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*Original Article*

# Comparison of Three Different Methods of Transfection for the Production of Recombinant Adenovirus Expressing Human Carcinoembryonic Antigen Gene

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**Abstract**

Adenoviral vectors (AdVs) are widely used as a gene delivery vehicle and vaccine design due to their genetic stability, transfer capacity of large genes, production at high titers, and remarkable efficacy of transduction. One of the most important applications of AdVs is in cancer immunotherapy. Tumor-associated antigens are overexpressed in cancer cells; however, they cannot induce immune responses sufficiently. Therefore, the immune system must be stimulated against these antigens to kill the cancer cells. This study described the construction steps of a recombinant AdV expressing human carcinoembryonic antigen (CEA) gene. Furthermore, in order to achieve a high titer of the virus, an efficient transfection was required. Three various transfection reagents were compared to achieve the best method of transfection. Carcinoembryonic antigen was cloned into the pAdV and transfected into the A293 cells using three different reagents, including polyethylenimine (PEI), calcium phosphate, and DMRIE-C. The PEI had the highest transfection efficiency, which was selected for the transfection of the recombinant plasmid. It has low toxicity for cells and is suitable for large-scale transfection. The virus produced in this study can be applied as a vaccine in cancer immunotherapy for stimulating the immune system against CEA-expressing tumors.

**Keywords:** Adenoviral vector, Calcium phosphate, DMRIE-C, Polyethylenimine, Vaccine

## 1. Introduction

Vaccine development based on recombinant deoxyribonucleic acid (DNA) technology has quickly become an attractive approach for immune induction (1). Numerous cancer patients suffer from the absence of immune response against tumors as a result of immunological tolerance toward tumor-associated antigens (TAAs) (2). Tumor-associated antigens are overexpressed in tumor cells; however, they often fail to induce sufficient immune responses. Therefore, one of the main purposes in the cancer vaccine fields is to enhance TAA-specific cellular immune responses via allowing the delivery of different TAAs that can stimulate effective antitumor immunity (3, 4). The results of various types of research have confirmed that the infiltration of T cells into the tumor microenvironment is associated with increased survival without recurrence in patients.

Several genetic vaccine platforms have been applied, including viral vectors, that have received attention as an effective gene delivery vehicle. Recombinant adenoviruses have long been considered a viral vector for applications in gene therapy and vaccines (5, 6). Adenoviruses consist of a 36kb linear double-stranded DNA (dsDNA), which is surrounded by an icosahedral protein capsid. There are more than 100 serologically

different types of adenoviruses divided into seven species (i.e., A-G) (7, 8). Most human adenovirus infections are asymptomatic and rarely cause serious complications. Consequently, adenovirus vectors (AdVs) offer several advantages that make them attractive to transfer and introduce functional genes into cells (9). The vector engineering (deleting the essential E1/E3 early gene) has led to the extension of the cloning capacity (10, 11), a large transfer of gene capacity without integrating into the host genome, easy infection of cells with negligible apparent side effects, simultaneous expression of more than one protein in a large scale, and efficient stimulation of immune system (12). Mainly, AdVs are divided into two groups, namely replicative-oncolytic that can selectively lyse the cancer cells via intratumoral amplification and replicative-deficient adenoviruses that have been designed for vaccine carriers without replication into the cells. Furthermore, adenoviruses via infection of dendritic cells are able to upregulate the presentation of TAAs to the immune system (13). In this respect, it may be useful for attempts to enhance the immune response and the number of potential effector T cells against TAAs. An option to achieve this purpose is the design of AdVs-based vaccines expressing TAAs (14, 15). One of the TAAs is a carcinoembryonic antigen (CEA)-related cell adhesion molecule that significantly elevates in a number of cancers, including colorectal, gastric, lung, pancreatic, and skin carcinoma. It has been established that CEA molecules play functional roles in cell adhesion, cancer progression, angiogenesis, inflammation, signaling, and metastasis. Therefore, these proteins are considered attractive therapeutic targets for tumor immunotherapy (16).

### 1.1. Objectives

In this study, an adenoviral vector-based vaccine candidate was designed to express the CEA as a tumor antigen, and subsequently, generate large numbers of effector T cells in vivo. This research described the preparation steps of the adenovirus vector and optimized the recombinant adenovector production protocol. As the transfection step in the construction of recombinant adenovectors is an important step to achieve a high titer of the virus, three different reagents for transfection were compared to achieve the best method of transfection.

## 2. Materials and Methods

### 2.1. Carcinoembryonic Antigen Cloning

#### 2.1.1. Preparation of the Human Carcinoembryonic Antigen Gene

Human CEACAM5/CEA/CD66e Gene ORF cDNA clone expression plasmid, C-GFPSpark tag (Cat Number: HG11077-ACG) was purchased from Sino Biological (Beijing, China). To make multiple copies of the plasmid, it was transformed to the bacteria using the heat shock method. Briefly, the plasmid was added to the competent *Escherichia coli* strain DH5α. The reaction was heat-shocked and then immediately incubated on ice. After the transformation of the CEA plasmid, it was extracted (GeneAll ExprepTM Plasmid SV mini, Korea).

The CEA gene (2838bp) was inserted into the plasmid between KpnI and NotI restriction sites, which were also presented in the multiple cloning sites of the adeno-transfer plasmid. About 1.5-2µg of the plasmid containing the CEA gene was digested with KpnI and NotI (Takara, Japan) according to the protocol. Agarose gel electrophoresis was run to check the product, and gel purification (Gene All Expin Gel SV kit, Korea) was performed to isolate and purify the desired fragment based on the size.

#### 2.1.2. Cloning the Carcinoembryonic Antigen into the Transfer Plasmid

To ligate the transfer plasmid (pAdenoVator-CMV5-IRES-GFP, Qbiogene) with CEA, the plasmid was digested with the same enzymes that cut the CEA from the cloned plasmid as described before. Afterward, the CEA was cloned into the transfer plasmid (3:1 and 5:1 molar ratio) using 2 µl 10x ligase buffer and 1 unit of T4 DNA Ligase (5 U/µL) (Thermo Fisher). The total volume was increased to 20 µl with distilled water. The reaction was incubated at 16°C overnight. Once the ligation process was completed, the CEA expressing

plasmid was transformed to DH5α bacteria and the ligated clones were screened.

**2.2. Co-Transformation**

Before co-transformation, the recombinant plasmid was linearized with the PmeI restriction enzyme. The digested product was verified on an agarose gel, and gel purification was performed subsequently. pAdenoVator ΔE1/E3 (AdenoVator system, Qbiogene) is a deficient E1 and E3 deleted human adenovirus type 5 backbone vector whose E1 functions can be complemented in the Human Embryonic Kidney A293 (A293) cells and it was used to construct the AdV. pAdenoVator containing most of the adenoviral genome in supercoiled plasmid form was co-transformed with recombinant transfer plasmid into *E. coli* strain BJ5183 by electroporation method. Briefly, 40 µL of BJ5183 competent cells were divided into electroporation cuvettes on ice. About 1 µg of linearized recombinant transfer plasmid and 100 ng/µL of pAdenoVator were added to the cuvette. In the next step, BJ5183 bacterial cells, which are more susceptible to recombination, were transformed according to the instructions provided by the electroporator supplier (Eppendorf). The transformation mix was immediately suspended in 1 mL of Luria-Bertani (LB) and transformed to a microtube. It was incubated at 37°C for 60 min with agitation. Afterward, the cells were cultured onto 3-5 LB/kan (50 µg/mL) plates, incubated at 37°C, and shaken at 250 rpm for a minimum of 24 h. Small scale colony was amplified in LB broth with kanamycin. After the extraction of the recombinant vector, in order to screen the correct clones, the plasmids were run on 0.7% agarose gel to confirm the approximate size of 40 kb that showed the production of the recombinant genome of adenovirus; subsequently, it was cleaved with PacI restriction enzyme.

**2.3. Comparing three Transfection Reagents Efficiency**

The PacI linearized plasmid prepared in the previous step to expose viral inverted terminal repeats (ITR) was transfected into the cells using three different reagents, namely polyethylenimine (PEI), calcium phosphate, and DMRIE-C, and then, the results were compared according to the amount of green fluorescent protein (GFP) expression. The steps of each method are described below.

**2.3.1. Polyethylenimine Transfection**

The day prior to transfection (50-70% confluency), A293 cells were seeded at a density of $5\text{-}8\times10^4$ in a volume of 700 µl complete growth medium per well in a 24-well plate. An amount of 1 µg of the plasmid for each well was diluted in serum-free Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco, UK), pipetted gently, and incubated for 10 min at room temperature. Afterward, 2 µl of PEI reagent (1 µg/µl) was added to the diluted plasmid, vortexed for 10 sec immediately, and then, incubated for 15-30 min at room temperature. The media on the cells was replaced with 150 µl of fresh serum-free DMEM medium, and the transfection complex was added to the cells in a drop-wise manner. The plate was shacked gently to ensure even distribution over the entire plate and was incubated for 16 h. After transfection, the medium was changed to 500-µl DMEM supplemented with 2% fetal bovine serum (FBS). The plate was incubated for 72 h, at 37°C, and 5% $CO_2$. Green fluorescent protein expression was examined by fluorescent invert microscopy, which confirms cell transfection.

**2.3.2. Calcium Phosphate Transfection**

After the preparation of cells, as described above, 44 µl of sterile water, 1 µg (6 µl) of the plasmid, and 6 µl of 2 M $CaCl_2$ solution was added in a 1.5-microtube and mixed by pipetting. Subsequently, the prepared solution was added, drop by drop, into a microtube containing 50 µl of the 2X HBS buffer solution while HBS buffer was shaking on a microfuge vortex and continued mixing for an additional 20 sec. The mixture was incubated at 23°C for 10 min. The old medium on the cells was exchanged to a 150-µl fresh serum-free DMEM medium, and the rest of the steps were performed as described earlier.

### 2.3.3. DMRIE-C Transfection

DMRIE-C (Invitrogen, USA) is a lipid-based reagent that is suitable for the transfection of DNA and ribonucleic acid (RNA) into eukaryotic cells. The process of the preparation of the cells before and after the transfection was the same as the two above-mentioned methods. The mixture of 1 µg of the plasmid and 125 µl of DMEM medium and the mixture of 2 µl of DMRIE-C transfection reagent and 125 µl of DMEM medium were incubated at room temperature for 30-45 min and the contents of the two tubes were mixed and incubated again for 45 min. At the end of the incubation time, the mixture was used for cells transfection.

### 2.4. Evaluation of Carcinoembryonic Antigen Gene Expression

To ensure the CEA gene expression in the cells, the cloned plasmid was transfected into the A293 with the three mentioned methods, and then, the cells were harvested for 44 and 68 h after transfection (a well of the plate that seeded with the cells was considered negative control). Total RNA was extracted (BioFACT Total RNA prep Kit, Korea) from cells immediately. For each sample, cDNA was synthesized using a cDNA synthesis kit (BioFACT, Korea). The primers were designed for CEA and hypoxanthine-guanine phosphoribosyltransferase (HPRT; internal control) genes and evaluated by the Applied Biosystems StepOne Real-Time PCR Systems using TaqMan assay (2Step 2x RT-PCR Pre-mix Taq, BioFACT kit) and stepone software version 2.3.

### 2.5. Transfection and Transduction of Recombinant Adenoviral Vector

To produce an adenoviral vector, the recombinant AdV construct should firstly cleave with PacI to linearize. Therefore, a 3-5-µg linear plasmid was transfected using 10 µl of PEI reagent, as previously described, into A293 cells, which were cultured at a 6-well plate and were incubated at 37°C. After 7 days of transfection, the full cytopathic effect was achieved, and the cells were then collected and lysed by three freeze/thaw cycles at -80°C/37°C. The cells were centrifuged at 2,000 g for 15 min. Afterward, 1 ml of the supernatant was transduced into the A293 in a 25-$cm^2$ flask and incubated for 90 min at 37°C. The supernatant was discarded and replaced with 2-ml DMEM with 1% FBS, followed by incubation at 37°C for 72 h. The cells were analyzed for GFP expression using a fluorescence microscope. Furthermore, to confirm protein production, the supernatant containing the AdV was collected and the amount of CEA protein was measured by the electrochemiluminescence immunoassay (Elecsys CEA, Roche, Germany).

## 3. Results

### 3.1. Generation of Adenoviral Vector

The overall strategy of constructing AdV involves three steps. First, the gene of interest (i.e., CEA) is cloned into a transfer plasmid. Second, the resultant construct is digested with a restriction endonuclease enzyme to linearize the plasmid and co-transformed with a supercoiled adenoviral backbone vector into *E. coli* strain BJ5183 (the selective antibiotic is kanamycin), and then, the recombinants are screened by restriction endonuclease digestion. Third, the adenoviral construct is cleaved with PacI to expose its ITRs, and subsequently, is transfected into A293 cells line (a packaging cell line) (10, 17). The process of adenoviral production can be conveniently followed in the cells by visualization of the GFP reporter that is incorporated into the adenoviral backbone vector. pAdenoVator-CMV5-IRES-GFP contains the cytomegalovirus (CMV) promoter and a consensus Kozak signal sequence for efficient transgene expression. The GFP expression using the three different transfection reagents is shown in figure 1. Following the mentioned process, after 7 days, adenoviruses were collected and used for amplification by transducing into the A293 cells. Then the assessment of gene expression, production of CEA protein, or other confirmation experimentations were performed.

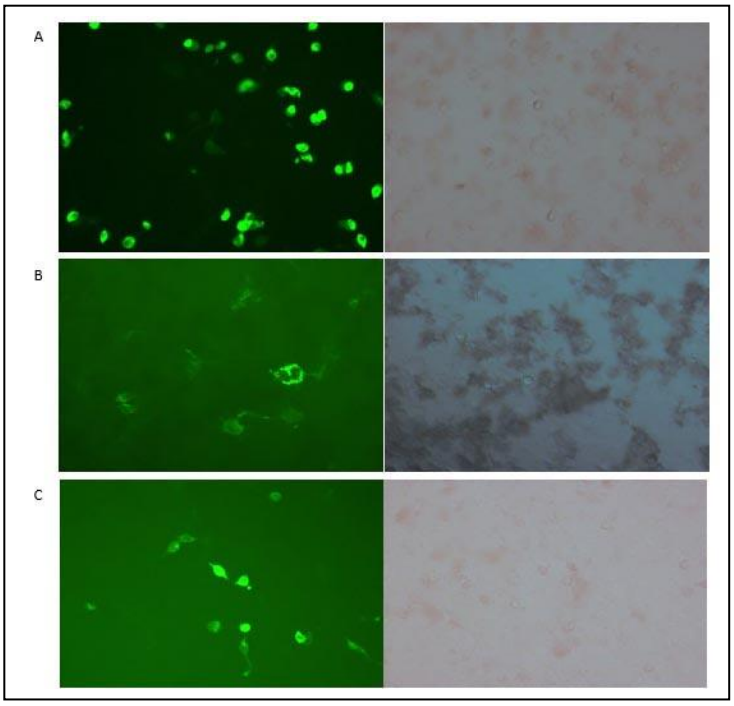


**Figure 1.** Comparison of plasmid transfection using three different reagents
Plasmid transfection was performed into A293 cells using different reagents, and then, the efficiency of the reagents was also compared with each other. A) gene transfection was performed with PEI, B) the process of transfection was conducted with calcium phosphate, and C) the gene was transfected with DMRIE-C. After transfection, the cells were observed with a fluorescence microscope and the GFP gene (as a biosensor) was expressed in the transfected cells, and the efficiency of the reagents in transfection was estimated according to the amount of GFP protein expression in the cells. In this study, it was concluded that the PEI had a higher transfection efficiency, compared to calcium phosphate and DMRIE-C

### 3.2. Carcinoembryonic Antigen Gene Expression Evaluation Using a Real-Time Polymerase Chain Reaction

This study aimed to test the CEA gene expression during the plasmid transfection and verify the functionality of the CMV5 promoter in the pAdenoVator-CMV5-IRES-GFP vector. The real-time polymerase chain reaction was performed on the samples that were harvested at different times, and gene expression changes were calculated by the comparative Ct (ΔΔCt) method. The amount of CEA gene expression at 68 h after transfection was about triple that of at 44 h, which indicated that the promoter had functioned correctly. The results are shown in figure 2.

### 3.3. Virus Transduction and Evaluation of Carcinoembryonic Antigen Protein

In order to produce the AdV, the supernatant of transfected cells was transduced into the A293 cells as described above. Green fluorescent protein expression in the produced virus was observed via a fluorescent microscope (Figure 3). Electrochemiluminescence immunoassay was used to quantify the level of in vitro CEA protein production from AdV. This assay was conducted using cells permissive to AdV infection. After 72 h, the cells were collected and analyzed. The amount of CEA protein in the transduced cells with AdV was 44.52 µg/µl and in the transfected cells with recombinant transfer plasmid as a positive control was 108.6 µg/µl. The cells without any plasmid inoculation were used as a negative control (<0.2 µg/µl). As a result, the production of CEA protein was confirmed in infected cells with AdV.

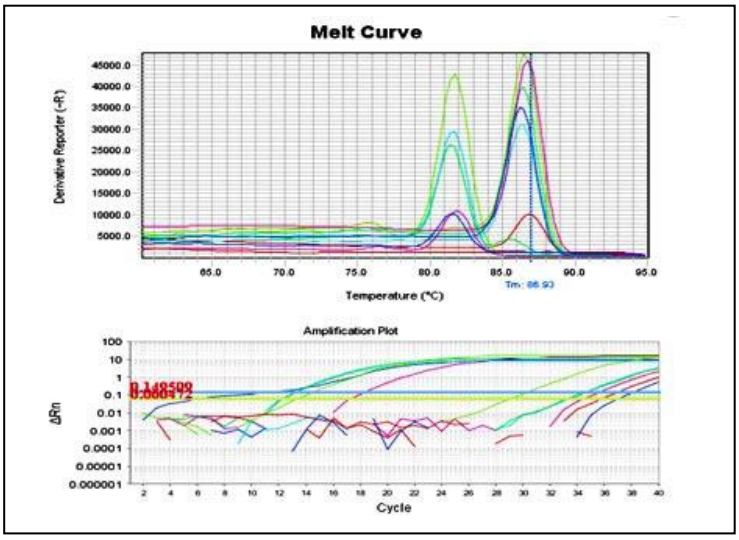


**Figure 2.** Melt curve of the amplified CEA and HPRT genes by real-time PCR
The left curve represents the CEA gene expression and the right curve belongs to the HPRT reference gene. The curves in the earlier cycles of the amplification plot represent CEA and the curves at the ending of the plot belong to HPRT genes expression

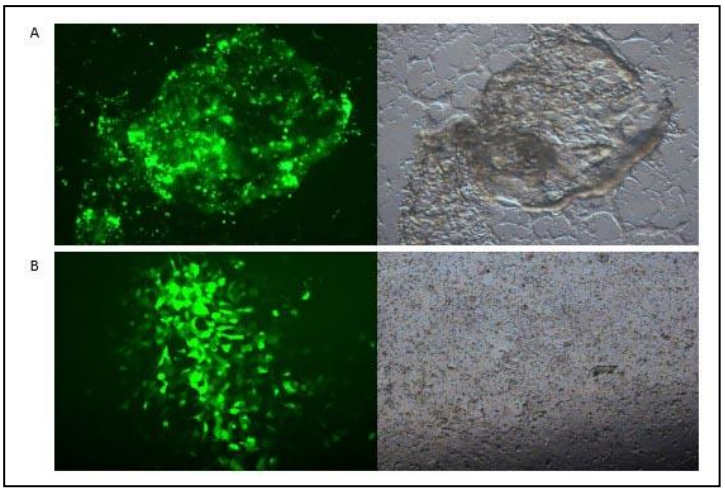


**Figure 3.** Production of the adenoviral vector
A293 cells were transfected with the cloned plasmid with CEA. After 7 days, the cells and media were collected, and the virus was released from the cells. The supernatant containing the AdV was transduced into the A293 cells. Cytopathic effect and GFP protein were observed using a fluorescence microscope. A) The produced virus on the second day and B) Cytopathic effects observed in infected cells on the third day

## 4. Discussion

Over the past decade, extensive experiences have been performed on the use of AdVs in different clinical applications, such as gene transfer and vaccination (6). The overall aim of this study was the construction of AdVs that transferred the CEA gene into the tumor cells to overcome the immune tolerance and trigger effective anti-tumor immunity. There are some considerable points in the production of AdVs, including the maximization and stability of virus particle yields and its cost-effectiveness. One mechanism that helps to achieve these goals is the use of an efficient transfection reagent. Although some transfecting agents, such as lipofectamine 3,000, have

been claimed to be highly efficient in transfection, the rather high cost has limited its use in large volume purposes. An important point about AdV production is to improve the quality of the transfection process.

Significant efforts have been made to overcome the limitations of the gene expression systems in a cost-effective manner. Higher transfection efficiencies of plasmid are achieved with some transfection reagents, including calcium phosphate, PEI, and DMRIE-C. This study examined the efficacy of these reagents in terms of the ability of plasmid transfection in A293 cells. Fluorescent microscope images of a transfected plasmid expressing CEA with each of the mentioned reagents showed the highest transfection efficiency achieved with PEI reagent according to the amount of GFP production in cells, in comparison to calcium phosphate and DMRIE-C. Polyethylenimine is a polycation with a high ionic charge density. The formation of positively-charged reagents and DNA complexes caused a higher transfection efficiency to be obtained. This reagent with low toxicity for cells is highly suitable for cases in which large-scale transfection with low toxicity is desired. Furthermore, PEI is a greatly cost-effective transfection factor that allows for external DNA to be endocytosed, and subsequently, gain access to mammalian host DNA (17).

Carcinoembryonic antigen gene cloned in the transfer plasmid was placed under the control of the modified CMV5 promoter; therefore, to evaluate the accuracy of the promoter performance after viral propagating, the amount of CEA protein with chemiluminescence immunoassay was examined. The results indicated that the promoter worked properly. The prepared AdV in this study would be used for immunotherapy of cancer in mice.

## Authors' Contribution

Study concept and design: T. B.
Acquisition of data: A. Y.
Analysis and interpretation of data: A. Y. and T. B.
Drafting of the manuscript: A. Y.
Critical revision of the manuscript for important intellectual content: T. B.
Technical and material support: T. B. and S. Y. H.
Study supervision: T. B. and S. Y. H.

## Conflict of Interest

The authors declare that they have no conflict of interest.

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