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

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

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Abstract

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

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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. A 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-3β. The levels of Bcl-2 and Bcl-xL expression were down-regulated, 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.

Keywords: cancer stem cell, CD44, adenovirus, RNA interference, colon cancer

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목차

표목차

그림목차

Introduction

Cancer is still a major life-threatening disease in the world. Because of population growth and aging, the global burden of cancer is rapidly growing (1). Colorectal cancer is one of the most common malignancies causing mortality worldwide (2). In Korea, colorectal cancer is the fourth greatest cause of cancer death, with its age-standardized incidence rate (34.0 per 100,000) being the third most common (3). And also, colorectal cancer has increased from 1999 to 2013 in Korea (3). 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.

1. Two major pathways in colorectal cancer

Colorectal cancer is believed to be a genetically heterogeneous disease. Most of the development of colorectal cancer can be explained by adenoma-carcinoma sequence (4). According to this basic principle, colorectal cancer is the result of genetic alterations causing ability of limitless proliferation and metastasis. Two different pathways, chromosomal instability pathway and microsatellite instability pathway, have been the commonly accepted view to explain this genetic alterations (4, 5). According to chromosomal instability pathway, accumulation of mutations of *APC*, *K-ras*, and *p53* genes results in the development of colorectal carcinoma. In contrast, mutations of mismatch repair genes or loss of expression of *MLH1* caused by epigenetic silencing are the key concept of microsatellite instability pathway (4, 5). It is important to understand the mechanisms of tumor initiation and progression more thoroughly for the development of novel treatment.

2. Models of colorectal carcinogenesis

There are two different models of tumor development. The first one is “stochastic model” (6). According to this traditional model of tumorigenesis, every cancer cell in a bulk of tumor has the ability of extensive proliferation and growth, which means any cell of the body can be changed into tumor following a series of mutations (6, 7). Heterogeneous tumor mass is explained to be the result of additional genetic and epigenetic changes. In contrast, hierarchical model of colorectal cancer development explains that only specific subset of tumor cells, which are called cancer stem cells (CSCs), have unlimited proliferative potential (7, 8). According to this theory, colorectal cancer is composed of normal cells as well as a rare subpopulation of undifferentiated CSCs having potential of self-renewal and multipotency, which are responsible for tumor initiation, maintenance, and spreading (8). Importantly, these CSCs are believed to be the cause of resistance to anticancer therapies, such as chemotherapy (6, 7). Consequently, the inhibition of CSCs by various ways have been widely studied as a target of novel therapy.

3. Markers of colorectal CSCs

To study the behavior of CSCs, markers for prospective isolation of CSCs are crucial. CD44 has been proposed as one of the CSC markers of colorectal cancer (6, 7, 9). 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 (6). Furthermore, CD44 is involved in fundamental aspects of cancer cell biology such as tumor stem cell phenotype, cell adhesion, invasion, and metastasis (10). Several studies have shown that CD44 expression was associated with tumor progression, metastasis, and poor prognosis (11-14). Another well-known CSC marker is CD133, a cholesterol-interacting pentaspan-transmembrane glycoprotein which belongs to the Prominin family (6). Colorectal cancer subpopulation with CD133+ was described to be enriched in tumor-initiating colon cancer stem cells, which had high tumorigenic potential. Several papers have investigated the prognostic role of CD133 expression, and reported that CD133 was associated with poor prognosis and metastasis (15-19). Some authors insisted that the interaction of CD133+ colorectal cancer cells with carcinoma-associated fibroblasts in tumor microenvironment is the cause of higher tumorigenic potential of CD133+ cells (20, 21). Based on the

results of these previous studies, we can assume that the inhibition of these CSC markers may have the possibility of new anticancer therapy, given that CSC markers play a key role in self-renewal and tumor progression.

4. Knockdown of CD44

Recent studies have reported that knockdown of CD44 resulted in the inhibition of tumor growth and metastasis (22-24). In our previous studies, we have shown that CD44 enhanced the epithelial-mesenchymal transition, which occurred because of the down-regulation of E-cadherin, and up-regulation of N-cadherin, α-actin, vimentin, fibronectin, and MT1-MMT (25). We suggested that CD44 might be an important mediator for invasion of colorectal cancer (25). And also, in another study, we showed that knockdown of CD44 expression using inducible short hairpin RNA (shRNA) significantly reduced cell proliferation, invasion, and migration, which is caused by down-regulation of the Wnt/β-catenin/PDK1/Akt signaling and induction of cell apoptosis (24). 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 (24); however, the efficiency of

plasmid delivery remains poor. Therefore, more efficient means of delivering therapeutic siRNA are necessary.

5. Oncolytic adenovirus

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 (26, 27). Oncolytic adenovirus is widely studied as a novel anti-cancer therapy which can selectively target the cancer cells (26, 28). Following viral infection, tumor cells with defective viral defense mechanism result in unchecked viral replication with consequent oncolysis caused by the production of cytotoxic proteins (28). Moreover, viral vectors may be used to deliver therapeutic genes to tumor cells, resulting in the expression of the protein encoded by the therapeutic gene which mediates an anti-cancer effect within the cell (28). There are numerous advantages of cancer virotherapy, including low resistance to therapy, low systemic toxicity, and increased potency of multimodal treatment. Moreover, therapeutic index of virotherapy is high due to the amplification of the initial virus within the tumor (28). That is the main reason why we focused on the oncolytic adenovirus for the delivery of therapeutic siRNA.

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.

Materials and Methods

1. 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, Nuaillé, France), 50 units/mL penicillin, and 50 μg/mL streptomycin (Lonza) at 37℃ in a humidified incubator with 5% CO2.

2. Construction of shRNA-CD44 plasmid

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

3. 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 (Enzynomics, Daejeon, Korea). Purified (Qiagen, Valencia, CA, USA) PCR products and adenovirus shuttle plasmids (Agilent Technologies, Palo Alto, CA) were digested with *kpnI* and *XbaI*, ligated with T4 DNA ligase (Promega, Madison, WI, USA), and then transformed into DH5a chemically competent *E. coli*. Miniprepped 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^{TM}20 RT (Sartorius Stedim Biotech, Göttingen, Germany) kit. The viral particle concentration was determined by measuring absorbance at 260 nm, and a standard $TCID_{50}$ (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.

4. Adenoviral infection

The HCT116 colon cancer cells were seeded onto a 6−well plate at a density of 0.1×10^{6} 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.

5. Reverse transcription PCR (RT−PCR) analysis

Total RNA was isolated from cells with TRIzol reagent (Invitrogen, Carlsbad, CA,USA) 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, Madison, WI, USA) and reverse transcriptase (Beams bio., Seongnam, Korea). PCR amplification of cDNA was performed using gene-specific primers (Table 1) and nTaq DNA polymerase (Enzynomics, Daejeon, Korea). PCR products were separated on a 1% agarose gel, visualized and photographed under UV light.

Table 1. 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’

6. Western blotting

Total cell extracts were lysed in Cell Lysis Buffer (Cell Signaling, Danvers, MA, USA) with a protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentrations were determined by a BCA protein assay (Thermo, 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% Tween20 (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, Danvers, MA) at 4° C overnight. After rinsing in TBST (0.1%), membranes were incubated with horseradish peroxidase-labeled anti-rabbit (Thermo Fisher Scientific, Rockford, IL, USA) or anti-mouse IgG secondary antibodies (Cell Signaling Technology, Danvers, MA) at room temperature for 1 h. The blot was detected

by ECL (enhanced chemiluminescence) of a HRP substrate (Millipore) on an image reader (Ras4000, Fujifilm, Tokyo, Japan).

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

8. 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 400μL of this cell resuspension was performed on a fluorescence active cell sorting (FACS) Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using the Cell-Quest version 3.3 software (Becton Dickinson).

9. Cell migration assay

Cells were cultured in 6-well plates and infected with the recombinant adenoviruses for 24-48 hours. The infected HCT116 cells were then seeded in Culture-Inserts (2×0.22cm^2; IBIDI GmbH, Martinsried, Germany) at 5×10^4 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.

10. Cell invasion assay

Cell invasion assays were carried out using 24-well transwell filters with 8-μM pores (Coring Inc., NY, USA). Transwell filters were coated with 500 μg 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 2.5×10^5 cells in 120μL 0.2% BSA medium and then seeded into the upper chamber. Then 400 μL of 0.2% BSA medium containing 50 μg/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 mm^2 each) using a hematocytometer under a light microscope.

11. 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 (1.5×10^4 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 40x magnification.

12. Statistical analysis

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

Results

1. 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 down-regulated 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 ($p < 0.01$).

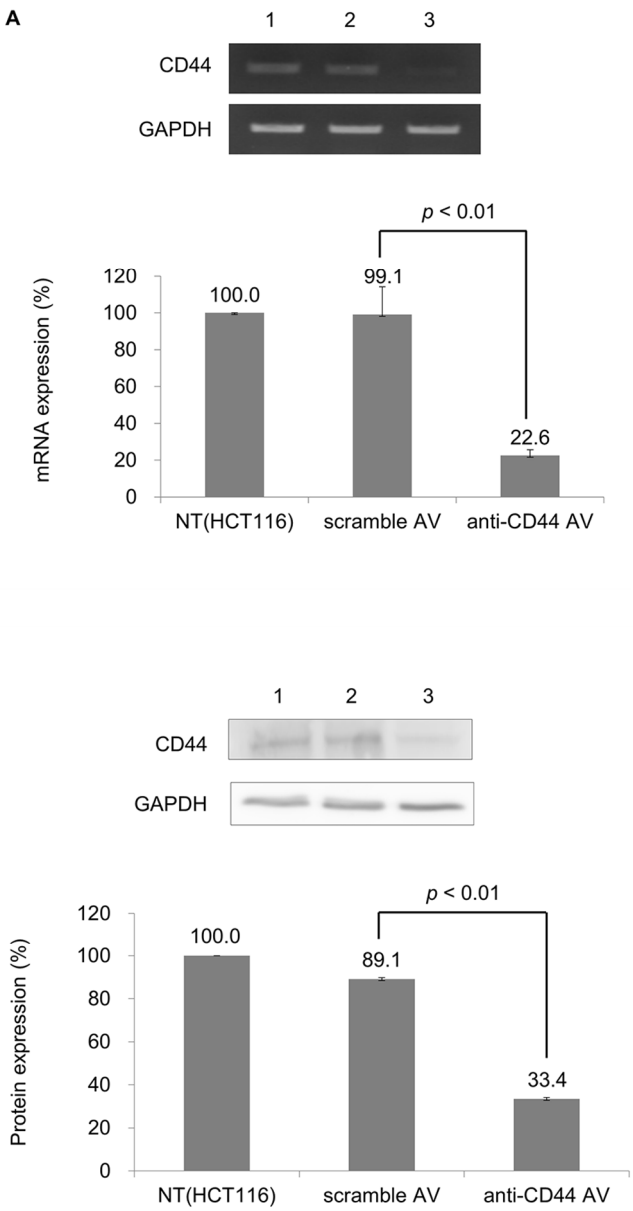

Fig. 1 CD44 expression detected by (A) RT-PCR and (B) western blotting, with GAPDH as an internal control. Lane 1, Nontreated WT cells (HCT116). Lane 2, Cells infected with scramble Ad. Lane 3, Cells infected with Ad-CD44-shRNA. Bands were analyzed and quantified by densitometry and the CD44/GAPDH ratio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

2. Cell viability

The results of the cell viability assay are shown in Fig. 2. Whereas the scramble-Ad showed little cytotoxicity, Ad-CD44-shRNA suppressed cell viability ($p < 0.05$) 4-5 days post-infection.

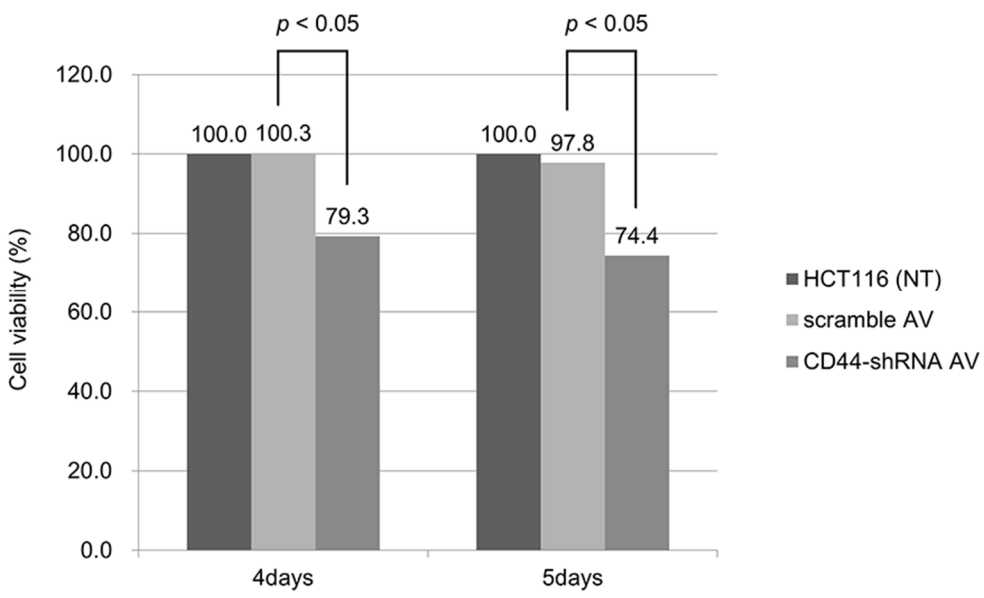

Fig. 2 Cell viability was determined using CCK-8 in 4-5 days after infection with scramble Ad or Ad-CD44-shRNA.

3. 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).

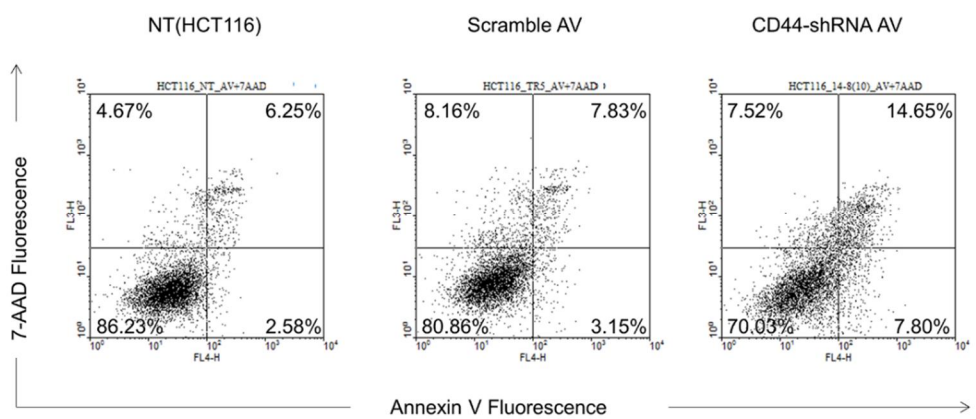

Fig. 3 Apoptosis was investigated using flow cytometric analysis at 72h after infection with scramble Ad or Ad-CD44-shRNA.

4. 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).

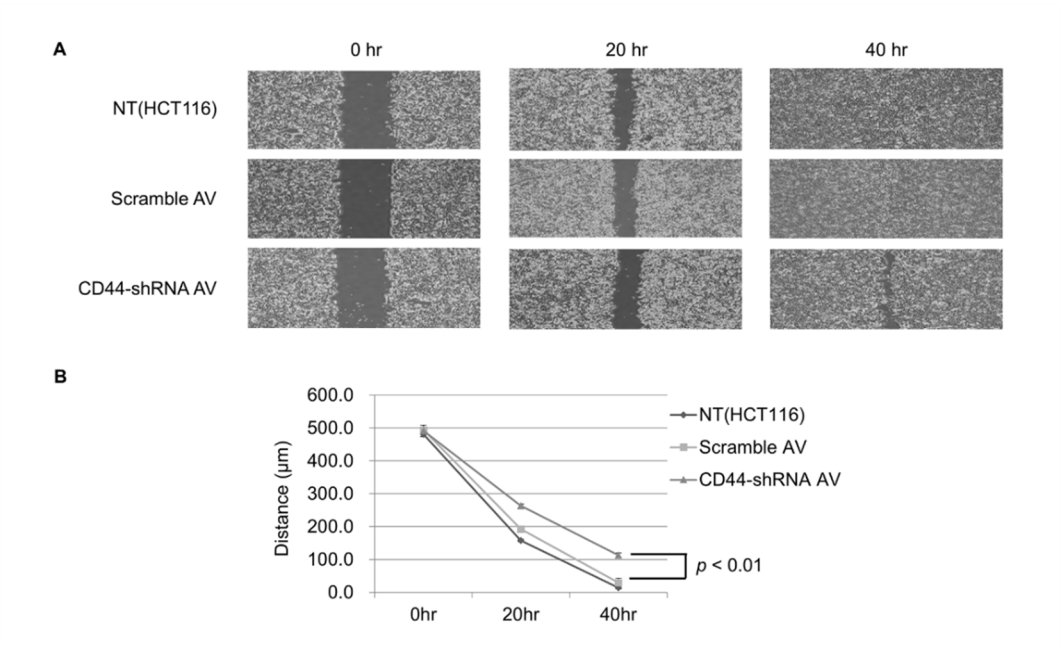

Fig. 4 The effect of CD44-shRNA recombinant adenovirus treatment on cell migration. (A) Cells were photographed at 0, 20, 40 hours after detach insert from plate. (B) Graphs of cell migration are displayed as relative healing distance.

5. 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).

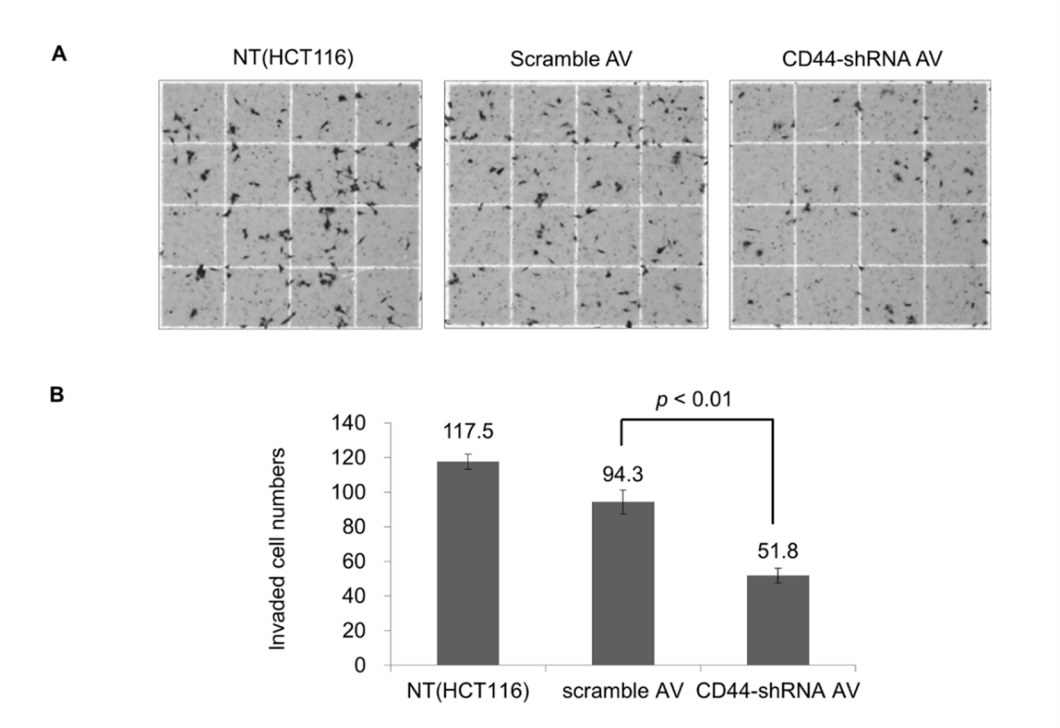

Fig. 5 Effects of CD44-shRNA adenoviral vector on invasion of HCT116 cells *in vitro*. (A) The invasiveness of colon cancer cells were assesses using a transwell invasion assay. The transmembrane cells were stained with Diff-Quick solution. (B) Number of invaded cells was counted at 24 hours.

6. 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-3β (Fig. 6A). In contrast, there was minimal change of β-catenin expression (Fig. 6A). The Ad-CD44-shRNA also resulted 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, caspase 9, and PARP (Fig. 6B).

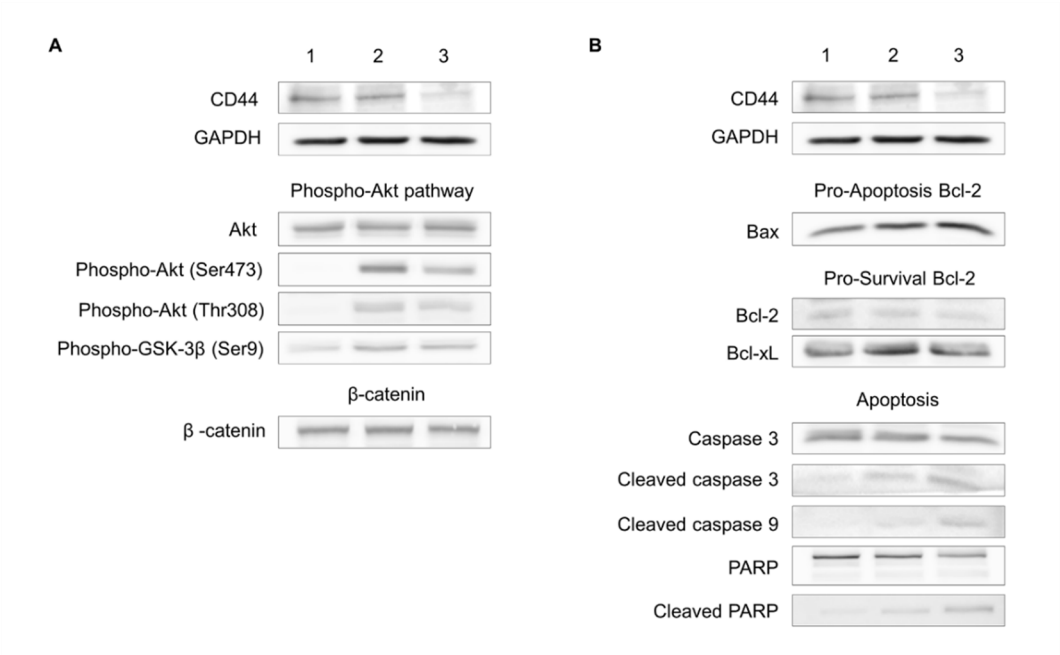

Fig. 6 Western blot analysis of (A) PI3-Akt signaling and (B) apoptosis. Apoptosis and survival-related proteins were detected at 72h after infection with recombinant adenoviral vectors. Lane 1, Nontreated WT cells (HCT116). Lane 2, Cells infected with scramble Ad. Lane 3, Cells infected with Ad-CD44-shRNA.

7. 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.

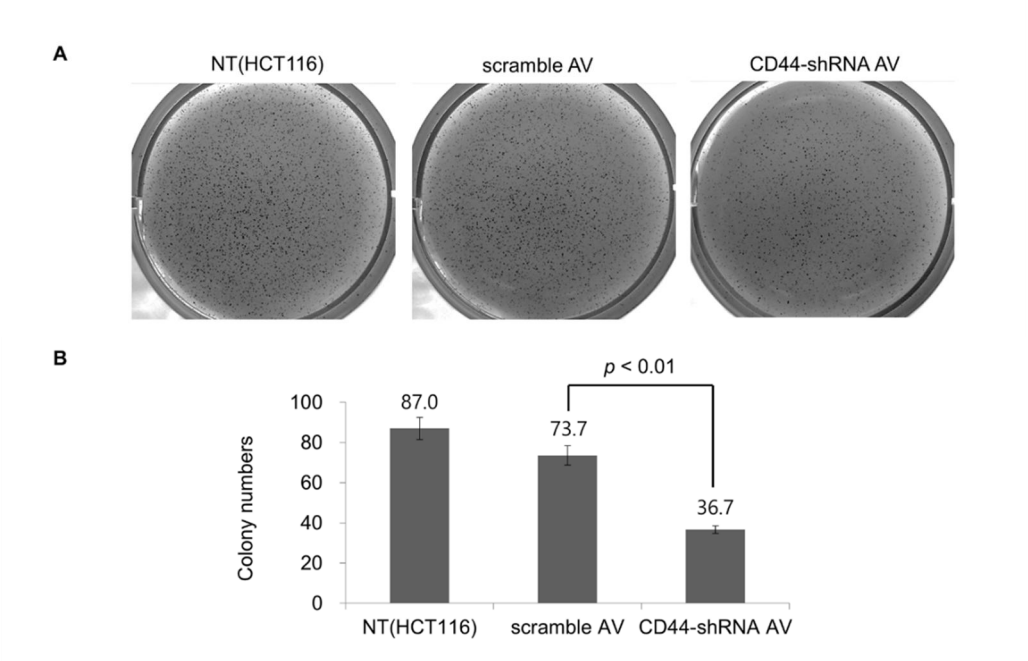

Fig. 7 Soft agar colony formation assay of CD44 knockdown. (A) HCT116 cells infected with scramble Ad and Ad-CD44-shRNA was used for the soft agar colony formation assay. Cells were cultured in 3D-agar for 2 weeks, and colonies were stained with crystal violet reagent and

photographed. (B) Quantitative analysis of colony numbers is shown in the lower panel.

Discussion

In the present 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.

1. Brief review of CSCs

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.

2. The meaning of the present study

2-1. Construction of CD44-shRNA recombinant adenoviral model

In the present study, we successfully constructed a recombinant adenoviral model to knockdown 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).

2-2. Mechanism of the inhibition of colon cancer cells by CD44-shRNA recombinant adenovirus

The result of the present 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 (34, 35). We demonstrated that Ad-CD44-shRNA infection inhibited Akt phosphorylation (Fig. 6A), which is one of the most important Wnt-target genes for the survival of cancer cells (36, 37). When Akt phosphorylation is inhibited, Akt signaling pathway is deactivated (37). We also showed the down-regulation of GSK-3β (Fig. 6A), 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 (38). The loss of GSK3 protein is associated with the stable accumulation of non-phosphorylated β-catenin, which is accessible to the adherens junction at the cell periphery (24). The Wnt/β-catenin pathway was suggested to protect cells from p53-mediated

FoxO1-induced apoptosis, which involved the activation of Akt signaling pathway (37). 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 (35).

In addition, we showed decreased expression of Bcl-2 and Bcl-xL; increased expression of Bax; and cleavage of caspase 3, caspase 9, and PARP (Fig. 6B). 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 (39-41). Increased expression of Bax induces apoptosis via a caspase-mediated mitochondrial pathway (40). 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 (42, 43). Death receptors and their ligands (Fas/Fas-L and DR5/TRAIL) induce caspase-8 activation, which cleaves Bid and activates caspase-3 (43). When Bid is cleaved, it migrates to the mitochondria and induces cytochrome c release and caspase-9 cleavage (43). Overall, the results of our study demonstrated that Ad-CD44-shRNA infection induces apoptosis in HCT116 colon cancer cells, suggesting reduced clonogenic ability.

2-3. Close mimicry of *in vivo* environment by soft agar colony formation assay

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. Actually, proliferation in a 2D monolayer culture which we generally utilize is quite different from that which occurs *in vivo* (34). 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). 3D culture is different from 2D culture systems because it quantifies tumorigenicity by measuring a cell's ability to proliferate within a semi-solid agarose gel (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.

3. Future perspectives

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. The present 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.

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요약(국문초록)

배경: 세포표면 당단백질(glycoprotein)인 CD44는 암줄기세포(cancer stem cell)의 주요 표지자 중 하나로서, 세포의 증식, 종양의 침습과 전이와 밀접한 관련을 가지고 있다. 이전 연구들에 따르면 short-hairpin RNA (shRNA)를 이용하여 CD44의 발현을 억제(knockdown)함으로써 대장암 세포의 증식과 이주를 억제하고 세포자멸사를 유도시킬 수 있다고 보고하였다. 그러나 소간섭 RNA (small interference RNA, siRNA)를 대장암 세포 내로 보다 효율적으로 도입시킬 수 있는 방법의 개발이 여전히 요구된다.

방법: 본 연구에서 우리는 CD44-shRNA 재조합 아데노바이러스 (Ad-CD44-shRNA) *in vitro* 모델을 개발하고 그것이 대장암 세포의 침습, 이주, 증식을 억제시키는지 여부와 세포자멸사를 촉진시키는지 여부를 알아보고자 하였다. CD44 발현 억제를 위해 shRNA를 이용하였고 AD293 세포를 이용하여 재조합 아데노바이러스를 만들었다. 이를 HCT116 대장암 세포에 도입하여 세포의 침습, 이주, 증식 능력과 세포자멸사의 변화를 확인하였다.

결과: Ad-CD44-shRNA 처리를 한 HCT116 대장암 세포에서 CD44의 발현이 감소됨을 확인하였다. Ad-CD44-shRNA 처리를 한 HCT116 대장암 세포는 세포의 증식, 이주, 침습 능력에 있어서 유의한

감소를 보였으며, 세포자멸사는 증가하는 결과를 보여주었다. 또한 Ad-CD44-shRNA는 Akt와 GSK-3β의 인산화를 감소시켰으며, Bcl-2와 Bcl-xL의 발현은 감소된 반면, Bax, cleaved caspase 3, cleaved caspase 9, PARP의 발현은 증가하였다.

결론: 본 연구는 아데노바이러스를 매개로 한 RNA 간섭으로 CD44의 발현을 억제시키는 것이 대장암의 증식과 침습 능력을 감소시키고 세포자멸사를 증가시킴을 확인하였다. 이러한 결과는 향후 암줄기세포를 표적으로 하는 치료제의 개발을 위한 초석으로 이용될 수 있을 것으로 기대된다.

주요어: 암줄기세포, CD44, 아데노바이러스, RNA 간섭, 대장암

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