Development of retargeted adenovirus to tumor-associated antigen sialyl Lewis X

Thesis by
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Development of retargeted adenovirus to tumor-associated antigen sialyl Lewis X

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`Abstract

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(Directed by Professor Joo-Hang Kim)

Sialyl Lewis X (sLewX) is a tumor-associated carbohydrate antigen. Several studies indicate that sLewX serves as a ligand in the adhesion of cancer cells to endothelial cells, resulting in hematogenous metastasis of human cancer cells. Increased expression of sLewX has been reported to correlate with poor prognoses in various cancers including gastric, colorectal, lung and ovarian cancers. In this perspective, we adopted an *in vitro* random phage display library panning on sLewX to develop adenoviral vectors capable of the selective infection of the cancer cells with respect to the normal cells. More than 20 individually isolated phage were tested for the ability to bind specifically to sLewX and the sequences of these novel peptides were identified.

The development of tissue/cell selective targeting adenovirus requires the generation of adenovirus vectors which lack native receptor binding and additionally contain domains which redirect the vector to tissue/cell specific receptors. Towards this goal, we have generated CAR binding-ablated adenovirus YKL-1/420A using the backbone of an E1B55kD deleted oncolytic adenoviral vector YKL-1.

Ykl-1/420A adenovirus exerted dramatic reduction of transduction efficiency compared to control adenovirus. Among the peptides isolated by *in vitro* random phage display, 3 peptides were chosen and fused to the C-terminus of the fiber protein of YKL-1-420A, generating three sLewX-targeted adenoviruses of YKL-1-420A/7mer, YKL-1-420A/12mer, and YKL-1-420A/p30. YKL-1-420A/p30 exerted about 10-100 times greater cytolytic ability than control adenovirus YKL-1-420A. Furthermore, treatment with YKL-1-420A/p30 significantly suppressed tumor growth in xenograft tumor model when compared with control adenovirus YKL-1-420. Taken together, these studies demonstrate that strategy to alter adenovirus tropism may allow greatly improved utilities of adenovirus for gene therapy applications. In particular, sLewX-specific targeted adenovirus YKL-1-420A/p30 allowed CAR-independent gene delivery as well as cell-specific gene delivery, and showed significant promise as a targeting replicative adenovirus vector in cancer gene therapy.

Key Words : adenovirus, phage display, targeting peptide, sialyl Lewis X, gene therapy

Development of retargeted adenovirus to tumor-associated antigen sialyl Lewis X

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Ⅰ. Introduction

Gene therapy has by now proved its usefulness in a number of clinical trials aimed to develop a cure for several inherited and acquired genetic diseases including various types of cancer, coronary artery disease, and cystic fibrosis[1-3]. For the effective application of gene therapy strategies to human disease, certain stringent criteria should be met, namely, that vectors should deliver a therapeutic gene specifically to a target cell, that resultant gene expression should be stable *in vivo* for time periods sufficient to achieve efficient gene delivery, and be achieved within an acceptable safety margin[4]. Therefore, human adenovirus(Ad) is one of the best gene delivery vector developed so far. Ad vectors derived from the human group C adenovirus 5 serotype are efficient gene delivery vehicles which readily transduce many nondividing cells. Adenovirus infects a broad range of cells and tissues, including lung, liver, endothelium, and muscle[5]. For transient high-level expression of therapeutic gene products, as required in cancer treatment, adenoviruses seem to be an ideal

tool[6,7]. Several adenovirus-based approaches of gene therapy for various cancer are currently under investigation in clinical trials, including a phase I/II and a phase II/III trial using p53 as a therapeutic gene[8-14]. A variety of antitumoral genes including cytokines, immune-costimulatory factors, suicide genes and tumor suppressor genes have been applied, which has resulted in some clinical response[15-20].

E1/E3-deleted replication-defective adenovirus derived from human adenovirus type 5 has been widely used for cancer gene therapy because it offers, in contrast to other vectors, much higher gene transfer efficiency and transgene expression in a broad spectrum of cell types[6,15]. Therapeutic genes are generally inserted in place of the E1 gene, which is esential for viral replication[16,17,21].

E1 gene, one of the adenovirus early genes, encodes multiple open reading frames, including E1A and E1B55KDa[16,22]. E1A gene product, a transcription factor, associates with pRB, p300 and other proteins, and is largely responsible for driving infected cells into the S phase to allow the synthesis of viral genome[22,23]. E1A expression and unexpected foreign DNA synthesis triggers the expression/ activation of p53[24,25]. However, the therapeutic efficacy of E1-deleted replication-incompetent adenovirus is limited by its inability to spread in and infect neighboring cancer cells subsequent to the initial infection event.

In contrast, adenovirus encoded E1B55KDa physically associates with and inactivates p53[26,27]. By using this mechanism, wild-type adenovirus can manipulate host cells to provide the optimal

conditions for effective virus replication and production. Therefore, E1B55KDa-attenuated recombinant adenovirus should not be able to replicate in normal human cells, whereas it would be able to replicate in cells lacking functional p53[28].

Despite tremendous progress made in attempts to further improve vector system for gene therapy applications, some disadvantages of adenovirus remain, i.e.,(1) vector-induced immunogenicity, (2) the inability of adenoviral vectors to efficiently infect certain cell types, some of which are of significant clinical importance, and (3) the promiscuous tropism of adenoviral vectors, which results in uncontrolled gene transfer to both target and nontarget cells, thereby compromising the overall efficiency of the therapy[29]. Thus, a means must be developed to redirect its tropism to specific cell targets for the treatment of disseminated diseases. The entry mechanism of adenovirus, including primary adenoviral receptors, are now well understood and allow for a rational approach to the targeting of adenoviral vectors[30].

Adenovirus was one of the first viruses shown to associate with distinct cell receptors that facilitate either attachment[31,32] or internalization[33]. Most adenovirus types bind to cells via a 46-kDa membrane glycoprotein that is widely expressed on different tissue types *in vivo*. This protein is a member of the immunoglobulin superfamily and is referred to as CAR(coxsackie adenovirus receptor) since it also mediate attachment of coxsackie B viruses. Recent studies have suggested that heparan sulfate proteoglycans[34,35] or sialic acid[36] may also facilitate adenovirus attachment.

CAR binding is mediated by the adenovirus fiber protein, a homotrimeric molecule composed of a N-terminal region (tail) that anchors the fiber to the penton base capsid protein, an elongated central domain (shaft)[37], and a C-terminal CAR-binding domain (knob). While fiber interaction with CAR clearly facilitates virus attachment to cells, this is not generally sufficient to allow rapid virus uptake. Instead, integrin αvβ3 or αvβ5 association with penton base protein promotes virus internalization. αv integrins recognize a highly conserved RGD motif that is present on an extended loop in the penton base of many but not all Ad serotypes[38,39]. Li and his collegues[40] lately revealed that the human embryonic kidney (HEK293) cell line, commonly used for recombinant adenovirus propagation, does not express the adenovirus coreceptor αvβ3 or αvβ5 integrins, yet these cells are efficiently infected by adenoviral vectors. Instead, adenovirus binds to HEK293 cells via the fiber receptor CAR and is subsequently internalized via interaction with integrin αvβ1.

The new structure, in combination with mutagenesis studies[41], revealed that amino acid residues located in several extended loops on the lateral surface of the fiber knob mediate CAR binding. Several amino acid residues in the fiber knob (i.e. AB loop) that are involved in CAR interactions are also highly conserved among different adenovirus serotypes. These new studies have provided important leads for retargeting adenoviral vectors to different cell receptors. This has been accomplished by removing critical CAR-binding residues in the knob and inserting new sequences that

faciliate binding to alternative receptors. While true Ad retargeting has not been fully accomplished for *in vivo* applications, fiber modifications have the potential to increase the efficiency of adenovirus-mediated gene delivery to specific cell types. Increasing the tissue selectivity of adenovirus for gene therapy has the potential to make these therapies safer, reduce humoral and CTL response against adenovirus, and to better enable the systemic administration of adenovirus.

Sialyl Lewis X (sLewX) is a well-established tumor-associated carbohydrate antigen, which is frequently up-regulated in breast, stomach, and colon cancers. Several studies have shown that sLewX serves as a ligand in the adhesion of cancer cells to endothelial cells, resulting in hematogenous metastasis of human cancer cells. In breast and colonic carcinoma patients, the expression of sLewX was reported to be associated with a poor prognosis and with an enhanced metastatic potential[42-44]. A recent study demonstrated that expression of sLewX in mucin-type *O*-glycans is highly correlated with lymphatic and venous invasion[42].

The use of adenoviral vectors for cancer gene therapy applications is currently limited by several factors, including broad adenovirus tropism associated with the widespread expression of CAR in normal human tissues, as well as limited levels of CAR in tumor cells. In this perspective, we developed adenoviral vectors capable of CAR-independent gene delivery to sLewX-expressing tumor cells, allowing the achievement of both cell-specific gene delivery as well as gene transfer efficiency augmentations (Fig 1).

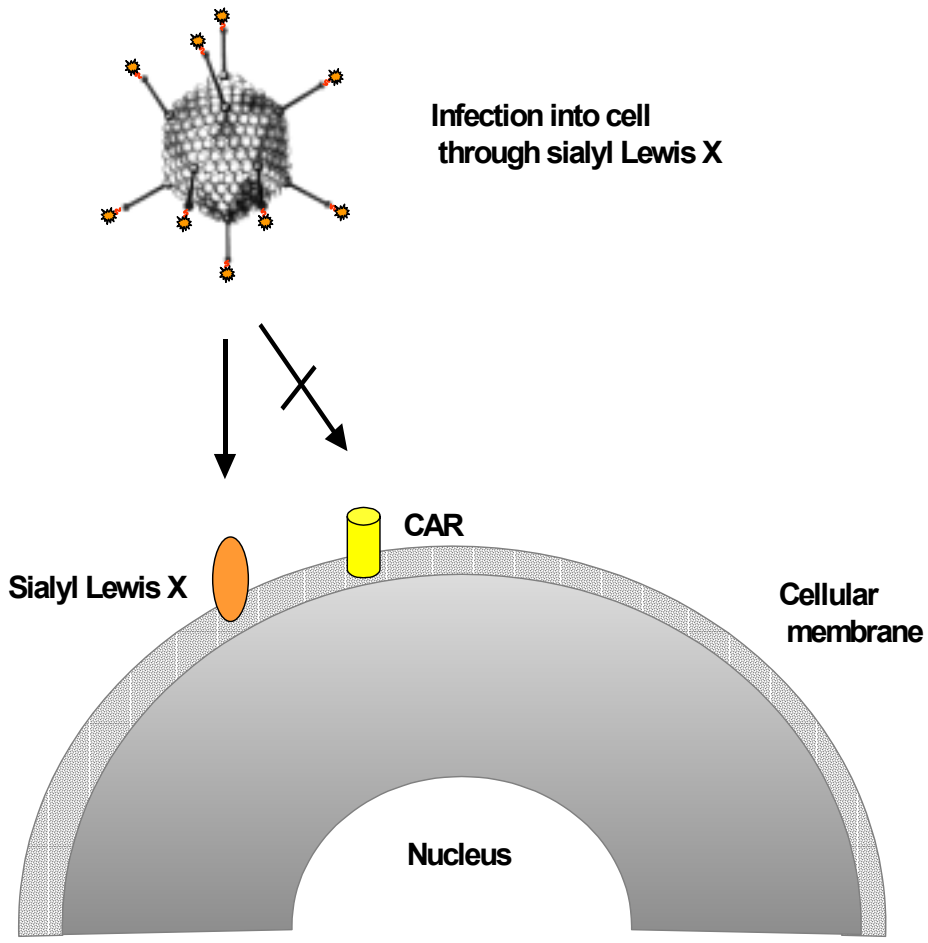


Fig 1. The strategy of targeting sLewX-expressing cancer cells by sialyl Lewis X-retargeted adenovirus (Ad). Most Ad types attach to cells via a coxsackievirus-adenovirus receptor (CAR) that is widely expressed on different tissue types *in vivo*. Thus, targeted and cell-specific gene delivery is limited for low expression of CAR on tumor cells in *vivo*. To address this issue, true retargeting can be accomplished by ablating native tropism and targeting cognate receptor. Because sialyl Lewis X (sLewX) is tumor-associated antigen, it is used as an alternative receptor and Ad is infected into cells through sLewX instead of CAR.

Ⅱ. Materials and methods

1. Cell lines and cell culture

All cell lines with except Hep3B were cultured in DMEM supplemented with 10% fetal bovine serum, and penicillin-streptomycin (100 IU/ml). Hep3B cell line was maintained in modified eagles medium supplemented with 10% fetal bovine serum and penicillin-streptomycin (100 IU/ml). HEK293 (human embryonic kidney cell line expressing the adenoviral E1 region), liver cancer cell lines (SK-Hep1, HepG2, Hep3B), lung cancer cell lines (A549, H460, H1299), gastric cancer cell line (AGS), cervical cancer cell lines (C33A, HeLa, SiHa, Me180), colon cancer cell line (HCT116), brain tumor cell lines (U343, U251N, U118MG), breast cancer cell line (MCF-7), and Chinese hamster ovary cell lines (CHO-K1, Pro5, Lec2) were purchased from the American Type Culture Collection (ATCC, Rakville, MD, USA).

2. Selection of sLewX binding phage

To identify sLewX-specific peptides, *in vitro* random phage display library panning was performed (Fig 2). Three kinds of phage display libraries, composed of a filamentous phage displaying random peptides (7mer, C7Cmer, 12mer) on its surface, was amplified in *E. coli*. For better coating efficiency, sLewX-BSA (Oxford Glycosystem, Bedford, MA, USA) conjugate was used for immobilization on a 6-well plate. Each library of phage was exposed to a plate coated with the sLewX-BSA, and unbound phage was washed

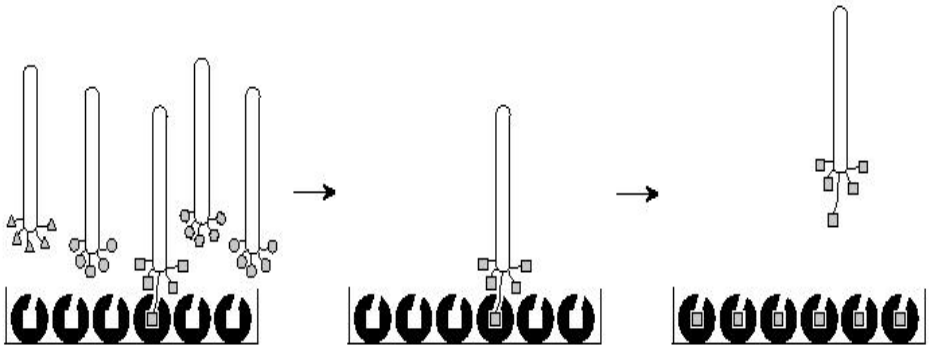


Fig 2. Isolation of tumor-associated antigen sLewX-specific peptides by *in vitro* random phage display library panning. To identify sLewX-specific peptides, sLewX-BSA was coated on 6-well plate, and three kinds of filamentous phage libraries containing random peptides (7mer, C7C, 12mer) were added into sLewX-coated plate. Unbound phage was washed away, and bound phage was collected. After these steps were repeated 3times, sLewX-specific peptides were isolated and sequenced.

away. Then, the bound phage was eluted by lowering pH. and, sLewX-specific peptides were isolated and sequenced.

3. ELISA

Solid-phase binding enzyme-linked immunosorbent assay (ELISA) was performed by a method previously described. sLewX-BSA conjugate at a concentration of 5 μg/ml in 0.1 M $NaHCO_3$ (pH 8.6) were immobilizd on microtiter plates overnight at 4℃. Remaining binding sites were blocked with PBS, 3% BSA for 1 hr at 37℃. Peptides diluted in PBS to concentrations ranging from 0.5 to 500 ng/ml was added, and then incubated for 1 hr at 37℃. After washing with PBS, bound peptides was detected by incubation with HRP-conjugated streptoavidin diluted 1/2500. Following incubation at 37℃ for 1 hr, the cells were then washed and incubated with a ABTS as substrate for 45 min. Plates were read in a microtiter plate reader set at 405 nm; results are presented as mean ± standard deviation (SD).

4. Construction of CAR binding ablated adenoviruses

To construct CAR binding ablated adenoviruses, genome encoding the fiber in Ad5 (from nucleotide 26173 to 33594 except 28592 to 30470) were cloned into the *Sac*II/*Kpn*I sites of pBluescript SK II (+) (Stratagene, La Jolla, CA, U.S.A) to create pSK5543. pSK5543 was then digested with *Stu*I/*Kpn*I, and the resulting DNA fragment containing adenoviral fiber was cloned into the pBluescript SK II (+), generating pSK1638.

To eliminate a CAR binding site on the fiber, mutagenesis was

performed by the QuickChange site-directed mutagenesis system (Stratagene, La Jolla, CA, U.S.A) (Fig 3). The oligonucleotide primers used for the incorporation of amino acid change are following: 5′-GCAGAGAAAGATGCT**GCA**CTCACTTTGGTC-3′ and 5′-GACCAAAGTGA G**TGC**AGCATCTTTCTCTGC-3′. The primers were designed to replace the lysine with alanine (bold) in the region of fiber. Later, the amino acid changes were identified by sequencing.

In order to incorporate a targeting motif into the C-terminus of fiber, stop codon (TAA) in fiber was changed to sequence (TCC) encoding a glycine by PCR mediated site-directed mutagenesis. For the easy of incorporating a targeting motif into the downstream of glycine, new *BamH* I and *Sac* I sites were also generated following glycine by PCR mediated site-directed mutagenesis. For the PCR mediated site-directed mutagenesis, following primer set was used: 5′-AACAAAGGCCTTTACTTGTT-3′ as a sense primer and 5-′GGG**GAGCTCGGAT CCTCC**TTCTTGGGCAATGTATG-3′ as an anti-sense primer. After PCR amplification, the resulting mutated PCR product (850bp) was digested with *Stu* I /*Sac* I, and subcloned into pSP72 (Promega, Madison, WI, U.S.A), generating a pSP72[835]. Three sLewX-specific peptides isolated by *in vitro* phage display library panning were phosphorylazed for incorporation into the fiber knob with T4 polynucleotide kinase (New England Biolabs, Beverly, MA, U.S.A) before use. The sequences of epitopes were following; p30: 5′-GATC CGGCGGGGGCGGTGGAGGAGGGCCTCAACCCGGTATGGCGCCTCGTCCAGGTATGCCGTGAGCT-3′ and 5′-CACGGCATACCTGGACGAGGCGCCATACCGGGTTGAGGCCCTCCTCCACCGCCCCCGCC G-3′, 7mer: 5′-GATCCGGCGGGGGCGGTGGAGGAGGGCCACGACCTGGTCTCAAACCCTGAG

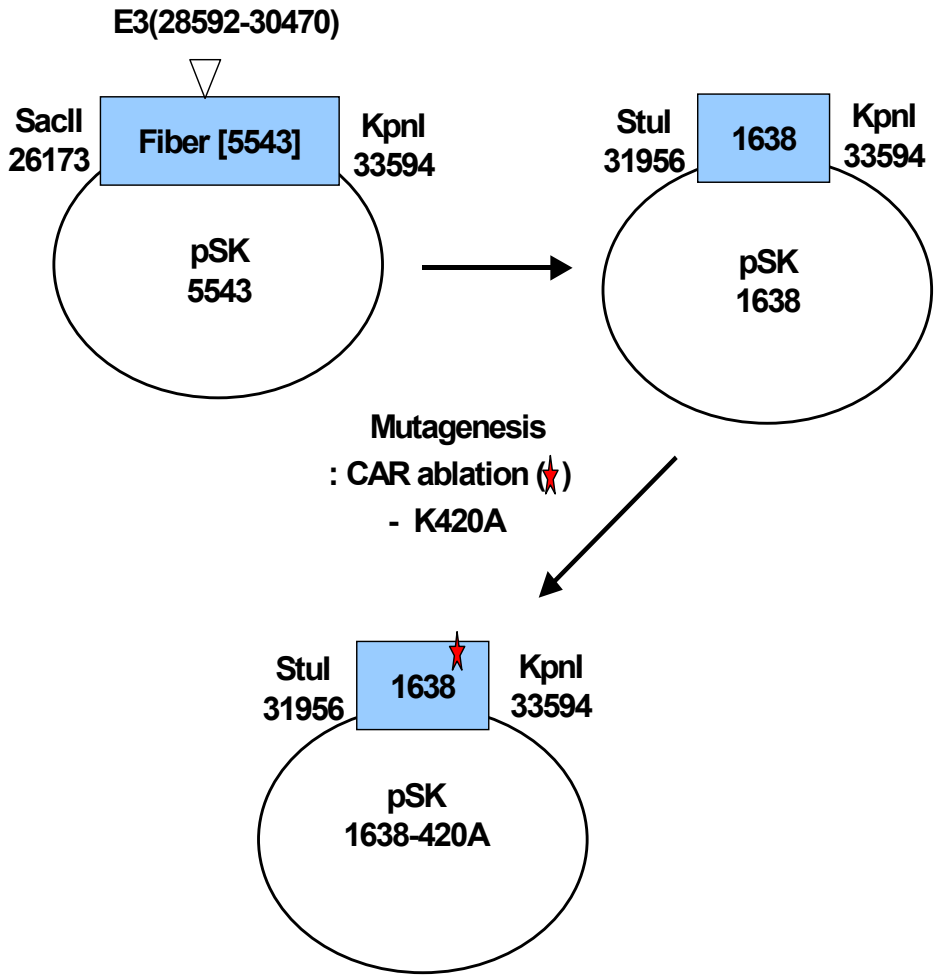


Fig 3. Site-directed mutagenesis of adenovirus fiber for CAR binding ablation. Genome encoding the fiber in adenovirus type 5 (from nucleotide 26173 to 33594 except 28592 to 30470) were cloned into pBluescript SK II (+) to create pSK5543. For CAR binding ablation, DNA fragment from nucleotide 31956 to 33594 were transferred to pBluescript SK II (+), and referred to pSK1638. CAR binding ablation (★) was achieved by changing 420th amino acid lysine to alanine with site-directed mutagenesis.

CT-3′ and 5′-CAGGGTTTGAGACCAGGTCGTGGCCCTCCTCCACCGCCCCCGCCG-3′, 12mer: 5′-GATCCGGCGGGGGCGGTGGAGGAGGGGCACATTGGATTCCGCGCTATTCCTCGCCT GCAACCAGAGCT-3′ and 5′-CAGGTTGCAGGCGAGGAATAGCGCGGAATCCAATGTGCCCCTC CTCCACCGCCCCCGCCG-3′. These peptides were cloned into the C-terminus of fiber knob site in pSP72[835] with 10 glycine linkers for the flexibility using *BamH*Ⅰ/*Sac*Ⅰ sites. The resultant plasmids were digested with *Stu*Ⅰ/*EcoR*Ⅰ, and then cloned into pSK5543 digested with *Stu*Ⅰ/*Mfe*Ⅰ, generating an adenovirus shuttle vector pSK[5543+epitope] (Fig 4).

5. Generation of the sLewX-retargeted adenoviruses

To generate sLewX-retargeted adenoviruses, this newly constructed shuttle vector pSK[5543+epitope] was then digested with *Sac*Ⅱ/*Kpn*Ⅰ, and viral vector YKL-1 or dl324/LacZ was digested with *Spe*Ⅰ for homologous DNA recombination in *E. coli* BJ5183 (Fig 5). YKL-1 is a E1B55KDa-deleted, tumor-selective, and oncolytic adenoviral vector. To verify the respected homologous recombinant, the plasmid DNA was amplified in *E. coli* DH5α and purified DNA from overnight *E. coli* culture was digested with *Hind*Ⅲ, then the digestion pattern was analyzed. The proper homologous recombinant adenoviral plasmid DNA was digested with *Pac*Ⅰ, and transfected into 293 cells to generate sLewX-retargeted adenoviruses. Plaques developed 7 to 10 days posttransfection. Recombinant adenoviruses were propagated on 293 cells, and purified by ultracentrifugation in CsCl gradients by a standard protocol. Determination of virus particle titer was accomplished spectrophotometrically, using a conversion factor of

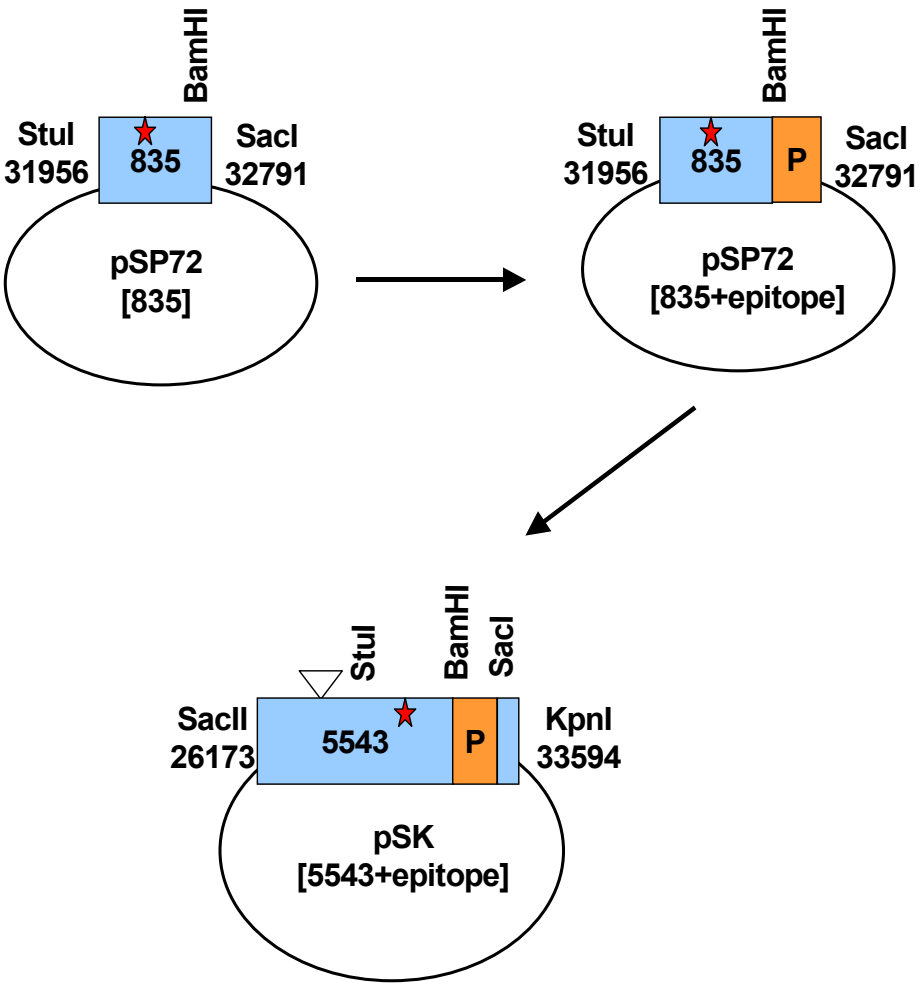


Fig 4. Addition of sLewX-specific peptides to the 3' fiber end of adenovirus type 5. Phosphorylized oligonucleotides which encode sLewX-specific peptides were ligated to *BamH*I/*Sac*I sites of pSP72[835]. The correct insertion of sLewX-specific peptides was confirmed by PCR. Then, pSP72[835+epitope] were digested with *Stu*I/*EcoR*I, and subcloned into pSK[5543], generating a shuttle vector pSK[5543-420A] containing CAR binding-ablated (★) fiber.

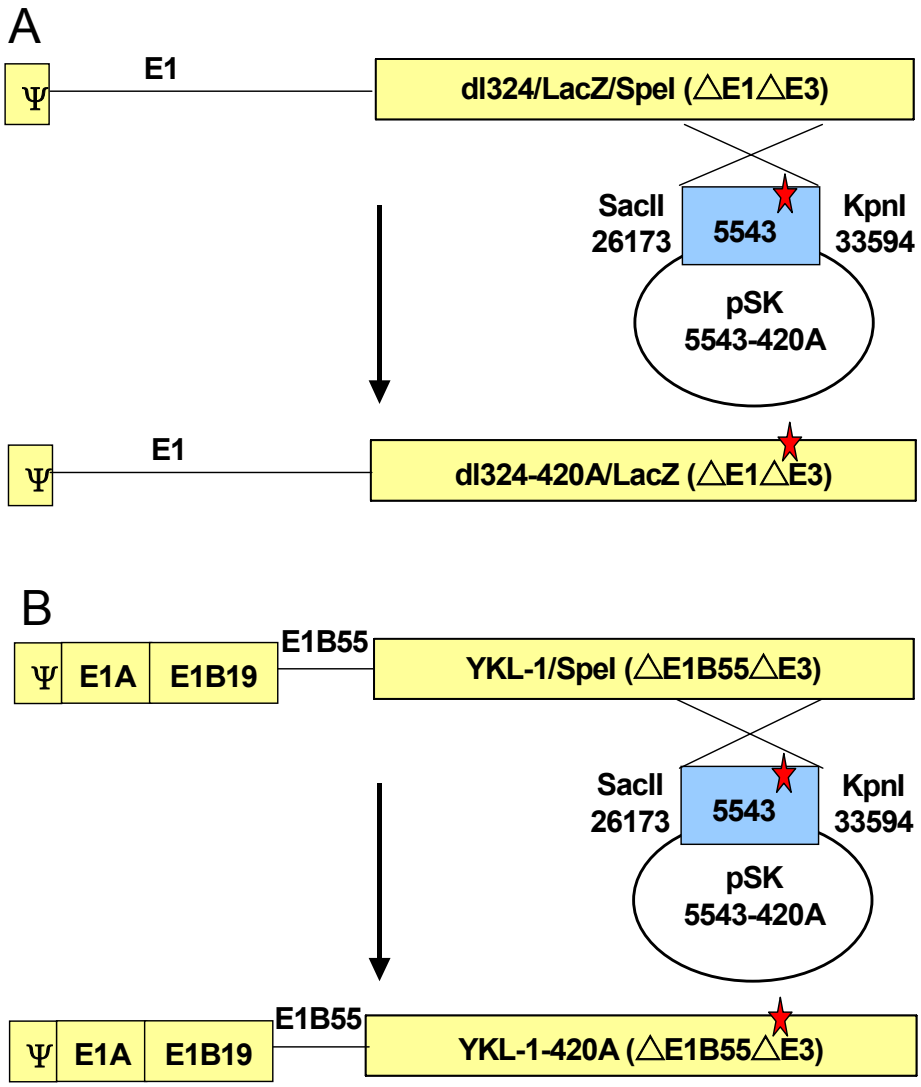


Fig 5. Generation of the sLewX-retargeted Ad. For homologous DNA recombination, shuttle vector pSK[5543-420A] was digested with *Sac*II/*Kpn*I, and viral vector dl324/LacZ (A) or YKL-1 (B) was digested with *Spe*I. They were then cotranformed into *E. coli* BJ5183, and recombinations were confirmed by *Hind*III or *Pac*I digestion. Star (★) denoted CAR binding ablation site.

1×10^{12} viral particles per absorbance unit at 260 nm. The titer of infectious viral particles was determined to virus particle titer$\times10^{-2}$. To construct a replication-deficient, sLewX-retargeted adenovirus, E1- and E3-deleted dl324/LacZ was used, and viral propagation was same as YKL-1/sLewX.

6. Fluorescence-activated cell sorting (FACS) analysis

To evaluate the expression level of CAR and sLewX on the panel of cancer and normal cells, FACS analysis was performed. Cells were briefly trypsinized with 0.25% trypsin, washed with 2% fetal bovine serum (FBS) in phosphate-buffered saline (PBS), and resuspended at a concentration of 10^6 cells/ml in RPMI1640 containing 10% FBS, 0.2% NaN_3, and 50 mM Hepes. A 50-$\mu\ell$ aliquot of the cell suspension was mixed with primary antibody. Monoclonal anti-CAR antibody RmcB (kindly provided by Dr. J. M. Bergelson, The children's hospital of Philadelphia, Philadelphia, PA, U.S.A) was used at a dilution of 1:2000 and monoclonal mouse anti-human sLewX antibody (US biological, Swampscott, MA, U.S.A) at a dilution of 1:10. Samples were incubated for 45 min at 4℃, and then washed with 3 ml of 2% FBS in PBS by centrifugation at 500×g for 5 min 2 times. Cell pellets were resuspended in 1 μg of a goat anti-mouse IgG antibody (DiNonA, Seoul, Korea) conjugated with phycoerythrin (PE) diluted with 50 $\mu\ell$ of RPMI1640 containing 10% FBS, 0.2% NaN_3, and 50 mM Hepes. After incubation for 30 min at 4℃, samples were washed with 3 ml of 2% FBS in PBS two times. Cells were resuspended in 500 $\mu\ell$ of RPMI1640 containing 2% FBS, 0.2% NaN_3, and 50 mM Hepes and

subjected to FACScan analysis (FACSCalibur flow cytometer; Becton Dickinson, Heidelberg, Germany).

7. *In vitro* transduction studies

To determine transduction efficiencies, rapidly proliferating cultures of cells were infected with either a non-targeted control virus, dl324/LacZ, or a CAR binding-ablated virus, dl324-420A/LacZ at various MOIs in minimal volume. At 2 days post infection, β-galactosidase activity was measured. Individual experiments were performed using three sets of cells, and all experiments were repeated at least three times.

8. β-galactosidase assay

For analysis of β-galactosidase expression, cells were plated into six-well plates at 5 X 10^5 cells per well then cells were infected 24 hr later with various viruses at an MOI of 0.1 to 10. β-galactosidase activity was visualized 48 hr later using 5-bromo-4-chloro-3-indolyl-β-D galactopyranoside (X-Gal; Life Technologies, Rockville, MD, U.S.A) staining according to standard techniques. To quantify β-galactosidase expression, the enzymatic activity of cell extracts was measured using o-nitrophenyl β-D-galactopyranoside (ONPG; Sigma, Deisenhofen, Germany) as substrate. The unit of ONPG assay was evaluated with ELISA reader at 450nm.

9. Oncolytic assay

To evaluate the cytopathic effect of modified viruses, $2\text{-}3\times10^4$ cells were plated in 24-well plates to about 60-80% confluences, and then infected with various viruses at an MOI of 0.1 to 10. Their killing effect was monitored day by day under a microscope. At the moment that the cells infected with any kind of adenoviruses were completely cytolysed, cells remained on the plate were then stained with 0.5% crystal violet in 50% methanol.

10. Anti-tumor effect of sLewX-retargeted adenovirus in human cancer xenograft

Male athymic *nu/nu* mice were obtained at 6-8 weeks of age from Charles River Japan Inc. Tumors were initiated on the abdomial wall of each animal by subcutaneous implantation of 1×10^7 cells in 100 $\mu\ell$ of PBS. Tumor growth was monitored at 2- to 3-day intervals by measuring the length(L) and width(w) of the tumor with a caliper and calculating tumor volume on the basis of the following formula: volume = $0.523 \times Lw^2$. When tumors were in the range of 60-70 mm^3 in volume, animals were randomized by tumor size into three groups of 6 animals each. The first day of treatment was designated as day 1. Tumor growth continued to be monitored by taking measurements at 2- to 3-day intervals. Adenoviruses were administered intratumorally (5×10^8 PFU per tumor in 50 $\mu\ell$ of PBS) on days 1, 3, 5, 7, and 9. When the tumors had reached the maximum acceptable size, the mice were sacrificed, and their tumors were excised and analyzed by hematoxylin and eosin staining. Tumor responses to each treatment were compared by the two-tailed *t*-student test.

Ⅲ. Results

1. Isolation of sLewX-specific peptides

To isolate sLewX-specific peptides, we utilized three bacteriophage libraries with random septapeptide, cyclic septapeptide, or dodecapeptide inserts at the N-terminus of pIII protein for *in vitro* random phage display library panning. After three rounds of selection, 23 positive phage clones were analyzed, and 18 different sLewX-specific peptide sequences were obtained. Among them, one 12mer-peptide sequence (#1 12mer-peptide) was found to be same in 6 clones, suggesting that this peptide might have the highest affinity to sLewX. When searched for #1 12-mer peptide (SHWDQ**PRPGLKP**) in online databases (through the National Center for Biotechnology Information (NCBI), it was found that p30 adhesion protein of *Mycoplasma pneumoniae* is highly homologous (86% amino acid sequence homology). Interestingly, 12 amino acid sequence was found to be repeated 8 times in the carboxy terminal of p30 adhesion protein, and this recurring sequence (PQPGMAPRPGMP) was selected as a targeting epitope and named as "p30". Interestingly, it was revealed that seven amino acids **PRPGLKP** in 12mer was highly conserved. Thus, this seven amino acids was named as "7mer" and used as a targeting epitope as well. With BLAST search, #2 12-mer sequence (AHWIPRYSSPAT) was found to be homologous to trans-sialidase of *Trypanosoma cruzi,* and this sequence was named as "12mer". The sequences of three selected peptides are following; 7mer: PRPGLKP, 12mer: AHWIPRYSSPAT, p30:

PQPGMAPRPGMP. Subsequently, these three selected peptides (12mer, 7mer, and p30) were then used as targeting epitopes for the construction of retargeted adenoviral vectors (Fig 6).

2. Evaluation of binding affinity of targeting peptides by ELISA

To evaluate binding affinity of selected peptides by phage display, ELISA was achieved. 12mer peptide was added to plate coated with sLewX-BSA. As peptide concentration was increased, binding affinity was also increased (Fig 7). Thus, it suggested that selected peptide 12mer had binding affinity to sLewX.

3. Ablation of CAR binding site in adenovirus fiber

The development of tissue-selective adenovirus requires the generation of adenovirus vectors which lack native receptor binding and additionally contain domains which redirect the vector to tissue-specific receptors. Towards this goal, we introduced mutation to change 420th amino acid, lysine (AAA), of fiber knob, to alanine (GCA). This amino acid change was then confirmed by sequencing (Fig 8).

4. Generation of sialyl Lewis X-specific adenovirus

As described in Materials and Methods, dl324/LacZ, dl324-420A/LacZ, dl324/LacZ/p30, YKL-1/7mer, YKL-1/12mer, YKL-1/p30, YKL-1-420A, and YKL-1-420A/p30 adenoviruses were generated. All viruses were propagated in HEK293 cells and purified

	Sequence	Homology search
1	SHWDQ*PRPGLKP*	P30 adhesion (*Mycoplasma pneumonia*)
2	AHWIPRYSSPAT	Trans-sialidase (*Trypanosoma cruzi*)

Amino acid sequence of p30 adhesion protein

EPAPQPVPVPPQ PQVQINFGPRTGFP *PQPGMAPRPGMP*
PHPGMAPRPGFP *PQPGMAPRPGMP* *PHPGMAPRPGFP*
PQPGMAPRPGMP *PHPGMAPRPGFP* *PQPGMAPRPGMQ*
PPRPGMPPQPGFP

↓

7mer : PRPGLKP
12mer : AHWIPRYSSPAT
p30 : PQPGMAPRPGMP

Fig 6. Analysis of sequences of selected peptides. The sequences of sLewX-specific peptides selected by phage display panning were BLAST searched against database in genbank. Among them, #1 12-mer sequence was found to be highly homologous to p30 adhesion protein of *Mycoplasma pneumoniae*, and #2 12-mer sequence to trans-sialidase of *Trypanosoma cruzi*. In addition, #1 12-mer amino acid sequence was found to be repeated 8 times in the carboxyl terminus of p30 adhesion protein. In #1 12-mer sequence homologous to p30 adhesion protein, PRPGLKP, was referred to 7mer. #2 12-mer sequence and repeated sequence of p30 adhesion were also selected, and named as 12mer and p30, respectively.

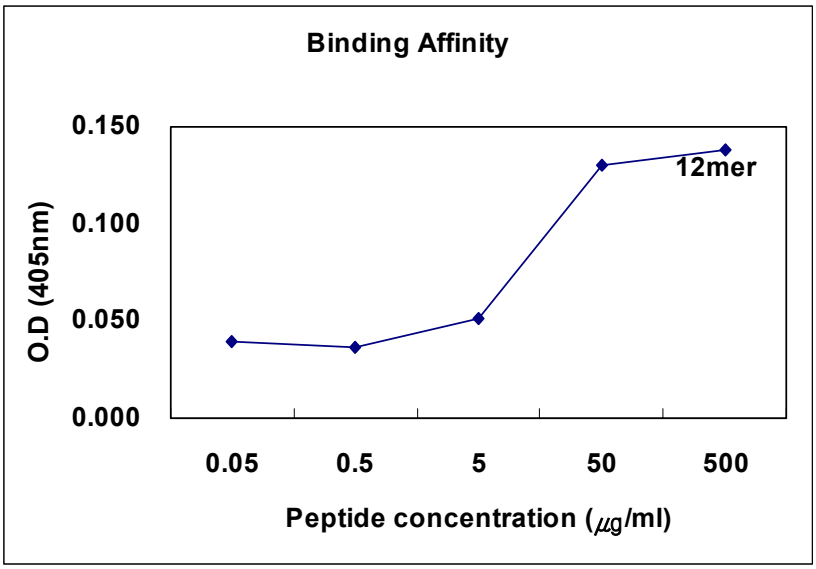


Fig 7. Evaluation of 12mer peptide binding by ELISA. sLewX-BSA conjugate at a concentration of 5㎍/ml in 0.1 M NaHCO3 (pH 8.6) were immobilizd on microtiter plates overnight at 4℃. Remaining binding sites were blocked with PBS, 3% BSA at 37℃ for 1 hr. Peptides diluted in PBS to concentrations ranging from 0.5 to 500 ng/ml was added, and then incubated at 37℃ for 1 hr. After washing with PBS, bound peptides was detected by incubation with HRP-conjugated streptoavidin diluted 1/2500. Following incubation at room temperature for 1 hr, the cells were then washed and incubated with a ABTS as substrate for 45 min. Plates were read in a microtiter plate reader set at 405 nm.

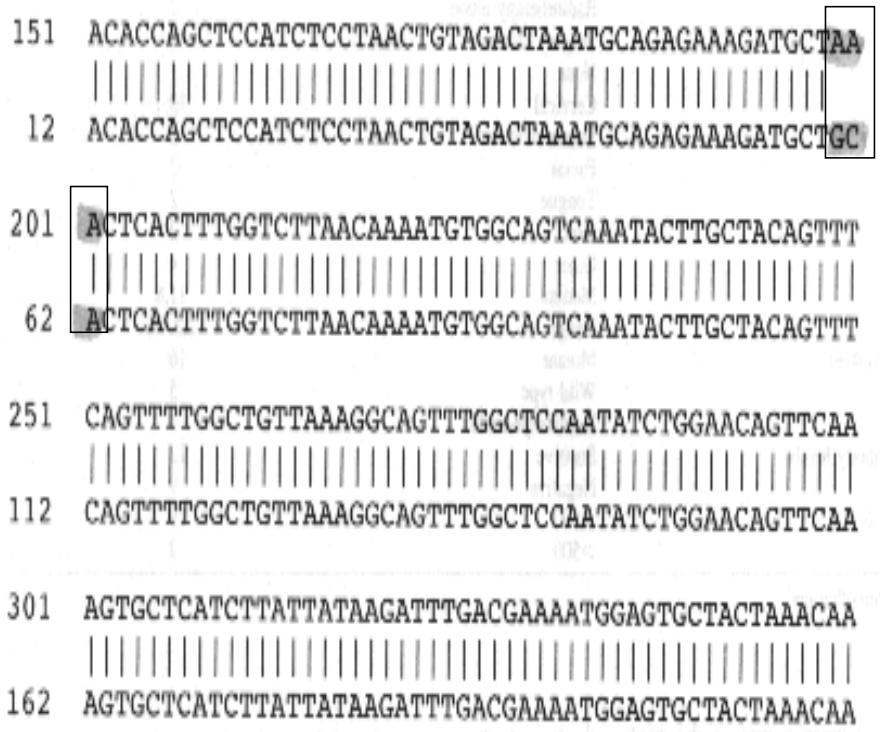


Fig 8. Confirmation of amino acid change for CAR binding ablation by nucleotide sequence analysis. Lysine (AAA) at 420th amino acid of fiber was changed to alanine (GCA) for CAR binding ablation by site-directed mutagenesis.

according to standard methods.

5. Evaluation of CAR and sLewX expression by FACS

To determine the expression level of CAR and sLewX on the cells used for oncolytic assay, FACS analysis was carried out (Table 1). The mean fluorescence intensity of cells incubated with both RmcB antibody and the FITC-conjugated secondary antibody was substracted by the mean fluorescence intensity of cells incubated with the FITC-conjugated antibody alone, and these are summarized in Table 1. While CAR expression of HCT116, Chang, and U251N were high, U118MG, MCF-7, and Lec2 showed low CAR expression (Fig 9). It was previously reported that U118MG[47], and Lec2[39] low or rarely express CAR. Moreover, Pro5, Ycc-2, and H460 exhibited low CAR, and C33A and Hep3B expressed CAR at high levels.

To evaluate the level of sLewX, cells were incubated with the anti-sLewX antibody followed by PE-conjugated secondary antibody. The determination of mean value was identical to that of CAR. SiHa and Ycc-1 significantly expressed sLewX, and MCF-7, U251N and U343 also showed high sLewX expression. On the other hand, CHO-K1, HeLa, and 173We expressed sLewX at very low level (Table 1). Especially, the expression of sLewX in CHO-K1 was about 200 times lower (mean value 2.14) than SiHa which expresses sLewX at high level (mean value 443.28).

This result showed relation of CAR and sLewX expression with cytopathic effect of sLewX-retargeted adenovirus. For example, MCF-7 was not infected with non-retargeted adenovirus (control

		Cell type	CAR	sLewX
1	SiHa	Cervical cancer	58.92	443.28
2	C33A	Cervical cancer	81.33	93.62
3	H460	Lung cancer	7.43	12.63
4	A549	Lung cancer	31.51	21.32
5	Hepl	Hepatoma	73.87	45.24
6	HCT116	Colon cancer	56.09	7.39
7	U343	Glioma	237.34	76.88
8	U251N	Glioma	88.81	68.5
9	Pro5	Hamster ovary	0.48	25.68
10	Lec2	Hamster ovary	0.36	60.72

Table 1. The expression level of CAR and sLewX determined by FACS analysis. FACS analysis was performed as described in materials and methods. The level of CAR and sLewX expression was expressed by mean value of fluorescence intensity.

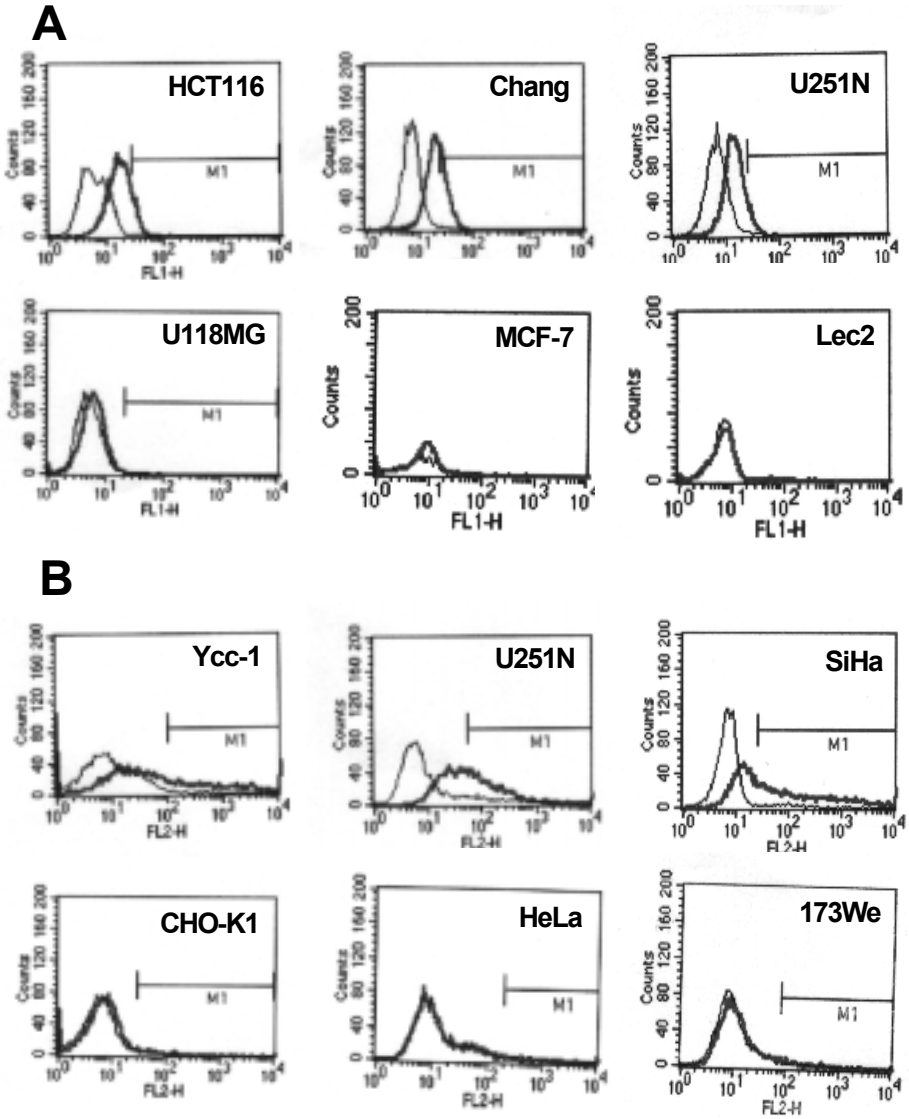


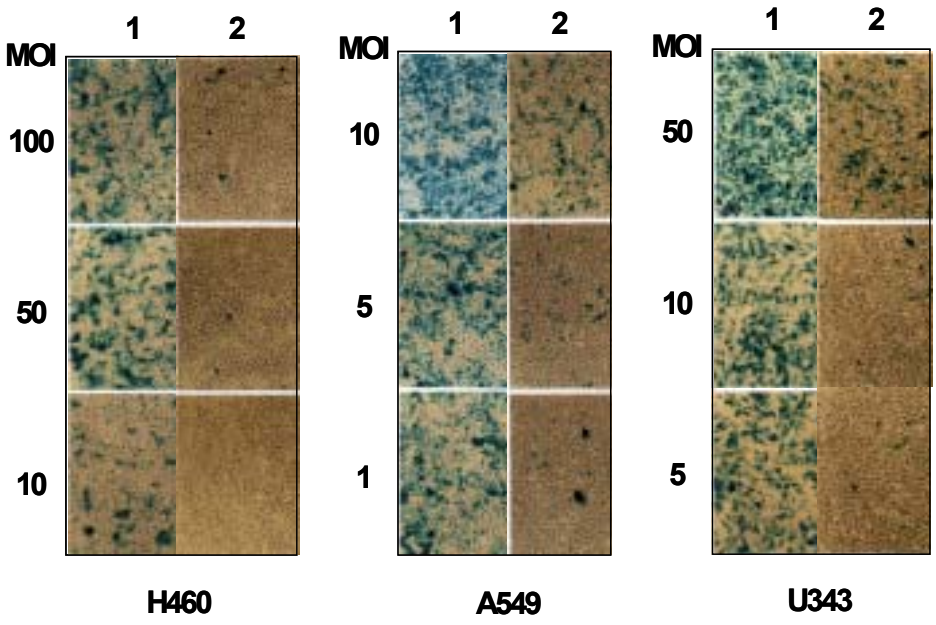
Fig 9. Flow cytometric analysis of CAR and sLewX expression in various cells. Cells were incubated with (A) anti-CAR (RmcB) or (B) anti-sLewX monoclonal antibodies, followed by incubation with secondary FITC or PE-labeled antibody as described in materials and methods. In both A and B, Upper panel shows cell lines expressing high CAR or sLewX and lower panel shows expressing low CAR or sLewX.

adenovirus) well, while it was infected with retargeted-adenovirus 5-10 times better than non-retargeted adenovirus (data not shown).

6. Analysis of adenoviral transduction efficiency

To evaluate transduction efficiency of CAR binding-ablated adenovirus, a panel of cells were infected with a E1, E3-deleted adenovirus expressing LacZ (dl324/LacZ) or a CAR binding-ablated adenovirus (dl324-420A/ LacZ) at an MOI of 0.1 to 100. Cells were then stained with X-Gal at 2 days post infection (Fig 10). When equal numbers of particles of adenovirus were used for the infection, the transduction efficiency of dl324-420A/LacZ was reduced substantially relative to that of control adenovirus dl324/LacZ. Most of cells were efficiently infected with dl324/LacZ, but not with CAR binding-ablated adenovirus dl324-420A/LacZ. At an MOI of 100, approximately 80-90% of H460 cells were transduced with dl324/LacZ, while less than 5% of cells were tranduced with CAR binding-ablated adenovirus dl324-420A/LacZ, suggesting that CAR binding site of dl324-420A/LacZ was efficiently ablated. Same result was seen to the other cells. In the case of the SiHa, more dramatic reduction of transduction efficiency of CAR binding-ablated adenovirus was observed in comparison to control adenovirus.

To quantify enzyme activity of β-galactosidase in cells infected with dl324/LacZ or dl324-420A/LacZ, β-gal assay was performed at 2 days post infection (Fig 11). When SiHa was infected with dl324/ LacZ, β-galactosidase enzyme activity was nearly 8-fold higher than


MOI
1
2
100
50
10
H460
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A549
MOI
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10
5
U343
MOI
1
2
50
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SiHa
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HCT116
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Ycc-2

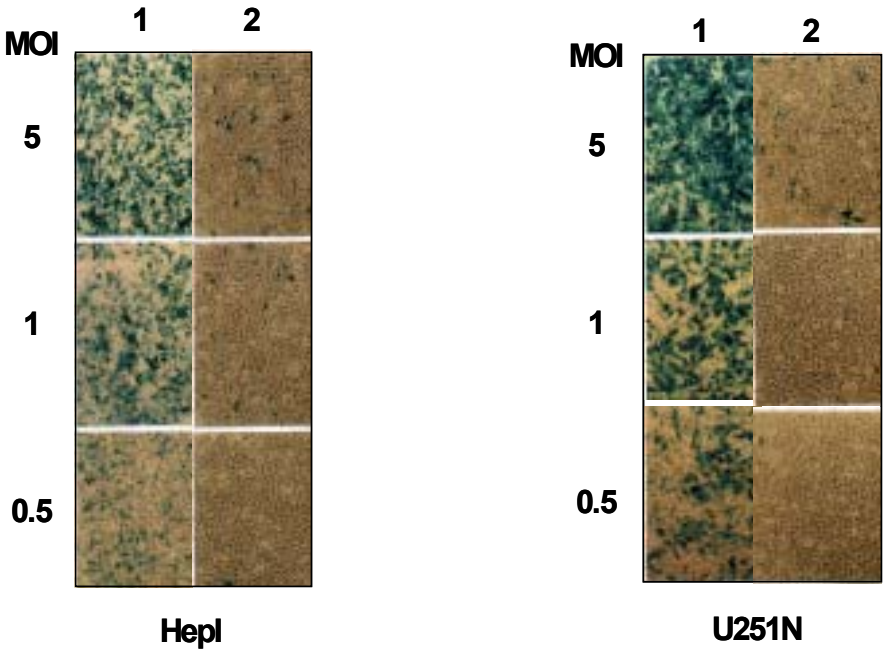

Fig 10. Cells were tansduced with the dl324/LacZ (Lane 1) or dl324-420A/LacZ (Lane 2) adenovirus, as indicated above the columns, and with different amounts of virus, as indicated besides the rows. After 24 h post infection, the cells were analyzed for β-galactosidase expression by staining the monolayers with X-Gal as described in materials and methods. CAR binding-ablated Ad showed significant decrease in transduction efficiency.

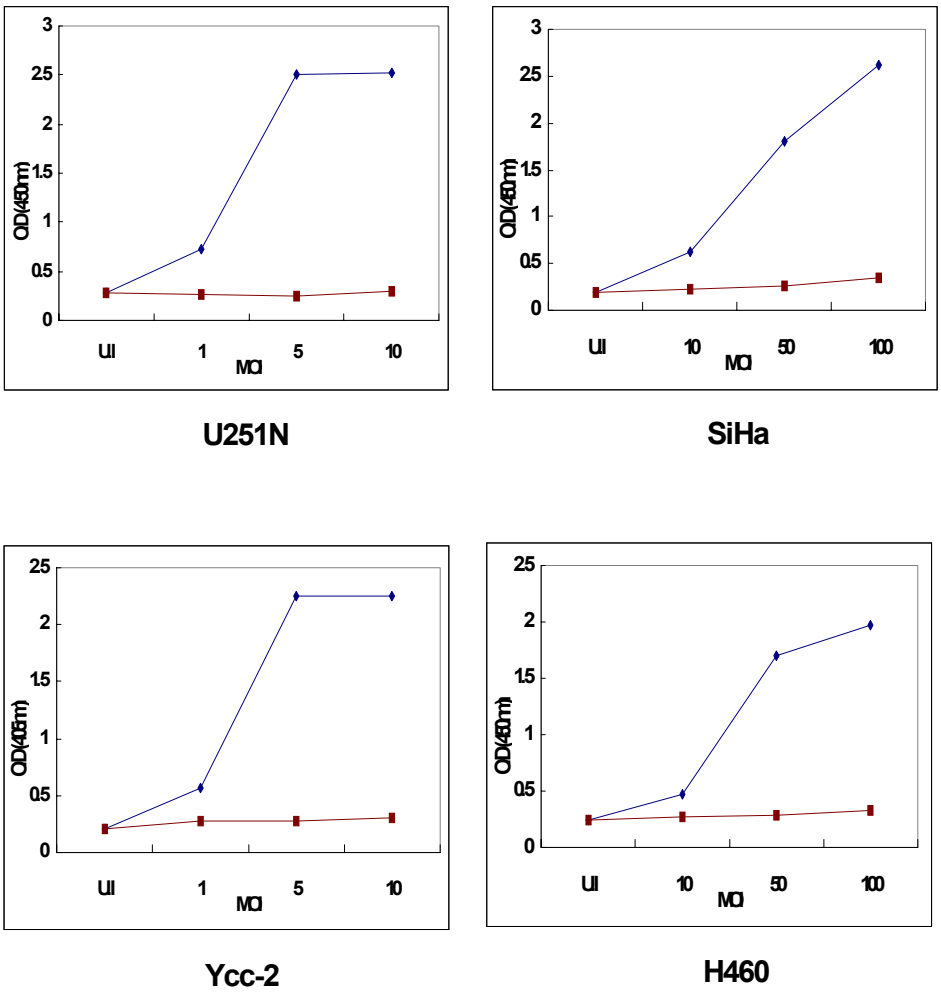

Fig 11. Adenovirus-mediated transduction properties of human cancer cell lines. Cells were transduced with indicated MOIs of dl324/LacZ (◆) or dl324-420A/LacZ (■) adenovirus. After 48 h post infection, β-gal assay was carried out. The β-galactosidase enzyme activity in CAR binding-ablated virus dl324-420A/LacZ transduced cells was as low as in uninfected cell, whereas β-galactosidase enzyme activity in dl324/LacZ trasnsduced cells was increased in response to increasing MOIs of virus treated .

cells infected with CAR binding-ablated virus dl324-420A/LacZ at an MOI of 100. The other cells showed similar results, too. Generally, cells infected with wild-type adenoviruses were tranduced 3-9 times better than CAR binding-ablated virus. Therefore, it was suggested that CAR is important to adenovirus infection, and amino acid change at 420 in fiber knob could abolish CAR binding site.

7. Oncolytic potential of CAR binding-ablated and sLewX-retargeted adenovirus

To determine which isolated peptide is best fit for targeting strategy to sLewX-expressing cancer cells, cell cytotoxicity was analyzed by an *in vitro* cytopathic effect (CPE) assay and compared in a range of different cancer and normal cells. Cells were grown to 70% confluence at which time cells were infected at an MOI of 0.1 to 10 with YKL-1/7mer, YKL-1/12mer, or YKL-1/p30, which incorporates sLewX-specific peptides 7mer, 12mer, or p30 in fiber knob of replication-competent oncolytic adenovirus YKL-1, respectively (Fig 12). As controls, cells were infected with a non-replicating adenovirus dl324/LacZ in parallel. Cells were then monitored everyday and incubated until cells infected with any kind of adenoviruses were completely cytolysed by viral replication.

As shown in Figure 12, dl324/LacZ infected cells showed no CPE because there is no viral replication mediated cell lysis at all. YKL-1/p30 showed most marked CPE among three sLewX-specific peptides against the human cancer cell lines tested. The cell lysis capacity of YKL-1/p30 is about 5-10 times greater than either

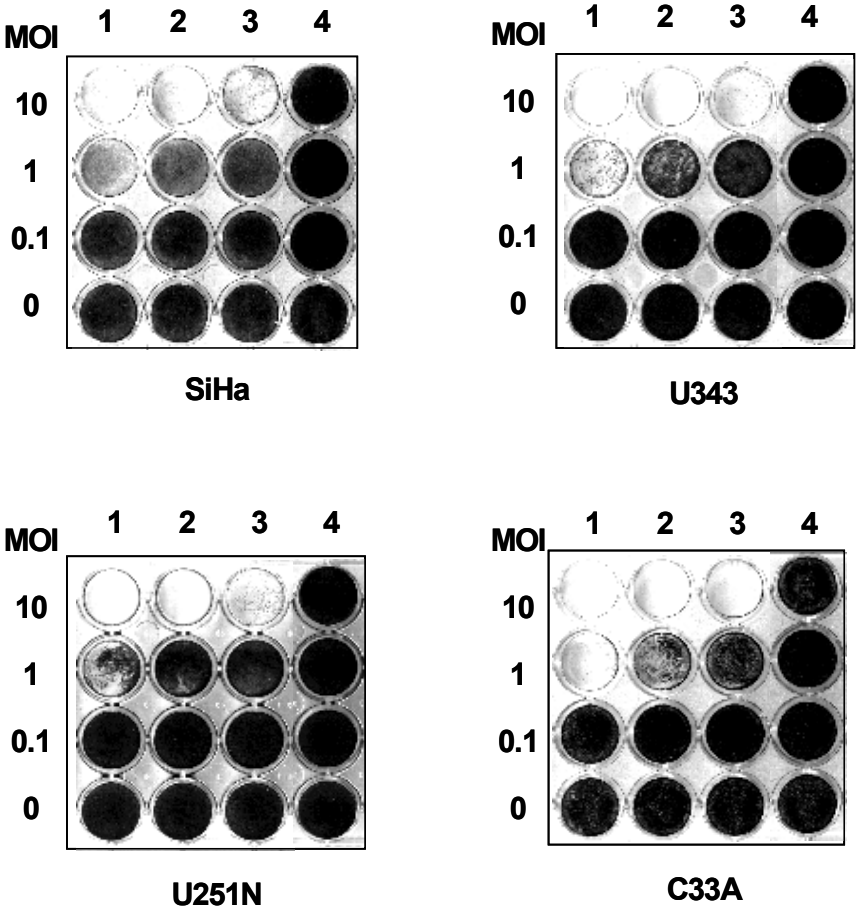

Fig 12. Oncolytic assay with YKL-1/p30, YKL-1/12s, or YKL-1/7s adenovirus for comparison of sLewX-specific peptides. Lane 1, YKL-1/p30; Lane 2, YKL-1/12s; Lane 3, YKL-1/7s; Lane 4, dl324/LacZ. Cells were infected at indicated MOIs. Replication incompetent adenovirus dl324/LacZ showed no CPE at all. NIH3T3 mouse fibroblast cells were not lysed by any of viruses tested in this study, whereas cancer cells were lysed by replication competent adenoviruses. YKL-1/p30 exerted most significant cell lytic effect among 3 different kinds of epitope containing oncolytic adenoviruses.

YKL-1/7mer or YKL-1/12mer in U343, U251N, and C33A cells. while NIH3T3 mouse fibroblast cells were not cytolysed by any replication-competent viruses. Theses results demonstrate that these three fiber modified-oncolytic adenoviruses retain activity of killing cancer cells. As YKL-1/p30 showed most efficient CPE among three sLewX-specific peptides tested, it was selected for the next following studies.

To investigate the ability of cytolysis of CAR binding-ablated and sLewX retargeted virus, various kinds of cells were infected with YKL-1-420A or YKL-1-420A/p30, along with a replication-incompetent adenovirus dl324/LacZ as a negative control (Fig 13). As YKL-1 is E1B55KDa-deleted adenovirus, p53-defective cancer cells should be selectively cytolysed. As in Fig 13, sLewX-retargeted virus YKL-1-420A/p30 efficiently cytolysed most of cells 10-100 times better than non-retargeted virus YKL-1-420A. Because YKL-1-420A is a CAR binding-ablated virus, it did not efficiently infect cells and no cytolysis was resulted like a replication-deficient virus dl324/LacZ. In the other hand, YKL-1-420A/p30, which incorporated a targeting peptide p30 in the fiber knob, could infect cells well so as to cytolyse efficiently.

8. Antitumoral efficacy of sLewX-retargeted Ad in *nu/nu* mice

To further assess the anti-tumor activity of sLewX-retargeted adenovirus YKL-1-420A/p30, the growth of U343 tumors treated with YKL-1-420A/p30 were compared with the growth of tumor treated with YKL-1-420A as a control in *nu/nu* mice. In tumors derived from U343

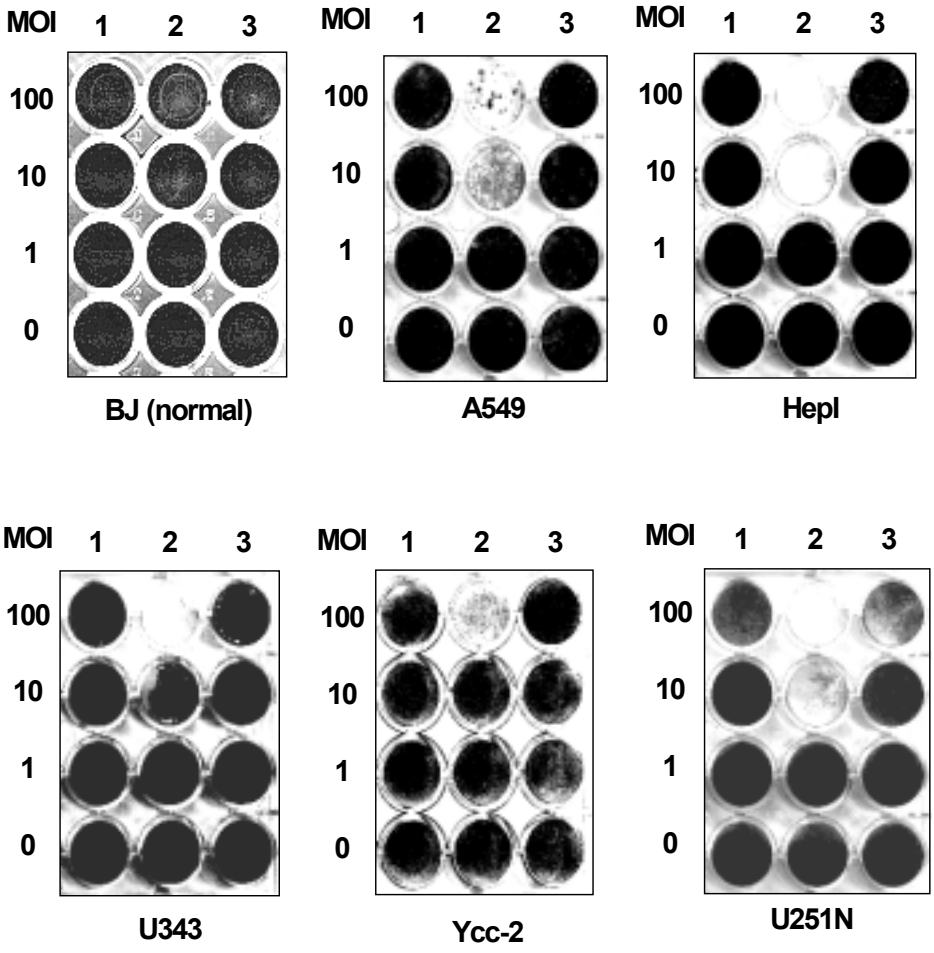

Fig 13. Cytopathic effect of fiber modified Ad on a various human cancer cell lines. Monolayers of cells were infected with YKL-1-420A (Lane 1), YKL-1-420A/p30 (Lane 2), or dl324/LacZ (Lane 3) with different amounts of virus, as indicated besides the rows. Replication-incompetent adenovirus dl324/LacZ served as a negative control. Retargeted Ad (YKL-1-420A/p30) cytolysed cells about 10-100 times better than CAR binding ablated Ad (YKL-1-420A).

cells, treatment with the YKL-1-420A/p30 exerted more significant suppression in tumor growth when compared with that of YKL-1-420A. By day 15, YKL-1-420A/p30 treated tumors were less than one-fourth of the size of YKL-1-420A treated tumors (YKL-1-420A/p30, 50 ± mm^3; YKL-1-420A, 165± mm^3) (Fig. 14).

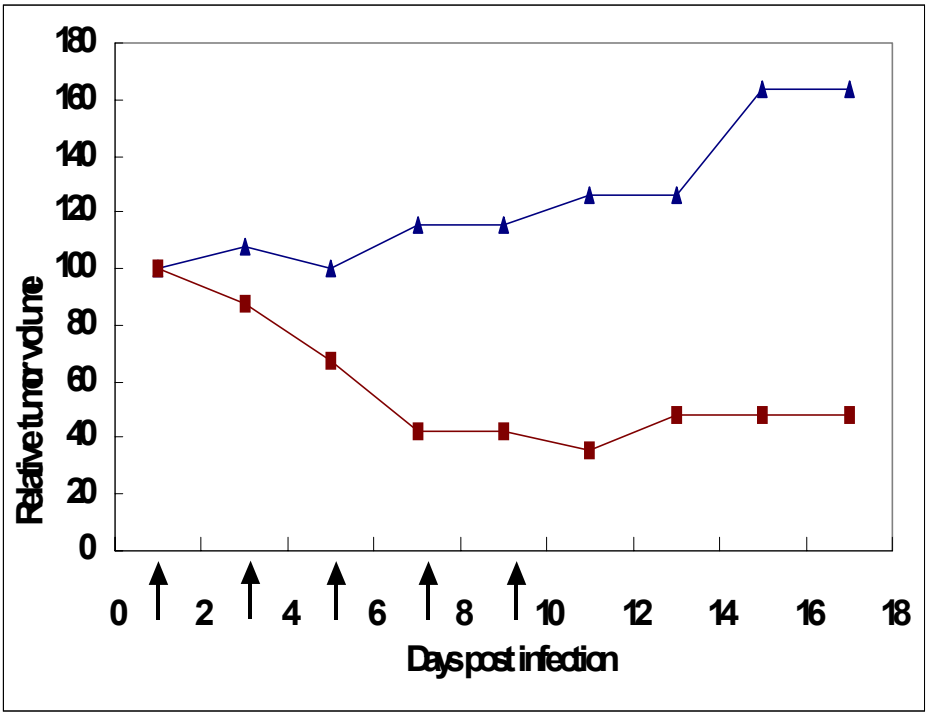

Fig 14. Anti-tumoral effect of sLewX-retargeted Ad in U343 xenograft model. Tumors were established in female nude mice by subcutaneous injection of 1×10^7 cells and allowed to grow to an average size of 60-70 mm^3. Animals with established tumors were randomized into two treatment groups. Each group received intratumoral injection of YKL-1-420A (▲) or YKL-1-420A/p30 (■) adenovirus (5×10^8 PFU) in 50 μl of PBS on days 1, 3, 5, 7, and 9 (vertical arrows). Relative tumor volume was analyzed using the following equation: tumor volume at the time point of analysis/tumor volume at the time of the first virus injection.

Ⅳ. Discussion

The development of tissue/cell selective Ad requires the generation of Ad vectors which lack native receptor binding and additionally contain domains which redirect the vector to tissue/cell specific receptors. Based on the knowledge of native viral binding, a rational place to begin in the development of genetically targeted vectors is with the knob domain, and most strategies have so far focused on this region. Wickham and colleagues reported a number of carboxy terminal modifications, including one containing an RGD motif (21 amino acids) to target cell surface integrins[45] and one containing seven lysine residues to target cell surface heparan sulfates[46]. Following these previous reports, we have generated sLewX-retargeted Ad. First, CAR binding was ablated by site-directed mutagenesis. Roelvink et al.[41] reported a conserved receptor-binding region on the side of three divergent CAR-binding knobs through sequence analysis and mutagenesis. The feasibility of simultaneous CAR ablation and redirection of an Ad to a new receptor was demonstrated. CAR-binding site was revealed to consist of residues from the AB loop, the B β sheet, and the DE loop that is the fiber 5 knob residues S408, P409, K417, K420, and Y477. These sites are located on the side of the knob, and do not overlap two adjacent monomers. This finding implies that the trimeric knob binds to three CAR molecules independently. Among those sites, we chose K420 and changed lysine (K) at 420 to alanine (A). As a result, cells infected with wild-type CAR binding Ad were

transduced 3-9 times better than CAR binding-ablated virus. Therefore, it was suggested that CAR is important to Ad infection, and amino acid change at 420 in fiber knob could abolish CAR binding site.

As progress is being made in the development of retargeted vectors, the importance of identifying truly cell-specific ligands has been highlighted. Although there are many established ligands and antibodies that may be candidates in certain settings, in many cases, such as mature airway epithelium, truly specific targets have yet to be discovered; therefore, further target definition is required. This is especially relevant in the context of those genetic retargeting strategies that attempt to target with small peptide ligands. In this regard, the use of bacteriophage panning techniques have shown potential utility in target definition[47,48].

We have adopted an *in vitro* random phage display library panning on sLewX to develop Ad vectors capable of the selective infection of the cancer cells with respect to the normal cells. Using the strategy, more than 20 individually isolated phage were tested for the ability to bind specifically to sLewX and the sequences of these novel peptides were identified. Among them, three peptides were selected and fused to the C-terminus of the fiber protein of the Ad vectors.

The mechanism of Ad binding and internalization suggests that in order to be susceptible to Ad infection, a target cell should express sufficient levels of both CAR and integrins. Cells with down-regulated CAR expression poorly support Ad infection,

resulting in low viral yields[49]. Our results also showed that MCF-7 cells and BJ normal cells, which express CAR at low level, were not infected efficiently by Ad with normal fiber. CAR deficiency has been reported to hinder Ad-directed gene transfer to various types of cancer cells, such as squamous cell carcinoma[50], glioma[51], melanoma[52], and bladder cancer cells[53]. In certain clinical settings, the CAR dependence of the Ad vector may result in sequestration of recombinant virions by nontarget, yet high-CAR-expressing cells, whereas the true target cells, if low in CAR, will be poorly transduced. To compensate for this sequestration, a significant escalation in the dose of administrated vector is needed, which increases the risk of inducing both direct toxicity and host immune responses against the vector, thus further compromising the overall efficacy of the therapy. The most reasonable approach to this problem is to create a novel generation of Ad vectors capable of delivering therapeutic payloads in an addressed manner selectively to those cell types which constitute a target in the context of a particular disease. On this account, genetic targeting seems to be the most promising approach for generation of targeted Ad vectors for clinical gene therapy applications. Next, we incorporated three sLewX-specific peptides selected by phage display to C-terminus of fiber knob. sLewX is a well-established tumor-associated carbohydrate antigen, which is frequently up-regulated in breast, stomach, and colon cancers. Therefore, retargeted Ad vectors could be capable of CAR-independent gene delivery to sLewX-expressing tumor cells, allowing the achievement of both cell-specific gene

delivery as well as gene transfer efficiency augmentations.

Because genetic targeting of Ad vectors is based on the physical incorporation of ligands into the Ad particle during biosynthesis of viral proteins *in vivo*, these ligands should be compatible with both the structure of these proteins and the biosynthetic pathways characteristic of Ad capsid components. As the fiber normally mediates the initial binding of Ad to cell surface receptors, it would be reasonable to consider it the primary candidate for a ligand-carrying and its localization within the Ad virion favor the most efficient interaction with a receptor. Simple considerations of geometry suggest that the carboxy-terminal knob domain of the fiber, which is most distal from the surface of the capsid, has the best chance to come into contact with the cell surface. Thus, the knob should be considered the most rational locale in the fiber molecule for insertion of receptor-targeting ligands. Initially, the carboxy terminus of the molecule was proposed as a locale for incorporation of targeting ligands. Michael et al.[54] showed that coupling of the gastrin-releasing peptide (GRP) to the end of the fiber via a flexible linker allows for interaction between the GRP ligand and an anti-GRP antibody serving as a receptor mimic. Augmented gene transfer using Ad vectors with a carboxy-terminal polylysine modification of the fiber protein has been observed in a variety of CAR-deficient cell types, including macrophages[56], smooth muscle cells[55], fibroblasts[55,56], endothelial cells[55], T cells[55], glioma cells[57], acute myeloid leukemic cells[58], myeloma cells[59], and skeletal muscle cells[60,61]. Depending on the levels of CAR expression by these

cells, the targeted Ad vectors achieved up to 500-fold increased efficiency of gene delivery.

Okada Y et al[62] found that fiber-mutant Ad containing RGD sequence in the fiber knob remarkably augmented gene transduction efficacy in melanoma cells by targeting αV integrins. In addition, intratumoral injection of RGD fiber-mutant Ad containing the tumor necrosis factor α gene (Ad-RGD-TNFα) revealed dramatic anti-tumor efficacy through hemolytic necrosis in an established murine B16BL6 melanoma model. Ad-RGD-TNFα required one-tenth the dosage of Ad-TNF α to induce an equal therapeutic effect. Mizuguchi H et al[63] also use a fiber-modified Ad vector containing an RGD peptide motif in the fiber knob and expressing HSV-tk(Herpes simplex virus-thymidine kinase). Ad-RGD-tk showed approximately 25 times more antitumor activity than Ad-tk. Histopathological studies suggested that liver damage in mice injected with Ad-RGD-tk was significantly lower than that in mice injected with Ad-tk. Magnusson MK et al[64] deleted the knob and the last 15 shaft repeats of the fiber gene and replaced with an external trimerization motif and a new cell-binding ligand, in this case the integrin-binding motif RGD.

E1B55KDa-deleted Ad YKL-1 was used as viral backbone to generate sLewX-retargeted adenovirus YKL-1-420A/p30. This virus showed selective cytolytic ability in p53-defective cancer cells like parental virus YKL-1[28]. sLewX-retargeted virus YKL-1-420A/p30 efficiently cytolysed various cancer cells about 10-100 times better than non-retargeted virus YKL-1-420A. Because YKL-1-420A is a CAR binding-ablated virus, it did not infect cells efficiently

and no cytolysis was resulted. In the other hand, YKL-1-420A/p30, which incorporated a targeting peptide p30 in the fiber knob, could infect cells well so as to cytolyse efficiently. In conclusion, these results demonstrate that p30 is a good candidate for targeting motif, and YKL-1/p30 can be used as an effective gene delivery vector for sLewX expressing cancer cells.

Ⅴ. Conclusions

To create a novel generation of Ad vectors capable of infecting selectively to target cell, it is required to eliminate native Ad tropism to CAR and to generate novel tropism to an alternative cellular receptor. In this perspective, we have developed sLewX-retargeted Ad. Among the three sLewX-specific peptides (12mer, 7mer, and p30) isolated by utilizing *in vitro* random phage display library panning, p30 peptide was superior to redirect Ad tropism to sLewX. The cell lysis capacity of YKL-1/p30 was about 5-10 times greater than either YKL-1/7mer or YKL-1/12mer and sLewX-retargeted virus YKL-1-420A/p30 efficiently cytolysed most of cells 10-100 times better than non-retargeted virus YKL-1-420A. Therefore, p30 is a good candidate for targeting epitope, and YKL-1/p30 can be used as an effective gene delivery vector for sLewX expressing cancer cells.

In conclusion, amino acid change at 420 in fiber knob could abolish CAR binding site, and p30 was demonstrated to be a good candidate for targeting sLewX expressing cancer cells. In particular, sLewX-specific targeted adenovirus YKL-1-420A/p30 allowed CAR-independent gene delivery as well as cell-specific gene delivery, and showed a significant promise as a targeting replicative adenovirus vector for cancer therapy.

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국문요약

종양관련항원 sLewX 표적화 아데노바이러스벡터의 개발

Sialyl Lewis X (sLewX)는 종양관련항원으로 암세포가 내피세포에 부착될때 리간드로 작용하여 암세포의 전이를 돕고, sLewX 발현이 증가될수록 위암, 대장암, 폐암, 난소암과 같은 다양한 암의 예후가 나쁘다는 보고가 있다. 따라서 우리는 sLewX에 대한 *in vitro* random phage display법을 이용하여 20여개의 phage를 분리하여 sLewX 특이적 결합능과 펩타이드들의 염기서열을 확인하였고, sLewX가 많이 발현되는 암세포에 선택적으로 감염되는 아데노바이러스벡터를 개발하였다.

특정조직 및 세포로 표적화된 아데노바이러스를 개발하기 위해서는 바이러스 본래의 세포수용체와의 결합능을 없애고, 새로운 세포수용체와의 결합능을 첨가해주어야 한다. 우리는 CAR 결합능이 배제된 아데노바이러스인 YKL-1/420A를 개발하였고, 그 결과 비교군의 아데노바이러스에 비해 유전자 전달효율이 급격히 감소하는 것을 확인하였다. 여기에 phage display법으로 분리한 펩타이드 중 3개를 선택하여 바이러스의 fiber C 말단에 결합시켜 YKL-1-420A/7mer, YKL-1-420A/12mer, YKL-1-420A/p30을 만들었다. YKL-1-420A/p30은 YKL-1-420A에 비해 10-100배 가량 증가된 세포살상능력을 보였으며, xenograft 종양모델에 처치한 결과 YKL-1-420A에 비해 급격하게 종양크기가 감소하였다. 따라서 아데노바이러스의 재표적화는 유전자치료에 매우 유용하게 이용될 수 있으며 특히 sLewX에 특이적으로 결합하는 아데노바이러스인 YKL-1-420A/p30은 세포특이적이며 CAR에 비의존적인 유전자전달을 가능하게 하였다.

핵심되는말 : 아데노바이러스, sialyl Lewis X, phage display,
재표적화, 유전자치료, 표적화 펩타이드