CD44 protein was also detected in Ad-CD44-shRNA-infected cells compared with scramble-Ad-infected cells ($p < 0.01$).

Cell viability. The results of the cell viability assay are shown in Fig. 2. Whereas the scramble-Ad showed little cytotoxicity,

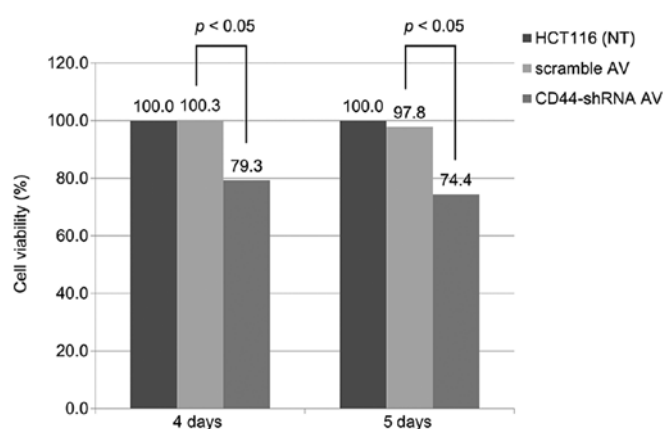


Figure 2. Cell viability was determined using CCK-8 in 4-5 days after infection with scramble Ad or Ad-CD44-shRNA.

Ad-CD44-shRNA suppressed cell viability ($p < 0.05$) 4-5 days post-infection.

Flow cytometry analysis. The early (7.80%) and late (14.65%) apoptotic rate of Ad-CD44-shRNA-infected cells was increased compared with parental (2.58 and 6.25%) and scramble Ad-infected (3.15 and 7.83%) cells ($p < 0.01$) (Fig. 3).

Cell migration assay. The Ad-CD44-shRNA-infected cells showed much lower migratory capacity than scramble-Ad-infected cells at 40 h after plating ($p < 0.01$) (Fig. 4).

Cell invasion assay. The invasion activity of Ad-CD44-shRNA-infected cells was significantly decreased compared with scramble-Ad-infected cells (51.8 vs. 94.3, $p < 0.01$) (Fig. 5).

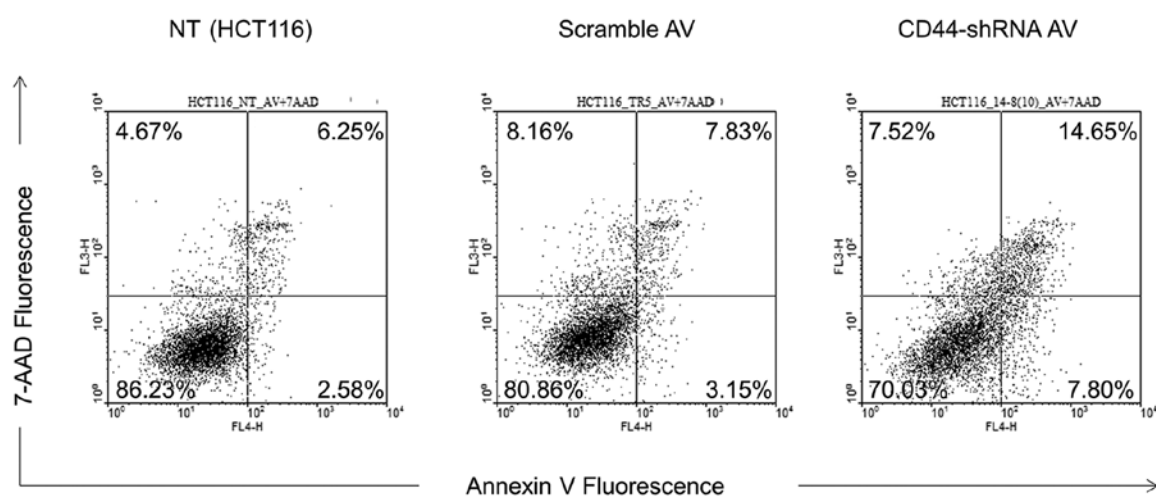


Figure 3. Apoptosis was investigated using flow cytometric analysis at 72 h after infection with scramble Ad or Ad-CD44-shRNA.

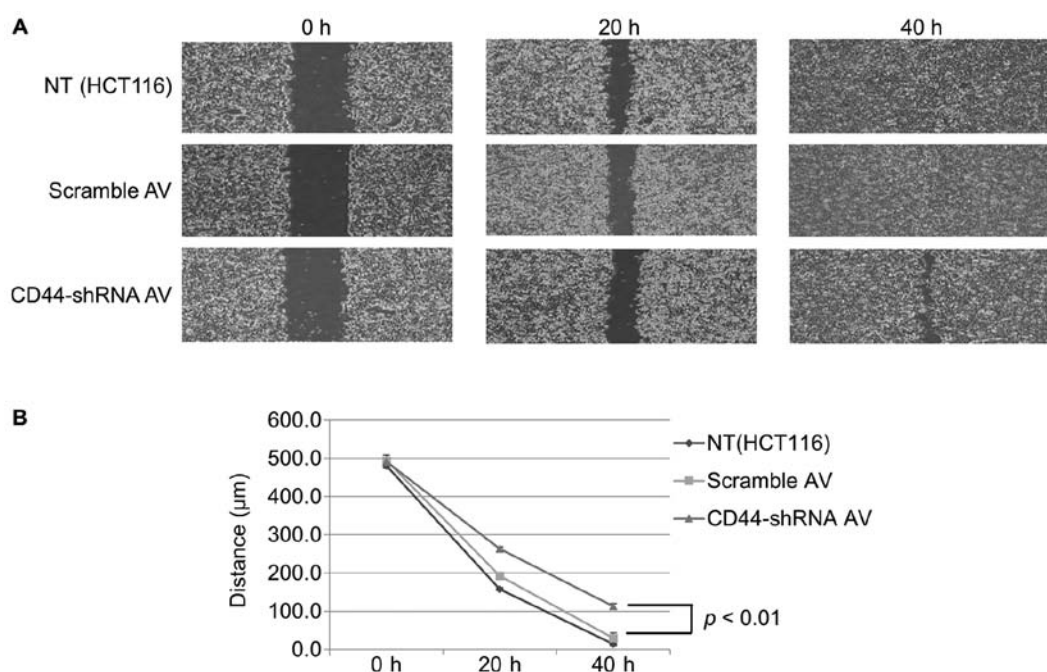


Figure 4. The effect of CD44-shRNA recombinant adenovirus treatment on cell migration. (A) Cells were photographed at 0, 20, and 40 h after detachment of insert from the plate. (B) Graphs of cell migration are displayed as relative healing distance.

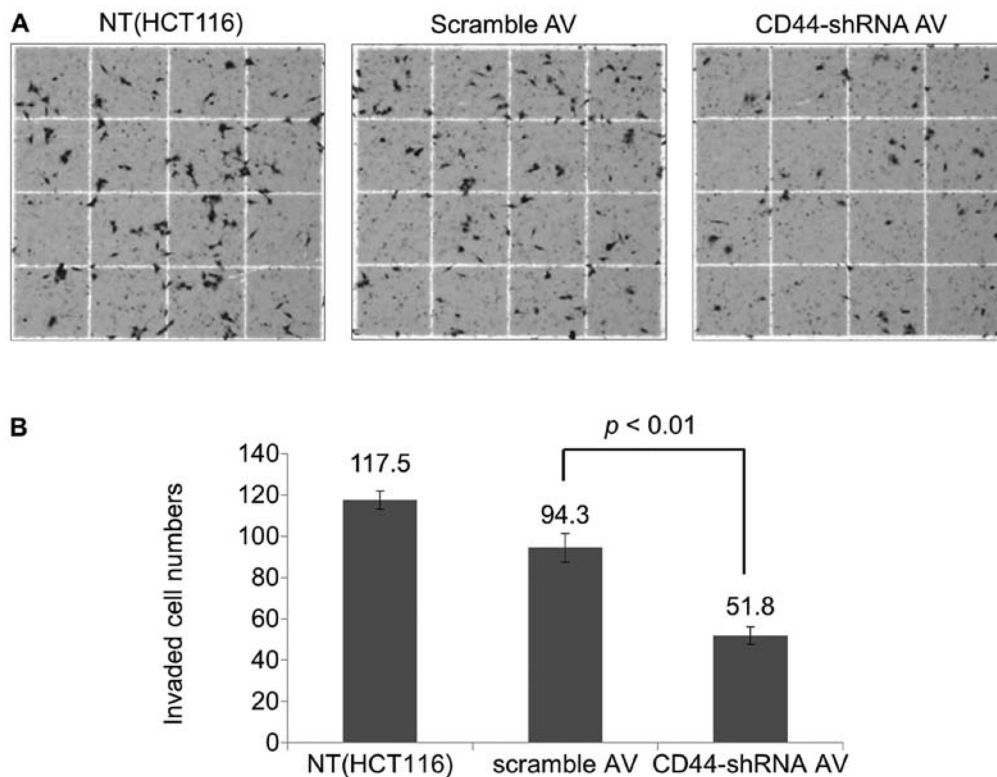


Figure 5. Effects of CD44-shRNA adenoviral vector on invasion of HCT116 cells *in vitro*. (A) The invasiveness of colon cancer cells were assessed using a Transwell invasion assay. The transmembrane cells were stained with Diff-Quick solution. (B) Number of invaded cells was counted at 24 h.

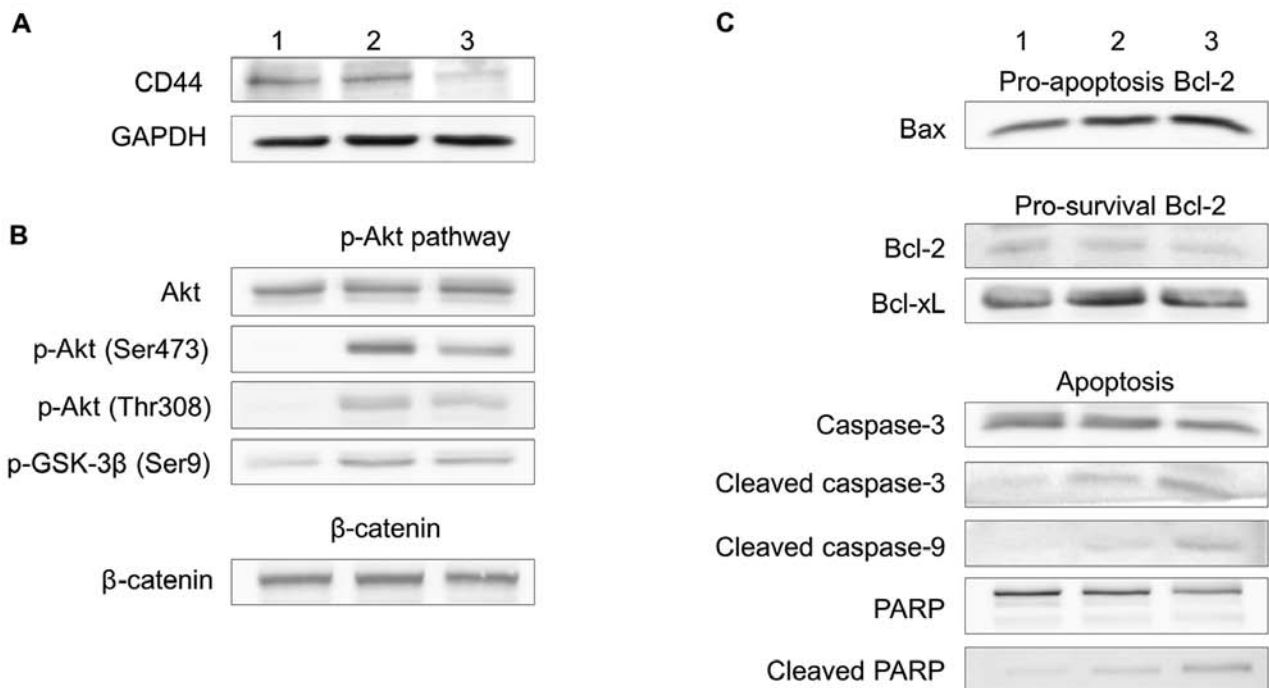


Figure 6. Western blot analysis of (A) CD44 and GAPDH, (B) PI3-Akt signaling, and (C) apoptosis. Apoptosis and survival-related proteins were detected at 72 h after infection with recombinant adenoviral vectors. Lane 1, non-treated WT cells (HCT116). Lane 2, cells infected with scramble Ad. Lane 3, cells infected with Ad-CD44-shRNA.

PI3-Akt signaling and apoptosis. Western blot analysis for expression of PI3-Akt signaling and apoptotic molecules is shown in Fig. 6. The Ad-CD44-shRNA resulted in a decrease

in the expression of phospho-Akt and phospho-GSK-3 β (Fig. 6B). In contrast, there was minimal change of β -catenin expression (Fig. 6B). The Ad-CD44-shRNA also resulted

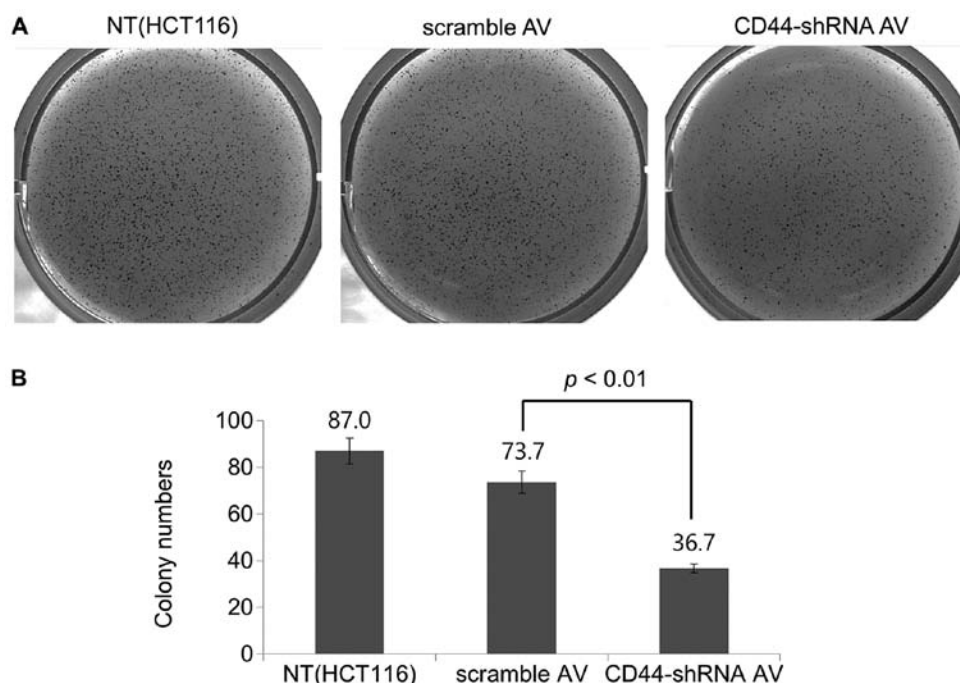


Figure 7. Soft agar colony formation assay of CD44 knockdown. (A) HCT116 cells infected with scramble Ad and Ad-CD44-shRNA was used for the soft agar colony formation assay. Cells were cultured in 3D-agar for 2 weeks, and colonies were stained with crystal violet reagent and photographed. (B) Quantitative analysis of colony numbers is shown in the lower panel.

in a decrease in the expression of Bcl-2 and Bcl-xL, but an increase in the expression of Bax, and promoted the cleavage of caspase-3, -9 and PARP (Fig. 6C).

Soft-agar colony formation assay. The Ad-CD44-shRNA-infected cells showed a marked decrease in colony formation (Fig. 7). When quantified, there was a 50.2% decrease in colony formation units in the presence of Ad-CD44-shRNA compared with scramble-Ad (Fig. 7B), suggesting a significant tumorigenic inhibition of HCT116 colon cancer cells by Ad-CD44-shRNA.

Discussion

In this study, we have constructed a recombinant adenoviral model to reduce the expression of CD44. We showed that Ad-CD44-shRNA inhibited cell proliferation, migration, and invasion in HCT116 colon cancer cells, which supports the feasibility of an adenovirus-mediated RNA interference therapy targeting colon cancer via the CD44 antigen.

Colorectal cancer is the result of genetic alterations that lead to a transformation of normal colonic epithelial cells into cancer cells. Currently, radical surgery followed by adjuvant chemotherapy is recommended to high risk patients for management of colon cancer (18). However, this standard treatment is not ideally effective because of the recurrence of the cancer and toxicity of the chemotherapeutic agents. CSCs are believed to be the reason of resistance to the conventional chemotherapy and radiotherapy that targets the bulk of cancer, leaving the stem cells unaffected (2-4). In the traditional stochastic model, every cancer cell from the bulk tumor has a carcinogenic potential. According to the hierarchical model,

however, only a small proportion of tumor cells are actually cancer stem cells (4). In contrast with the stochastic model, slowly proliferating CSCs displaying multipotency and self-renewal are only responsible for tumor initiation, maintenance, and metastasis (4). These CSCs are hypothesized to be spared from the chemotherapy that interferes with the ability of rapidly growing cells to divide (2).

To identify and isolate CSCs, there have been many efforts to identify specific CSC markers. Well-known CSC markers for colorectal cancer include CD44, CD133, EpCAM, CD24, and CD29 (4). Above all, CD44, a transmembrane glycoprotein functioning as a cell adhesion protein and a signaling receptor (6), is one of the most well-studied CSC surface markers. CD44 enhances the epithelial-mesenchymal transition, which is related to cancer cell migration and invasion (14), and therefore is associated with tumor progression, metastasis, and poor prognosis in colon cancer (9,10,19,20). Importantly, inhibition of these CSC surface markers may result in the inhibition of tumor cell proliferation, invasion, and metastasis (3). We have previously developed a CD44 knockdown model using plasmids for RNA interference and reported that shRNA against CD44 inhibited cell proliferation, invasion, and migration (13). However, as mentioned above, more efficient means of delivering therapeutic siRNA are still needed because of the limited efficiency of delivery via plasmid.

In this study, we successfully constructed a recombinant adenoviral model to knock down CD44 using adenoviruses, which are among the most widely used vectors for gene therapy (21). Oncolytic virotherapy using recombinant adenoviruses has a number of potential advantages. It can be used to specifically target cancer cells while leaving normal tissue stem cells unharmed, thus minimizing systemic toxicity (15,22). There is

also a low possibility of resistance because of the diverse ways it induces oncolysis (22). Above all, therapeutic genes, such as inhibitory RNA against specific oncogenes, can be delivered using recombinant adenoviral vectors (22). Because of the high efficiency of transduction *in vivo*, the adenoviral system has been used for virus-based therapies (15,23).

The result of this study demonstrated that reduced cell proliferation, migration, and invasion, and enhanced apoptosis were likely to be a result of the Ad-CD44-shRNA infection. Also, we showed differential expression of PI3-Akt signaling and apoptotic molecules in colon cancer cells treated with Ad-CD44-shRNA. Tumor proliferation, differentiation, and apoptosis are known to be under the control of several signaling pathways such as the Wnt signaling pathway (24,25). We demonstrated that Ad-CD44-shRNA infection inhibited Akt phosphorylation (Fig. 6B), which is one of the most important Wnt-target genes for the survival of cancer cells (26,27). We also showed the downregulation of GSK-3 β (Fig. 6B), the target of PDK1/Akt signal transduction, inactivates various proteins involved in cell proliferation and survival such as β -catenin, cyclin D1, c-jun, and c-myc (28). β -catenin is a downstream molecule in the Wnt signaling pathway and plays an important role in cell-to-cell adhesion, tumor invasion, and metastasis (25). In addition, we showed decreased expression of Bcl-2 and Bcl-xL; increased expression of Bax; and cleavage of caspase-3, -9, and PARP (Fig. 6C). The Bcl-2 family proteins are key regulators of apoptosis, with a pro-survival subfamily including Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and A1; and a pro-apoptotic subfamily including Bax, Bak, and Bok (29-31). Apoptosis is precipitated by the activation of cysteine proteases of the caspase family, including caspase-3, -8 and -9, and their cleavage is considered the primary hallmark of apoptosis (32,33). The results of our study also demonstrated that Ad-CD44-shRNA infection induces apoptosis in HCT116 colon cancer cells, suggesting reduced clonogenic ability.

We also utilized the soft agar colony formation assay, or 3D culture, as a novel modality to identify the inhibition of tumorigenesis by Ad-CD44-shRNA. Because of the intrinsic difficulties in investigating the tumor progression *in vivo*, the soft agar colony formation assay, which is a close mimicry of the 3D cellular environment *in vivo*, has recently been used (34). With this assay, we assessed the effects of Ad-CD44-shRNA on cell proliferation and migration. The result of the assay provided us with a straightforward and intuitive result, as well as a quantitative assessment of the inhibitory potential of Ad-CD44-shRNA.

Until now, there have been several clinical trials using oncolytic adenoviruses (22); however, regarding colorectal cancer, it is rarely reported (35-38). Although there is an increasing demand for novel therapeutic modalities, such as non-pathogenic viruses in the treatment of colorectal cancer, clinical evidence of oncolytic virotherapy is still lacking (22). Our results support the feasibility of an adenovirus-mediated RNA interference therapy targeting colon cancer via the CD44 antigen, which can be used as a therapeutic intervention with the anti-survival/pro-apoptotic machinery in human colon cancer. This study is also meaningful as a cornerstone to potential future gene therapies using oncolytic adenoviruses against colorectal cancer. Oncolytic adenoviral therapy, despite its limited efficacy as a single agent, has a potential

role in combination therapy with conventional chemotherapy (22). Further translational studies and clinical trials focusing on the administration of cancer virotherapy in combination with conventional chemotherapy are needed.

Acknowledgements

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