의학 박사학위 논문

A Novel Gene Therapy for Uterine Cervical Cancer and Renal Cell Carcinoma Using MN/CA9 Promoter-based Replication Competent Adenovirus

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안 미 원

MN/CA9 프로모터를 가진 Replication-competent 아데노바이러스를 이용한 자궁경부암과 신세포암에 대한 새로운 유전자 요법

지도교수 김 세 중

이 논문을 의학 박사학위 논문으로 제출함.

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감사의 글

이제 한고비를 넘기고 또 다른 세계의 문을 열게 해주신 선생님들과 사랑하는 나의 가족들에게 감사를 드립니다.

늘 격려와 꼼꼼함으로 지도해 주신 김 세중 지도 교수님과 늘 넉넉한 웃음으로 감싸주신 임 호영 교수님께 깊은 감사 드립니다.

학위논문에 많은 조언을 해 주신 최 진혁 교수님, 심사를 해 주신 김 영수, 문 창현 교수님께 감사 드립니다.

다양한 분야까지 공부할 수 있는 기회를 주신 연세 암전이 연구센터의 정 현철 교수님께 진심으로 감사 드립니다.
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학교에만 있어 세상 물정 모르는 딸을 위하여 늘 기도로 눈물 흘린 것 감사해요. 앞으로 더 열심히 해서 멋진 엄마 아빠의 딸이 될께요.

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멀리 외국에서 공부하며 선교하는 동생 명원, 이제 갓 사회 초년생인 된 막내 명옥! 언니를 위해 기도해 줘서 늘 고마워.

그리고 나의 친구들, 함께 해줘서 너무 고마워.

이 작은 자리를 통하여 저의 삶과 함께 해주신 모든 분들에게 감사의 마음을 전합니다.

마지막으로 이 모든 나의 삶을 주관하고 계신 하나님 아버지께 이 작은 성과를 드립니다.

안 미 원 드림

A Novel Gene Therapy for Uterine Cervical Cancer and Renal Cell Carcinoma Using MN/CA9 Promoter-based Replication-competent Adenovirus

by

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- ABSTRACT -

A Novel Gene Therapy for Uterine Cervical Cancer and Renal Cell Carcinoma Using MN/CA9 Promoter-based Replication-competent Adenovirus

Purpose: Recombinant adenoviral vectors have been widely used in preclinical models for *in vitro* and *in vivo* gene transfer. Major obstacles of cancer gene therapy are low efficiency of gene transfer and low selectivity to target cells. To circumvent these problems, selective replication-competent oncolytic virus may be a promising strategy by selectively replicating in the tumor and amplifying the viral dose throughout the tumor.

MN/CA9 is a transmembrane glycoprotein originally detected in highly malignant human cervical carcinoma cell line, HeLa. While the expression of MN/CA9 protein is found in several types of malignancies including uterine cervical cancer and renal cell carcinoma, its expression in normal tissues is restricted to the epithelial cells of gastrointestinal tract.

The author constructed a tumor-specific replication-competent oncolytic adenoviral vector system using MN/CA9 promoter, which replicates only in MN/CA9-expressing cells and demonstrated its therapeutic growth inhibitory effect

in uterine cervical cancer and renal cell carcinoma cell lines.

Materials and Methods: Recombinant adenoviral vector, Ad-MN/CA9-E1A, was constructed utilizing MN/CA9 promoter to regulate adenoviral early protein E1A, which enables the conditional replication of the adenoviral vector in cells that permit MN/CA9 transcriptional activity. Total RNA was extracted from human cervical (HeLa, C-33 A, and SiHa) and renal (SK-RC-52 and SK-RC-29) cancer cell lines grown in culture. Expression of MN/CA9 in cells was determined by RT-PCR. Adenoviral E1A protein production in cells following infection with Ad-MN/CA9-E1A was determined by western blot analysis. Cytotoxicity of tested cell lines with Ad-MN/CA9-E1A was evaluated with *in vitro* killing assays. Subcutaneous tumor of HeLa cell line was established in athymic nude mice and Ad-MN/CA9-E1A virus or PBS was intratumorally injected.

Results: RT-PCR assay identified the expression of MN/CA9 cDNA message in HeLa, C-33 A, SiHa, and SK-RC-52 cell lines. The MN/CA9 expression was not detected in SK-RC-29. After Ad-MN/CA9-E1A infection, MN/CA9-positive cells (HeLa, C-33 A, SiHa, and SK-RC-52) showed strong expression of E1A protein, ranging in size from approximately 35 to 46 kDa. On the other hand, MN/CA9-negative cell (SK-RC-29) showed a much lower level of E1A expression. *In vitro* killing assays revealed conditional replication and cytolytic effect of Ad-MN/CA9-E1A in MN/CA9-positive cell lines (HeLa, 0.1 MOI of virus; C-33 A, 0.01 MOI;

SiHa, 1 MOI; SK-RC-52, 1 MOI). However, the growth of SK-RC-29 was not inhibited with the similar concentration of virus and could be inhibited only with 100 MOI of Ad-MN/CA9-E1A. Ad-MN/CA9-E1A virus injection caused growth inhibition of subcutaneous tumors induced by HeLa cells, which showed significant decreases in tumor volume and extensive necrosis of cancer cells in histology.

Conclusions: The author has established a novel replication-competent adenoviral vector system with MN/CA9 promoter to drive the replication of adenovirus only in MN/CA9-expressing tumor cells with selective cytotoxicity. This novel vector system may have potential as a strategy for the treatment of uterine cervical cancer and renal cell carcinoma.

Key Words: Gene therapy, Replication-competent adenovirus, MN/CA9, Cervical cancer, Renal cell carcinoma

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I. INTRODUCTION

Uterine cervical cancer is still the leading cause of cancer deaths for women in many developing countries, although the incidence has been declining steadily in western countries.[1] Because the cytologic screening method is highly sensitive and has led to detect early stage of disease, the comprehensive screening programs have made early detection and high cure rate in cervical cancer.[2]

The choice of treatment may be decided by several factors, including stage, histopathologic features and the presence of lymph node metastasis. However, in general, intraepithelial lesions are treated with superficial ablative techniques and early invasive cancers are treated with surgery or radiotherapy. Although local treatments result in high cure rates in these stages of cervical cancer, some patients suffer from treatment complications such as ureterovaginal fistula, urinary tract infection, wound infection or bladder dysfunction.[3-5] These complications compromise the patient's quality of life, despite significant improvement of survival with local treatments. To circumvent these problems of conventional local treatments,

tumor-specific gene therapy using tissue-specific promoter can be a rational treatment strategy for uterine cervical cancer; moreover, uterine cervix is an organ to apply intratumoral approach readily without invasive techniques.

Comparing with the high response rates in localized uterine cervical cancer to treatments, patients with disseminated disease almost always have dismal results. Metastatic cervical cancer may respond to chemotherapy, but the response duration is usually very short and adverse effect of chemotherapy is sometimes serious.[6] Systemic administration of tumor-specific oncolytic adenovirus may be an effective and safe treatment option in patients with metastatic diseases.

Renal cell carcinoma accounts for 2-3% of adult cancers and approximately 85% of malignant renal tumors. Despite an expanding knowledge of renal cell tumor biology and histogenesis, the incidence of renal cell carcinoma has steadily increased. When it is detected in the early stages, radical nephrectomy can result in durable long-term survival. Unfortunately, symptoms rarely occur before extensive disease is present. Because metastatic renal cell carcinoma responds poorly to radiotherapy and chemotherapy, patients presenting with metastatic disease have a dismal prognosis with 0-20% 5-year survival rates.[7,8] Therefore, novel therapeutic approaches for metastatic renal cell carcinoma are needed and gene therapy with tumor-specific oncolytic adenovirus would be an effective and safe treatment option.

Recombinant adenoviral vectors have been widely used in preclinical models

for *in vitro* and *in vivo* gene transfer. Adenoviral vector-mediated therapeutic gene expression has been achieved in broad spectrum of eukaryotic cells and is independent of cell replication.[9] The main advantage of adenoviral vectors is that the efficiency of transduction is high, as is the level of gene expression, although this is only transient and can deteriorate rapidly within a few weeks. Most of the cancer gene therapy trials to date utilize replication-deficient viruses for gene transduction. Its problem is that the low percentage of cells in a tumor nodule eventually becomes infected by replication-deficient adenoviral vectors.[10,11] To overcome these problems, replication-competent adenovirus with tissue-specific promoters have been used to regulate the expression of viral genes that are necessary for the replication of adenoviral vector by selectively replicating in the tumor and amplifying the viral dose throughout the tumor.

Two approaches are currently being used to engineer tumor-specific adenoviral replication. One is to limit the expression of the E1A gene product to tumor tissues through the use of tumor-specific promoters. E1A functions to stimulate S phase entry and to transactivate both viral and cellular genes that are critical for a productive viral infection. A second approach to optimize tumor selectivity is to delete gene functions that are critical for efficient viral replication in normal cells but are expandable in tumor cells. Tumor-specific promoters can replace endogenous viral sequences in order to restrict viral replication to a particular target tissue.[12] For

example, the prostate-specific antigen (PSA) promoter/enhancer element has been inserted upstream of the E1A gene. The result is that viral replication correlates with the level of PSA expression in a given cell. This virus, CN706, is currently in a phase I clinical trial of intratumoral injection for patients with locally recurrent prostate carcinoma.[13]

It has been known that the most human renal cell carcinoma cells express an antigen recognized by monoclonal antibody G250. Molecular cloning has recently revealed that this antigen is homologous to MN/CA9.[14] MN/CA9 is a transmembrane glycoprotein with an apparent molecular weight of 54/58 kDa, which was first detected in the highly malignant human cervical carcinoma cell line HeLa.[15] This antigen is detectable in several types of malignancies including cervical, ovarian, colorectal, esophageal and bladder cancers. MN/CA9 expression in normal tissues is restricted to the epithelial cells of gastrointestinal tract.[14-21] High level of MN/CA9 protein expression is found in more than 90% of uterine cervical cancer and renal cell carcinoma,[17,18] but not in normal tissues of the cervix and kidney. This evidence provides the possibility of MN/CA9 to serve as a powerful biomarker tracking uterine cervical and renal cell carcinoma.

The author constructed the tumor-specific replication-competent adenoviral vector system, Ad-MN/CA9-E1A, using MN/CA9 promoter to drive the viral early E1A gene with restricted replication in cells that express MN/CA9 transcriptional

activity. Here, the author described the construction of tumor-specific oncolytic adenovirus and demonstrated its selective cytotoxicity toward MN/CA9-expressing uterine cervical cancer and renal cell carcinoma cells *in vitro* and *in vivo.*

II. MATERIALS AND METHODS

A. Cells and cell culture

The human cervical cancer cell lines HeLa, C-33 A, and SiHa cells were obtained from American Type Culture Collection (Manassas, VA). SK-RC-52 and SK-RC-29, human renal cell carcinoma cell lines, were generously provided by Dr. Neil H. Bander (New York Hospital-Cornell Medical Center, New York, NY).[22] 911, a transformed human embryonic retinoblast cell line, was obtained from Introgen (Leiden, Nethrelands).[23] PER.C6, a transformed human embryonic retinoblast cell line, was obtained from DirectGene (Annapolis, MD).[24] HeLa, C-33 A, and SiHa cells were cultured in minimum essential medium (MEM, GIBCO BRL, Grand Island, NY). SK-RC-52 and SK-RC-29 cells were cultured in RPMI 1640 (GIBCO BRL). 911 and PER.C6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL). All media were supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum (FBS) and maintained at 37°C in 5% CO_2.

B. Construction and production of the replication competent Ad-MN/CA9-E1A virus

All plasmids were constructed according to the standard protocols. Briefly, a pMN/CA9E1A was constructed by inserting a 550-bp fragment of MN/CA9 promoter, which was cut from pMN/CA9PGL3-P using EcoRI enzyme, into the unique EcoRI restriction site in pΔBPAE1II.[25] This pMN/CA9E1A shuttle vector was cotransfected with pJM17 into 911 cells by the N-[1-(2,3-dioleoyloxyl)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP, Boehringer Mannheim, Indianapolis, IN)-mediated transfection method[26] to generate a replication-competent adenovirus, Ad-MN/CA9-E1A. The culture medium of the 911 cells showing complete cytopathic effect was collected and centrifuged at 1000 × g for 10 min. The pooled supernatants were aliquoted and stored at −80°C as primary viral stock. Viral stocks were propagated in 911 cells, and selected clones of Ad-MN/CA9-E1A virus were obtained by plaque purification according to the method of Graham and Prevec.[27] One of the viral clones was selected, propagated in PER.C6 cells, harvested 36 to 40 h after infection, pelleted, resuspended in PBS, and lysed. Cell debris was removed by subjecting the cells to centrifugation, and the virus in the cell lysate was purified by CsCl gradient centrifugation. Concentrated virus was dialyzed, aliquoted, and stored at −80°C. The viral titer was determined by plaque forming assay and optical density measurements.

C. RNA extraction and reverse transcription of cDNA

Total RNA was extracted from the tested cell lines by disrupting the cells in Trizol solution (GIBCO BRL). RNA was precipitated by ethanol, dissolved in water treated with diethylpyrocarbonate (DEPC, Sigma, St. Louis, MO) and reverse transcribed with Moloney Murine leukemia virus (M-MuLV) reverse transcriptase (MBI fermentas, Hanover, MD) using random hexamer primer. Two μg of total RNA was added to 0.2 μg of random hexamer primer and brought to a final volume of 20 μl. The samples were placed at 70°C for 5 min and then cooled on ice. The primer RNA mixture was combined with 1 mM deoxynucleotide triphosphate, 20 U/μl ribonuclease inhibitor, 50 mM Tris-HCl, 50 mM potassium chloride, 4 mM magnesium chloride, and 10 mM DTT. The mixture was incubated at 25°C for 5 min and then cooled on ice. The master mixture was completed by adding 20 U/μl M-MuLV reverse transcriptase. The RT reaction was then carried out at 37°C for 1 hr. Samples were stored at –20°C.

D. PCR assay for MN/CA9

PCR reaction was performed using the cDNA-specific primers for MN/CA9 according to the methods described by Bartosova et al. with minor modifications.[28] The two primers (5'-CCGAGCGACGCAGCCTTTGA-3' and 5'-TAGTCGACTAG

GCTCCAGTCTCGGCTACTT-3') were chosen to amplify a particular 255-bp region of MN/CA9 cDNA. PCR was run with denaturation at 95°C for 20 s, annealing at 65°C for 30 s, and extension at 72°C during 40 s for a total of 35 cycles, and finally 5 min at 72°C. PCR products were analyzed on a 1% agarose gel. â-actin cDNAs were also amplified as an internal control.

E. Western blot analysis of adenovirus E1A protein expression

For detection of the E1A protein, 2×10^6 cells in 100-mm dishes were infected with Ad-MN/CA9-E1A at a concentration of 1 MOI for 14 h. Cells were collected and lysed in 200 μl of cell lysis buffer (0.1 M Tris-HCl (pH 7.4), 0.5% SDS, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 μM leupeptin) 2 days after viral infection. Lysates were centrifuged at 14,000 rpm for 20 min, and the supernatants were collected. Total protein was estimated by dye binding assay (Bio-Rad, Hercules CA). Protein (20 μg) was loaded onto 12% SDS-PAGE gel. The proteins were transferred to polyvinylidene difluoride membrane (PVDF, Scheleicher & Schuell, Dassel, Germany) and the membrane probed with an Ad5 E1A-specific antibody (MS-587-P1, NeoMarkers, Fremont, CA). Binding of the primary antibody was detected using a goat anti-mouse secondary antibody conjugated to horseradish peroxidase, and visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

F. *In vitro* cytotoxicity assay

HeLa, C-33 A, SiHa, SK-RC-52 or SK-RC-29 cells were seeded into 24-well plates at a density of 5×10^3 cells/well. After 24 h, the cells were infected with Ad-MN/CA9-E1A with a range of concentrations from 0.01 to 100 MOI. Half of the medium was aspirated on days 3 and 5 after viral infection (day 0) and replaced with fresh medium. The cell numbers per well were assessed at day 0, 1, 3, 5, and 7 by crystal violet assay. The data is expressed as percentage of cell numbers of Ad-MN/CA9-E1A infected group relative to those of uninfected control group.

G. *In vivo* animal studies

Athymic BALB/c nu/nu nude female mice (Japan SLC, Shizuoka, Japan), aged 6 week and weighing 20g, were inoculated subcutaneously with HeLa cells (2×10^6 cells) in 100 μl of 50% medium-50% MATRIGEL basement membrane matrix (Becton Dickinson, Bedford, MA). When the tumor became palpable (4-5 mm in diameter), the animals were randomly assigned to two experimental groups: group 1 (n = 7), Ad-MN/CA9-E1A; and group 2 (n = 7), PBS. A single dose of 50 μl Ad-MN/CA9-E1A virus (2×10^9 pfu) or PBS was injected intratumorally with a microliter syringe fitted with a 28-gauge needle. Tumor volume was measured every 5 days and calculated using the following formula: volume (a rotational ellipsoid) =

$M_1 \times M_2^2 \times 0.5236$ (M_1, long axis; M_2, short axis). Tumor volumes were normalized to 100% on day 0. At 30 days after intratumoral injection, the mice were sacrificed and the tumor was removed for histological examination.

III. RESULTS

A. RT-PCR analysis of MN/CA9 expression

Total RNA was extracted from human cervical (HeLa, C-33 A, and SiHa) and renal (SK-RC-52 and SK-RC-29) cancer cell lines grown in culture. HeLa and SK-RC-52 cells have been known to be MN/CA9-positive[16,29] and SK-RC-29 known to be MN/CA9-negative.[29] A distinct 255-bp band characteristic of MN/CA9 cDNA was detected in the reaction products of all three human cervical cancer cell lines tested and SK-RC-52. HeLa cell showed the strongest expression of MN/CA9. The MN/CA9 PCR product was not detected in the reaction product from SK-RC-29 (Fig. 1).

B. Western blot analysis of adenovirus E1A protein expression

Adenovirus E1A protein production in HeLa, C-33 A, SiHa, SK-RC-52, or SK-RC-29 cells following infection with Ad-MN/CA9-E1A was determined by western blot analysis. E1A protein was not detected in all cells prior to infection. After Ad-

MN/CA9-E1A infection, MN/CA9-positive cells (HeLa, C-33 A, SiHa, and SK-RC-52) showed strong expression of E1A protein, ranging in size from approximately 35 to 46 kDa. On the other hand, MN/CA9-negative cell (SK-RC-29) showed a much lower level of E1A expression (Fig. 2). The results of western blot analysis coincide

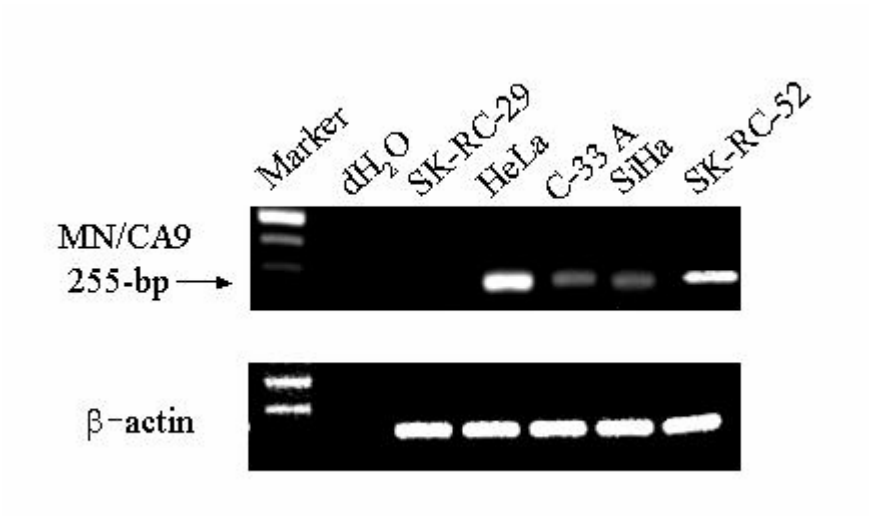

Fig. 1. RT-PCR assay identifies the expression of the 255-bp MN/CA9 cDNA message in HeLa, C-33 A, SiHa, and SK-RC-52 cell lines. No expression is seen in cDNA from SK-RC-29 cell line. β-actin cDNAs were used in a parallel reaction to confirm RNA integrity.

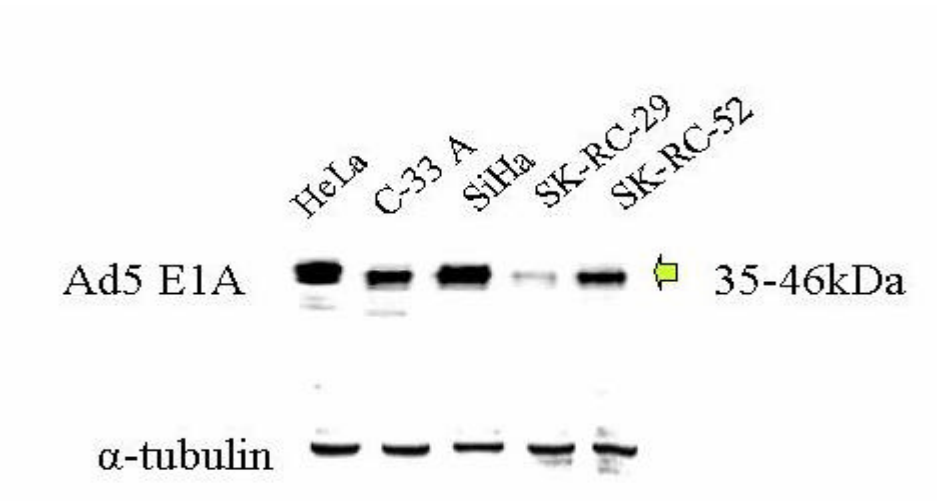

Fig. 2. Expression of adenovirus E1A protein by Ad-MN/CA9-E1A. Strong protein bands of MN/CA9, ranging in size from approximately 35 to 46 kDa, were found in HeLa, C-33 A, SiHa, and SK-RC-52 cell lines. SK-RC-29 showed a much weaker band.

with those of RT-PCR, suggesting a tissue-specific pattern of expression of this vector.

C. Selective cytotoxicity of Ad-MN/CA9-E1A to MN/CA9-positive cell lines *in vitro*

To assess the selective cytotoxicity of Ad-MN/CA9-E1A, we exposed the MN/CA9-positive (HeLa, C-33 A, SiHa, and SK-RC-52) and MN/CA9-negative (SK-RC-29) cell lines *in vitro* to a wide range (0.01-100 MOI) of Ad-MN/CA9-E1A. The growth of MN/CA9-positive cells was significantly inhibited with low concentrations of Ad-MN/CA9-E1A (HeLa, 0.1 MOI of virus; C-33 A, 0.01 MOI; SiHa, 1 MOI; SK-RC-52, 1 MOI). However, the growth of MN/CA9-negative cell line SK-RC-29 was not inhibited with the similar concentration of virus and could be inhibited only with 100 MOI of Ad-MN/CA9-E1A (Fig. 3).

D. *In vivo* growth inhibition of HeLa xegograft with intratumoral Ad-MN/CA9-E1A injection

Human cervical HeLa tumors were induced by subcutaneous injection of HeLa

cells in athymic mice. After tumor formation, animals were intratumorally injected with Ad-MN/CA9-E1A or PBS on day 0. Tumor volumes were measured at the indicated times. Ad-MN/CA9-E1A effectively inhibited the growth of HeLa tumors (Fig. 4). After 25 days, 2 of 7 mice were visually free of tumor (Fig. 5).

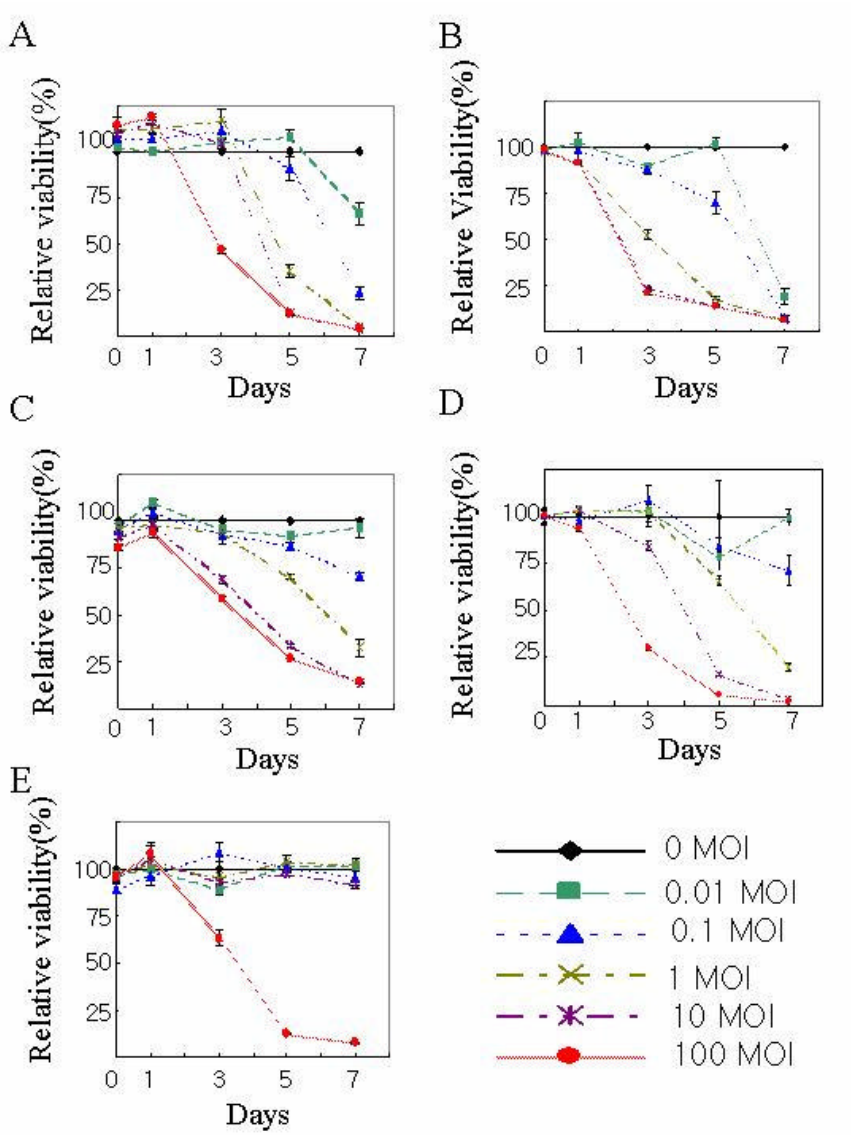

Fig.3. Selective inhibition of MN/CA9-positive cell growth *in vitro* by Ad-MN/CA9-E1A. After cells were exposed to a wide range (0.01-100 MOI) of Ad-MN/CA9-E1A, cell growth

was assessed *in vitro*. The data is expressed as percentage of cell numbers of Ad-MN/CA9-E1A infected group relative to those of uninfected control group. Whereas the growth of MN/CA9-positive cell lines, HeLa (A), G-33 A (B), SiHa (C), and SK-RC-52 (D), was significantly inhibited with low concentrations of Ad-MN/CA9-E1A, the growth of MN/CA9-negative cell, SK-RC-29 (E), could be inhibited only with 100 MOI of Ad-MN/CA9-E1A. The data represents mean; bars, ± SD.

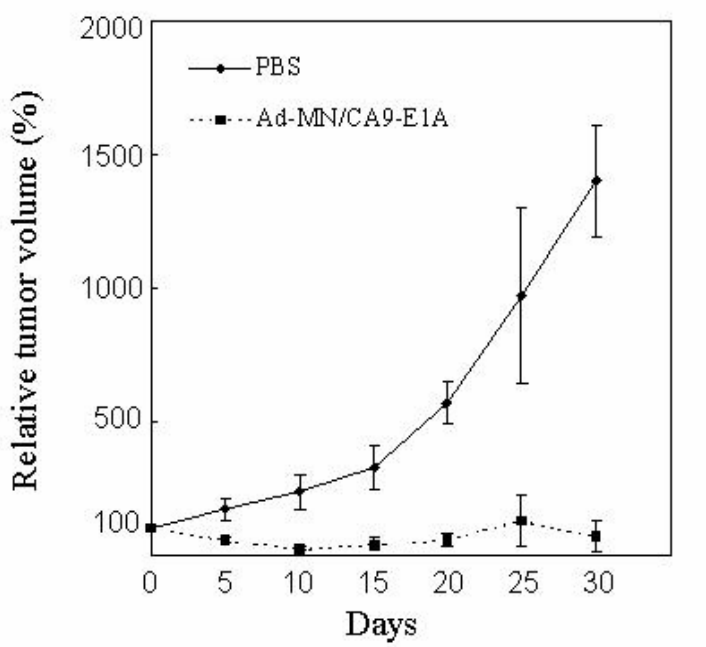

Fig. 4. Treatment of tumor xenografts with Ad-MN/CA9-E1A. HeLa tumor xenografts were grown s.c. in athymic nude mice. Tumors were treated with Ad-MN/CA9-E1A (n = 7) or PBS (n = 7) by intratumoral injection on day 0 and measured every 5 days. Tumor volumes were normalized to 100% on day 0. The data represents mean; bars, ± SD.

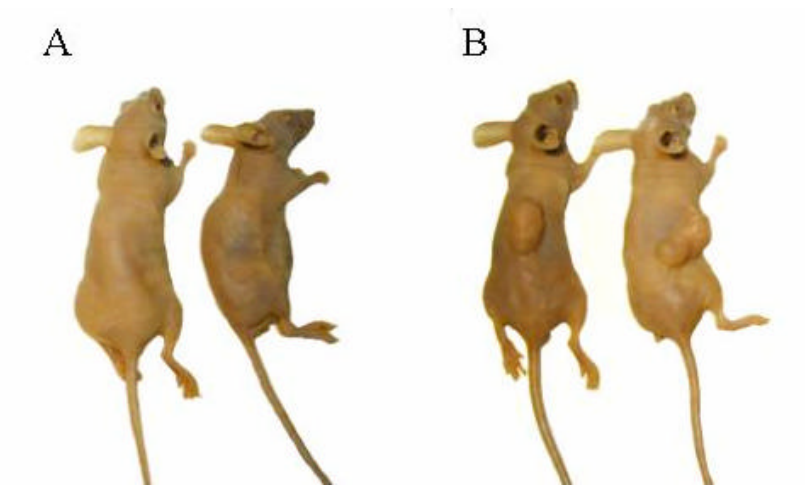

Fig.5. Inhibition of tumor growth by Ad-MN/CA9-E1A on subcutaneous HeLa tumor xenografts at 30 days after intratumoral viral injection. A. Tumor has regressed in mice treated with Ad-MN/CA9-E1A. Two of 7 mice with Ad-MN/CA9-E1A intratumoral injection were visually free of tumor. B. Big tumors were seen in mice treated with PBS.

a

Light microscopic finding of tumors in mice injected with Ad-MN/CA9-E1A showed that nearly all cancer cells were necrotized. On the other hand, tumors in mice injected with PBS showed that cancer cells were grown well (Fig. 6).

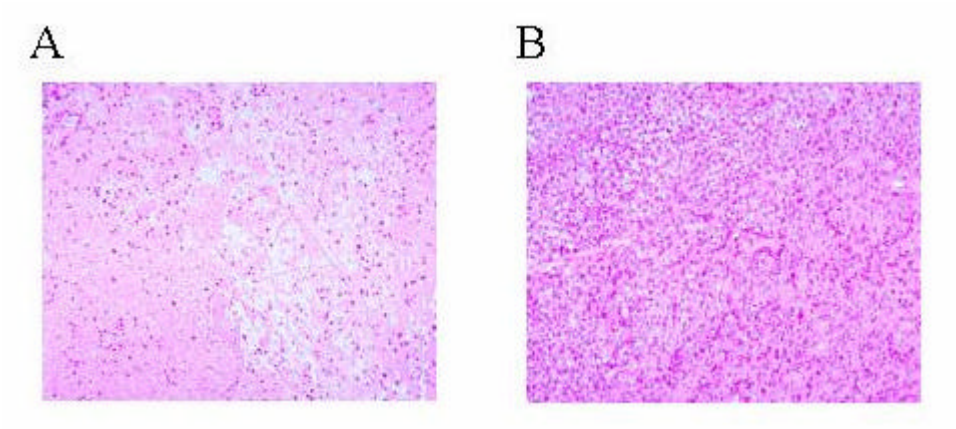

Fig. 6. Effect of Ad-MN/CA9-E1A on subcutaneous HeLa tumor xenograft at 30 days after intratumoral viral injection. Tumor was removed for histological examination after sacrifice of mice. A. Nearly all cancer cells were necrotized in mice treated with Ad-MN/CA9-E1A (H&E, x 200). B. Cancer cells were grown well in mice treated with PBS (H&E, x 200)

IV. DISCUSSION

Major obstacles of cancer gene therapy are low efficiency of gene transfer and low selectivity to target cells. To circumvent these problems, selective replication-competent oncolytic virus may be a promising strategy by selectively replicating in the tumor and amplifying the viral dose throughout the tumor. This replication-competent oncolytic adenovirus can kill the tumor cells by direct consequence of the viral replication. Furthermore, input viral dose amplifies with replication and spread to adjacent cells after lysis of tumor cells. Since replication-competent oncolytic gene therapy with these favorable properties has been highlighted for novel therapeutic strategy of cancer treatment, several clinical trials have been approved in human studies.[30-34] However, trials with replication-competent virus have not been investigated in the area of treatment for cervical cancer. This study was to create adenoviral vectors that displayed tumor-specific replication competency and that were directly cytotoxic to tumor cells.

The adenovirus early region 1 (Ad E1) is the first region transcribed from the

viral genome after infection of a cell. Two groups of transcripts are produced from this region, E1A and E1B that, together, encode proteins that function to modulate the cellular environment to enhance the ability to replicate. E1B proteins primarily function to inhibit p53, thereby preventing p53-mediated cell death. The primary function of E1A is to activate viral transcription and to reprogram cellular gene expression, which is accomplished through the ability of E1A proteins to interact with a variety of different cellular transcription factors and regulatory proteins, including p300/CBP, P/CAF, CtBP, and the pocket proteins RB, p107, and p130. E1A can activate or repress transcription, induce entry and passage through the cell cycle, and block differentiation.[35,36] In this replication-competent adenoviral vector, MN/CA9 promoter was used to achieve a tumor-specific viral replication for the treatment of MN/CA9-expressing cancer cells.

It has been reported that MN/CA9 may be useful as a biomarker for the diagnosis and treatment of cervical cancers and renal cell carcinoma. The monoclonal antibody G250 raised against a renal cell carcinoma has been shown to react with a large number of renal cell carcinomas.[14] Recently, G250 antigen was isolated and found to be homologous to the MN/CA9 gene originally identified in HeLa cell. MN/CA9 protein, a novel tumor-associated antigen, consists of 4 distinct domains, a secretary signal sequence, N-terminal helix-loop-helix domain, a carbonic anhydrase domain and a single transmembrane domain.[37] Although its role in

oncogenesis is unclear, MN/CA9 may be involved in the control of cell proliferation and transformation.[37] MN/CA9 expression correlates both with the density of HeLa cells and with the tumorigenic phenotype of CGL1 and CGL3 hybrids between HeLa and normal human fibroblasts.[38,39] Introduction of MN/CA9 cDNA into NIH 3T3 cells enhanced cellular proliferation and transformation.[37] Previous studies revealed that MN/CA9 expression was restricted only to certain malignant tumors, but not in the normal tissues except epithelial cells of the gastrointestinal tract.[15,38,39] Moreover, MN/CA9 protein is expressed in more than 90% of cervical cancer and MN/CA9 expression has significant prognostic values in patients with locally advanced cervical cancer.[40]

Currently, 293 cell, which was generated by transformation of human embryonic kidney cells by sheared Ad5 DNA, is the most widely used helper cell line for the production of recombinant adenoviruses.[38] However, the author used 911 and PER.C6 cell lines in this experiment because these cell lines possess several favorable characteristics compared to 293 cell. 911 cell line is Ad5 E1-transformed human embryonic retinoblast (HER) cell with a plasmid containing base pairs 79-5789 of the Ad5 genome. This cell line has comparable quality of 293 cells with respect to the transfection efficiency and the frequency of homologous recombination. It also exhibited a shortened plaque-forming time in plaque assay and enhanced viral yields in small-scale productions of adenoviral vectors in comparison with 293 cells.

Most importantly, because Fallaux et al.[23] mentioned that replication-competent adenoviruses were not generated in 911-produced replication-deficient adenoviral vector batches, the author used this cell line for making our replication-competent adenovirus, Ad-MN/CA9-E1A. However, 2 years later, the same group reported that 911 cells could also generate a replication-competent adenovirus in replication-deficient adenoviral vector batches and developed a new helper cell line PER.C6 cell. PER.C6 cell line, which contains the Ad5 E1A- and E1B-encoding sequences (Ad5 nucleotides 459-3510) under the control of the human phosphoglycerate (PGK) promoter, does not result in the generation of replication-competent adenoviruses.[24] Therefore, the author used PER.C6 cell line for the amplification of Ad-MN/CA9-E1A .

In the present study, the author constructed Ad-MN/CA9-E1A adenoviral vector using MN/CA9 promoter to achieve a tumor-specific viral replication for the treatment of MN/CA9-expressing uterine cervical cancer and renal cell carcinoma. Because the author used a novel tumor-specific MN/CA9 promoter to control the expression of E1A essential for viral replication, the author anticipated that this type of adenovirus could replicate preferentially in MN/CA9-expressing cancer cells, and kill those cells.

The tissue-specific replication and cytotoxicity of Ad-MN/CA9-E1A adenovirus was assessed with five different cancer cell lines, three cervical (HeLa, C-33 A, and

SiHa) and two renal (SK-RC-52 and SK-RC-29) cancer cell lines. All three of these cervical cancer cell lines and SK-RC-52 produced MN/CA9; HeLa cell line has been known to express MN/CA9 and this cell showed the strongest expression of MN/CA9 with RT-PCR assay. Because one of renal cell carcinoma cell lines, SK-RC-29, has been known to be MN/CA9-negative,[29] the author chose this cell line as a negative control for *in vitro* study and MN/CA9-negativity was confirmed in RT-PCR. By western blot analysis, the author proved tissue-specific promoter activity with demonstration of selective E1A expression in MN/CA9-expressing cells, which coincides with the results of RT-PCR assay.

The *in vitro* tissue-specific cytotoxicity of Ad-MN/CA9-E1A to MN/CA9-expressing cells was assessed. Whereas the growth of MN/CA9-positive cervical cancer cell lines and SK-RC-52 were significantly inhibited with low concentrations of Ad-MN/CA9-E1A, the growth of SK-RC-29 could be inhibited only with 100 MOI of Ad-MN/CA9-E1A. These results demonstrate that Ad-MN/CA9-E1A has a selective cytotoxicity in MN/CA9-expressing cells with good therapeutic window. Intratumoral administration of Ad-MN/CA9-E1A effectively inhibited the growth of HeLa xenograft in athymic nude mice. After 25 days of viral injection, 2 of 7 mice were visually free of tumor.

In conclusion, the author have established a replication-competent adenoviral vector system with MN/CA9 promoter to drive the replication of adenovirus only in

MN/CA9-expressing cancer cells with selective cytotoxicity and good therapeutic window.

V. CONCLUSIONS

The author has established a novel replication-competent adenoviral vector system with MN/CA9 promoter to drive the replication of adenovirus only in MN/CA9-expressing cancer cells with selective cytotoxicity.

This novel vector system may have potential as a strategy for the treatment of uterine cervical cancer and renal cell carcinoma.

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- 국문요약

MN/CA9 프로모터를 가진 Replication-competent 아데노바이러스를 이용한 자궁경부암과 신세포암에 대한 새로운 유전자 요법

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목적: 재조합 아데노바이러스 벡터 (vector)는 *in vitro* 혹은 *in vivo* 유전자 전달체로서 널리 이용되고 있다. 아데노바이러스 벡터를 이용한 암유전자치료에 있어서의 문제점은 유전자 전달의 효율이 낮고, 표적세포에 대해 선택적으로 작용하지 못한다는 것인데, 조직 특이적인 프로모토를

갖고 있으면서 복제 가능한 재조합 아데노바이러스를 사용한다면 이러한 문제점을 극복할 수 있다. 이 아데노바이러스는 그 단백질이 발현되는 세포에서만 선택적으로 프로모토가 작동하므로 원하는 세포만 선택적으로 죽일 수 있고, 적은 양의 아데노바이러스만 주입하여도 세포내에서 바이러스의 복제가 가능하므로 높은 효과를 볼 수 있다.

MN/CA9 는 막내외 (transmembrane) 당단백으로 인체 자궁경부암 세포주인 HeLa 세포에서 최초로 발견되었다. 그 후 MN/CA9 단백은 자궁경부암과 신세포암을 포함한 여러 종류의 암조직에서 발현되며, 정상조직에서는 오직 위장관 상피세포에서만 발현되는 것이 보고되었다.

이에 저자는 MN/CA9 프로모토를 이용하여 MN/CA9 가 발현되는 세포에서만 복제가 가능하도록 만든 아데노바이러스 (Ad-MN/CA9-E1A)를 만들었고, 이 아데노바이러스를 이용하여 자궁경부암과 신세포암에서의 치료효과를 알아보고자 하였다.

재료 및 방법: MN/CA9 이 발현되는 세포에서만 아데노바이러스 E1A 단백을 복제할 수 있도록 만든 MN/CA9 프로모토를 가진 재조합 아데노바이러스 즉, Ad-MN/CA9-E1A 을 만들어서 실험에 이용하였다. 자궁경부암 세포주 (HeLa, C-33 A, SiHa)와 신세포암 세포주 (SK-RC-52, SK-RC-29)에서 RNA 를 추출해서 RT-PCR 을 이용하여 MN/CA9 의 발현을 조사하였다. Ad-MN/CA9-E1A 아데노바이러스를 감염시킨 후 단백을 채취해서 western blot 분석을 이용하여 아데노바이러스 E1A 단백의 발현

정도를 조사하였다. 자궁경부암 및 신세포암 세포주에서 Ad-MN/CA9-E1A 아데노바이러스에 의한 *in vitro* 세포살상효과를 확인하였다. 무흉선 누드 마우스에 HeLa 세포를 피하주사하여 종양이 형성되었을 때 Ad-MN/CA9-E1A 바이러스 혹은 PBS 를 투여하여 *in vivo* 세포살상효과를 확인하였다.

결과: RT-PCR 결과 MN/CA9 양성 세포주인 HeLa, C-33 A, SiHa 및 SK-RC-52 세포에서는 MN/CA9 mRNA 의 발현을 확인할 수 있었으나 MN/CA9 음성 세포주인 SK-RC-29 세포에서는 발현되지 않았다. Western blot 분석 결과 Ad-MN/CA9-E1A 아데노바이러스 감염 후 MN/CA9 양성 세포주인 HeLa, C-33 A, SiHa 및 SK-RC-52 세포에서는 35-46 kDa 크기의 E1A 단백이 강하게 발현되었으나, MN/CA9 음성 세포주인 SK-RC-29 세포에서는 E1A 의 발현이 매우 미약하였다. 각 세포주에서 *in vitro* 세포살상효과를 확인하기 위하여 Ad-MN/CA9-E1A 아데노바이러스를 0.01-100 MOI 로 감염시킨 후 세포성장억제효과를 관찰한 결과 MN/CA9 양성 세포주인 HeLa (0.1 MOI), C-33 A (0.01 MOI), SiHa (1 MOI) 및 SK-RC-52 (1 MOI) 세포는 낮은 농도의 바이러스에서도 세포성장억제효과가 관찰된 반면, MN/CA9 음성 세포주인 SK-RC-29 는 매우 높은 농도인 100 MOI 에서만 세포성장억제효과가 나타났다. 누드 마우스의 피하부위에 형성된 HeLa 종양에 Ad-MN/CA9-E1A 아데노바이러스를 투여한 결과 종양 용적의 현저한 감소와 종양의 조직학적 검사에서 거

의 모든 종양세포의 괴사를 보여 종양의 성장억제효과를 관찰할 수 있었다.

결론: 저자는 MN/CA9 프로모토를 이용하여 MN/CA9 를 발현하는 종양세포에서만 선택적으로 복제할 수 있도록 만든 새로운 아데노바이러스 벡터인 Ad-MN/CA9-E1A 을 만들었으며, 이 아데노바이러스는 자궁경부암과 신세포암 세포주에서 MN/CA9 발현 양상에 따라 선택적인 세포살상 효과를 보여주었다. 따라서 이 아데노바이러스 벡터는 자궁경부암과 신세포암에 대한 새로운 효과적인 유전자치료법으로 이용될 수 있으리라 생각한다.

핵심되는 말: 유전자치료, Replication-competent 아데노바이러스, MN/CA9, 자궁경부암, 신세포암