

## PAPER DETAILS

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## Recombinant Adeno-Associated Viral Vector Transduction of Human Prostate Cancer Cell Lines

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### Abstract

At the core of gene therapy lies the use of viral vectors, engineered viruses serving as delivery vehicles to transport restorative genes into target cells. Therefore, the effect of 7 different rAAV serotypes and their different quantites was analysis here on human prostate cancer cell lines PC-3 and DU-145, which are hard to be transfected. PC-3 and DU-145 cell lines were infected with different multiplicity of infection (MOI) ratios of 7 rAAV serotypes, which were expressing the green fluorescent protein (GFP) transgene driven by the CMV promoter. The transduction efficiency was analyzed by fluorescent microscopy and flow cytometry. In addition, the cell viability of the infected cells was measured by Muse Cell Analyzer at the MOI of 10.000. rAAV 2/2 and rAAV 2/6 have the most significant ability to transduce PC-3 cells. Although rAAV 2/2 and rAAV 2/6 were also the most transducing serotypes in the DU-145 cell line, the transduction rates did not exceed 20% in this cell line. On the other hand, after viral infection, no difference in cell viability was observed in PC-3 cells compared to the mock group, while a significant decrease in viability was observed in DU-145 cells. This study determined the transduction efficiency of 7 different rAAV serotypes on human cancer cell lines. While rAAV 2/2 and rAAV 2/6 serotypes achieved more than 60% transduction efficiency in PC-3 cells, the transduction efficiency could not exceed 20% in DU-145 cells. Overall, this study demonstrated that rAAV 2/2 and rAAV 2/6 could mediate the expression of a transgene with a high transduction efficiency.

**Keywords:** Cancer, Gene therapy, rAAV, Recombinant adeno-associated virus, Transduction

### 1. Introduction

Gene therapy in cancer treatment is a favorable approach that aims to target and modify the genetic features of tumor cells to halt their growth or induce cell death [1]. The direct goal of gene therapy in cancer treatment is to correct or exploit the genetic aberrations that contribute to tumor formation and progression. However, challenges remain, including optimizing delivery methods, minimizing off-target effects, and addressing potential immune responses. Also, as research and technology advance, gene therapy can revolutionize cancer treatment and improve patient outcomes [2,3]. Cancer stays one of the most difficult challenges in modern medicine, with its intricacy and heterogeneity posing noteworthy limitations to efficacious treatment [4].

On the other hand, prostate cancer is a common types of cancer in men worldwide [5]. For this reason, a personalized approach to prostate cancer treatment,

which is resistant to therapy, can be needed. For this reason, gene therapy is a promising outcome for patients resistant to chemotherapy and radiotherapy [6,7].

One of the most significant characteristics directly connected with gene therapy is the effectiveness of the gene transfer method [8,9]. This challenge can be overcome by using AAV (adeno-associated virus) for gene therapy in cancer treatment [10]. AAVs have a broad tissue tropism, indicating they can infect different tissues [11]. The specific tissues infected by AAV rely on the serotype of the AAV used in treatment. Different AAV serotypes have further preferences for targeting distinct tissues, including the neuron, liver, skeletal muscle, heart, and lung [12-16]. Today, there are 11 defined serotypes for human tissues [16-18]. 80-90% of adult individuals are seropositive for AAV2; and no immune response, symptom, or disease associated with AAV has been identified [19,20]. In recent studies, the rep gene and

ITR (inverted terminal repeat) belong to the AAV2 serotype (2/). Still, the capsid morphologies (AAV -/1, -/2, -/3, -/5, -/6, -/7, -/8, -/9, -/11, -/12, -/13) AAV recombinant/hybrid serotypes with different AAV serotypes were found to have higher transduction efficiency [21-23]. However, the optimal amount of virus (multiplicity of infection/MOI) for transduction can vary depending on factors such as the AAV serotype used, the specific target cell type, the therapeutic gene being delivered, and the experimental objectives. Achieving an appropriate MOI is critical to obtaining reliable and consistent results in AAV-mediated gene therapy experiments [24-27].

AAV-mediated gene therapy has great potential to revolutionize cancer therapy by offering personalized, targeted, and potentially remedial approaches. For this reason, this study try to qualify the usability of different recombinant AAV (rAAV) serotypes in gene transfer in different human prostate cancer cell lines. Hence, we compared the transduction efficiency of rAAV 2/1, 2/2, 2/3, 2/5, 2/6, and 2/9 serotypes and MOI rates, by employing green fluorescent protein (GFP) reporter transgene in human prostate cancer cell lines PC-3 and DU-145. The study results provide valuable information for choosing the most relevant rAAV serotype for prostate cancer gene therapy.

## 2. Materials and Methods

### 2.1. Cell Line and Culture Conditions

The human prostate cancer cell lines PC-3 and DU-145 for rAAV-mediated transduction evaluation and HEK293T cell line for rAAV production were gained from ATCC (American Type Cell Culture). PC-3 and DU-145 cells grew as adherent monolayers in a culture flask, maintained in the complete medium of RPMI 1640. The related cells were incubated with 5% CO<sub>2</sub> at 37.0 °C in the humidified incubator (Thermo Fisher Scientific, USA). 5–12 passage numbered PC-3 and DU-145 cells were used for this study. HEK293T cells grew as an adherent monolayer in a culture flask, maintained in the complete medium of DMEM high glucose (Gibco, 11995073) medium. The related cells were incubated with 5% CO<sub>2</sub> at 37.0 °C in the humidified incubator. HEK293T cell with a passage number of 30 was used for this study.

### 2.2. Cell Count And Viability Analysis

After incubation of the cells, number and viability of the cells were defined with Muse Count & Viability reagent (Merck Millipore, USA) according to the manufacturer's instructions using the automated Muse® Cell Analyzer (Merck Millipore, USA), which stains viable and dead cells based on their permeability with the help of the two different dyes.

### 2.3. Production of the rAAV Serotypes

Seven different rAAV serotypes were used for this study. rAAV 2/1, 2/2, 2/4, 2/5, 2/6, 2/8, 2/9 serotypes carried the same GFP transgene driven by cytomegalovirus (CMV) promoter. rAAV serotypes were produced with tripartite transfection of the helper, RepCap and GFP transgene (transfer) plasmids in the HEK293T cell line. Hence, pAdDeltaF6 helper plasmid (112867, Addgene), pAAV2/1 (112862, Addgene), pAAV2/2 (104963, Addgene), pAAV2/4 (VPK-424, Cell Biolabs), pAAV2/5 (104964, Addgene), pAAV2/6 (VPK-426, Cell Biolabs), pAAV2/8 (VPK-428, Cell Biolabs), pAAV2/9 (112865, Addgene) RepCap plasmids and AAV-CMV-GFP plasmid (67634, Addgene) were used to produce rAAV 2/1, 2/2, 2/4, 2/5, 2/6, 2/8, 2/9 serotypes. All rAAV serotypes were purified using the iodixanol gradient ultracentrifugation method. The standard was prepared from a plasmid (Addgene, #59462) stock of  $2 \times 10^9$  molecules/ $\mu$ l to generate a standard curve, and then six different dilutions were made between  $2 \times 10^8$ - $2 \times 10^3$  molecules/ $\mu$ l from plasmid stock. The ITR regions of the plasmid were used as a proxy. Also, the fwd ITR 5'-GGAACCCCTAGTGATGGAGTT-3' and rev ITR 5'-CGGCCTCAGTGAGCGA-3' primer pair was used to amplify the related AAV2 ITR region of the plasmid using SYBR Master Mix 2X in qPCR analysis (GeneMarkBio, Taiwan) [28]. The titers of the rAAV serotypes were determined by qPCR analysis according to the above mentioned standards. The helper plasmid pAAV2/2 (104963, Addgene) without the GFP transgene and the RepCap plasmid were used to produce Mock AAV viruses.

### 2.4. rAAV-Mediated Transduction Assays

For transduction evaluation,  $10^5$  PC-3 and DU-145 cells were seeded in each well of 24-well plates and incubated at 37.0 °C for 16 hours. Cells were rinsed with PBS, and were then 1 ml full RPMI-1640 media was added to each well of plates. After plating, PC-3 and DU-145 cells were infected with MOI of 10,  $10^2$ ,  $10^3$ , and  $10^4$  rAAV particles/cells for each serotype. Forty-eight hours post-infection, the rAAV serotypes' transduction efficiency was evaluated in fluorescence microscope imaging and flow cytometer analysis. The cells were analyzed under an IX73 microscope (Olympus, Japan) for fluorescence microscope imaging analysis. The rAAV transduction efficiency was evaluated by GFP imaging using images from the different fields of control and infected cells. The images' GFP intensity (total area of green fluorescence) was analyzed by ImageJ analysis software (NIH, MD). The cells were examined by BD Accuri™ C6 (BD Biosciences, USA) for flow cytometer study.

## 2.5. Statistical analysis

The statistical package software (SPSS) was employed to investigate the differences controlled between the groups. The received results were experimented with Tukey post hoc test in conjunction with One-way ANOVA. The p-values less than 0.05 were assumed significant, and all data defined the duplicate experiments. Results were given as a mean  $\pm$  standard deviation (SD).

## 3. Results

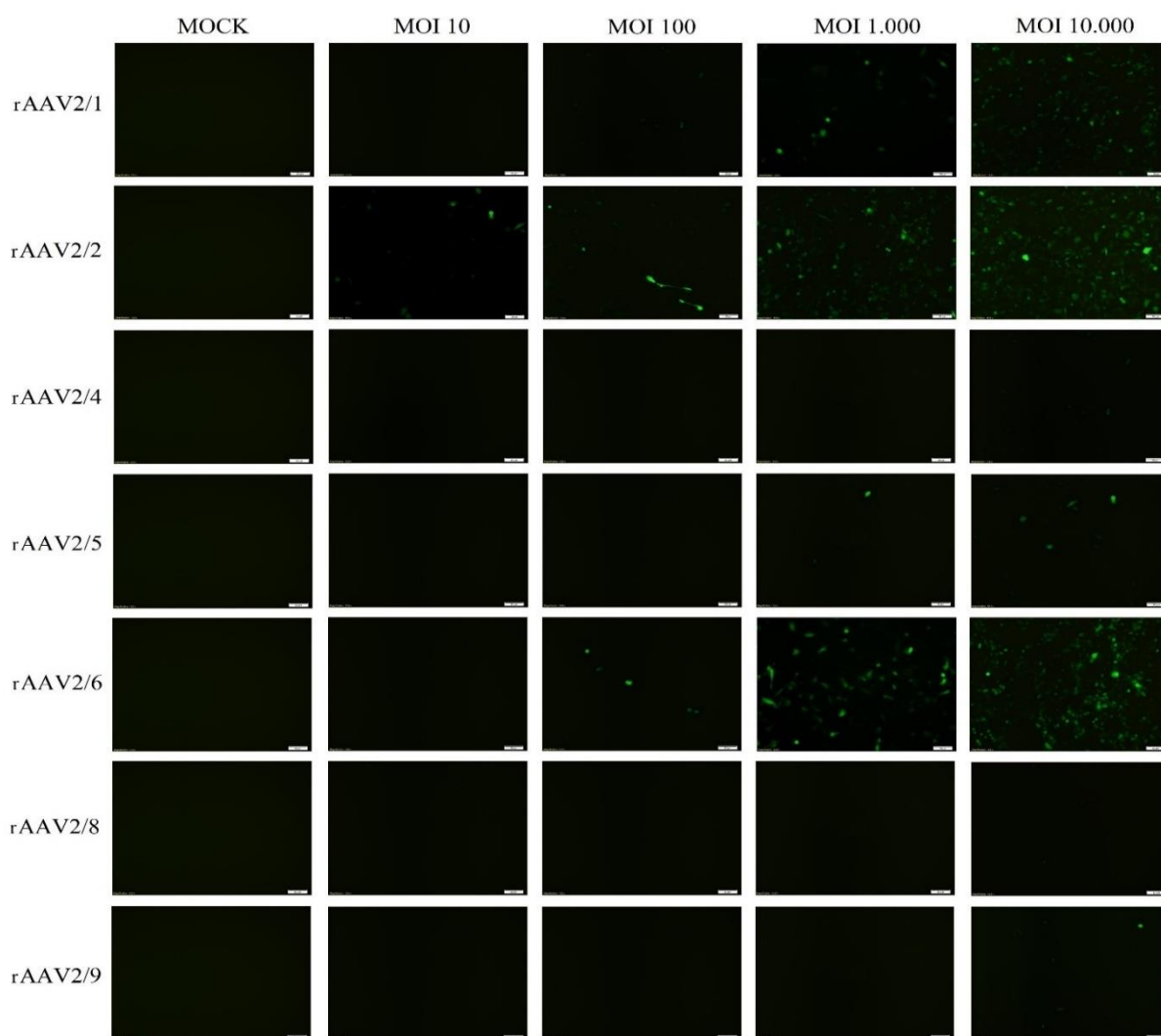
### 3.1. Fluorescence Microscopy Analysis of Seven Serotypes rAAV Vectors in PC-3 and DU-145 Prostate Cancer Cell Lines

In our study, to compare the transduction efficiency of 7 different rAAV vectors based on fluorescence microscopy, PC-3 cells were infected with MOI 10,

100, 1.000, and 10.000 of rAAV2/1, rAAV2/2, rAAV2/4, rAAV2/5, rAAV2/6, rAAV2/8, and

rAAV2/9 carrying the GFP transgene driven by cytomegalovirus (CMV) promoter. The transduction efficiency was measured at forty-eight hours post-infection, and the efficiency was increased with the increasing levels of MOI. As shown in Figure 1, an AAV MOI of 10.000 was given the highest GFP transgene expression level for all serotypes.

The transduction rates for MOI 10.000 rAAV2/1, rAAV2/2, rAAV2/4, rAAV2/5, rAAV2/6, rAAV2/8, and rAAV2/9 transduction rates were 52%, 76%, 5%, 11%, 64%, 2%, 3% compared to mock, respectively. Also, the highest transduction efficiency was obtained from AAV2/2 ( $p < 0.0001$ ), AAV2/6 ( $p < 0.0001$ ), and AAV2/1 ( $p < 0.0001$ ) in contrasted with the other AAV serotypes (lower than 50% transduction), respectively.



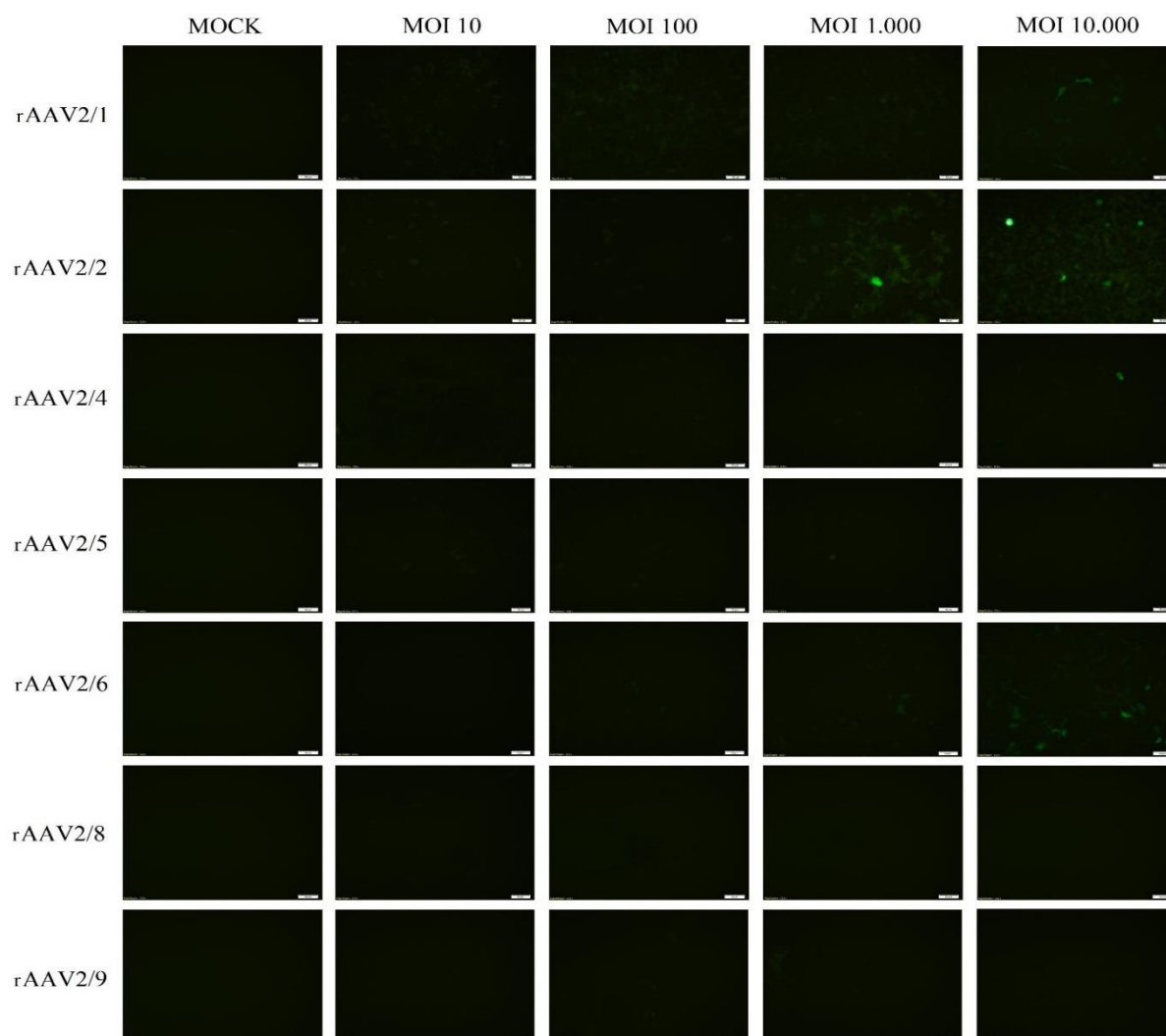
**Figure 1.** Fluorescent microscope image of PC-3 cells transduced with different rAAV serotypes.

On the other hand, these results revealed that the PC-3 cells are highly resistant to rAAV2/4, rAAV2/5, rAAV2/8, and rAAV2/9 infection. The GFP transgene expression could be first detected at forty-eight hours post-infection with fluorescent microscopy, and the signal persisted for 21 days.

DU-145 cells were also infected with MOI 10, 100, 1.000, and 10.000 rAAV2/1, rAAV2/2, rAAV2/4, rAAV2/5, rAAV2/6, rAAV2/8, and rAAV2/9 carrying the GFP transgene driven by the CMV promoter. The transduction efficiency was measured at 48 hours post-infection, and the efficiency was increased with the increasing levels of MOI, the same as PC-3 cells. As displayed in Figure 2, an AAV MOI of 10.000 was given the highest GFP transgene expression level for all serotypes.

The transduction rates for MOI 10.000 of rAAV2/1, rAAV2/2, rAAV2/4, rAAV2/5, rAAV2/6, rAAV2/8, and rAAV2/9 were 9%, 11%, 3%, 1%, 19%, 2%, 1%

(when the dead cells were rinsed away with PBS and the only live adherent cells were counted) compared to mock, respectively (Figure 2). The highest transduction efficiency was obtained from rAAV2/6 ( $p<0.0001$ ), rAAV2/2 ( $p=0.01$ ), and rAAV2/1 ( $p=0.001$ ) serotypes in contrast with the other rAAV serotypes. An increase in dead cell ratios in DU-145 cells was observed with the increasing levels of MOI in contrast to PC-3 cells. Also, the rAAV2/2 infection-related DU-145 cell death ratio was higher than the other rAAV serotypes, which could be noticeable by the microscopic analysis (Figure 2). This situation can be explained by the highest infection rate of rAAV2/2 on DU-145 cells, the same as PC-3 cells, as mentioned above. On the other hand, these results reveal that the DU-145 cells were highly resistant to rAAV2/4, rAAV2/5, rAAV2/8, and rAAV2/9 infection. The GFP transgene expression could be first detected at forty-eight hours post-infection with fluorescent microscopy and the signal persisted for 13 days.



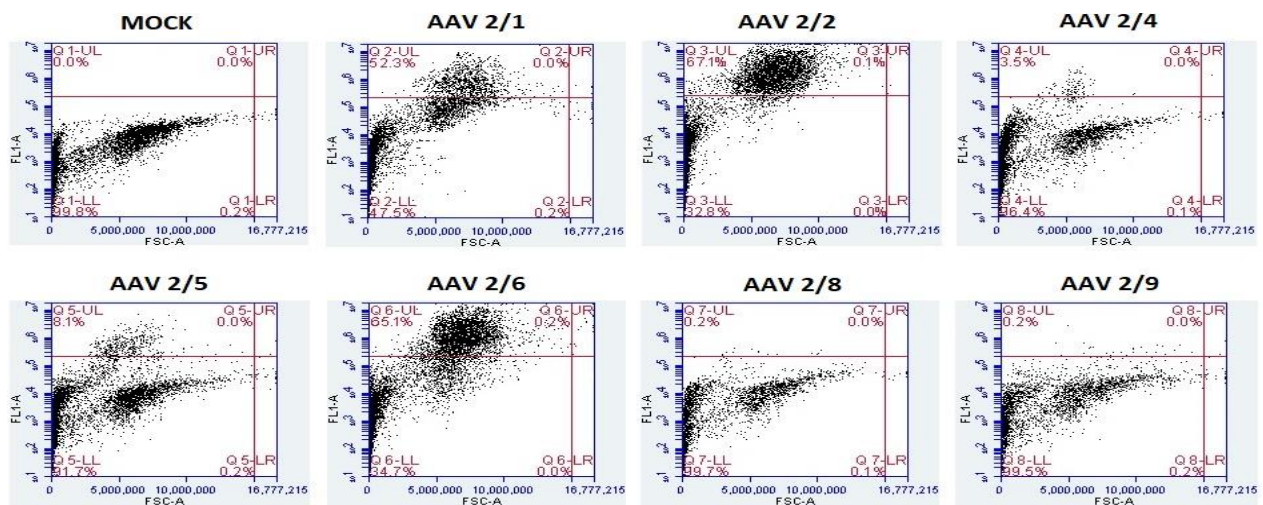
**Figure 2.** Fluorescent microscope image of DU-145 cells transduced with different rAAV serotype.



### 3.2. FACS Analysis of Seven Serotypes rAAV Vectors in PC-3 and DU-145 Prostate Cancer Cell Lines

Fluorescent microscope images showed the highest GFP transgene expression was at 10000 MOI in both PC-3 and DU-145 cell lines (Figure 3 and 4). Therefore, flow cytometry analyzes were performed at 10000 MOI forty-eight hours post-infection in both PC-3 and DU-145 cell lines. From the results displayed in Figure 3, the number of GFP-positive detected cells in the PC-3 cell line was significantly the highest in cells infected with rAAV2/1, rAAV2/2,

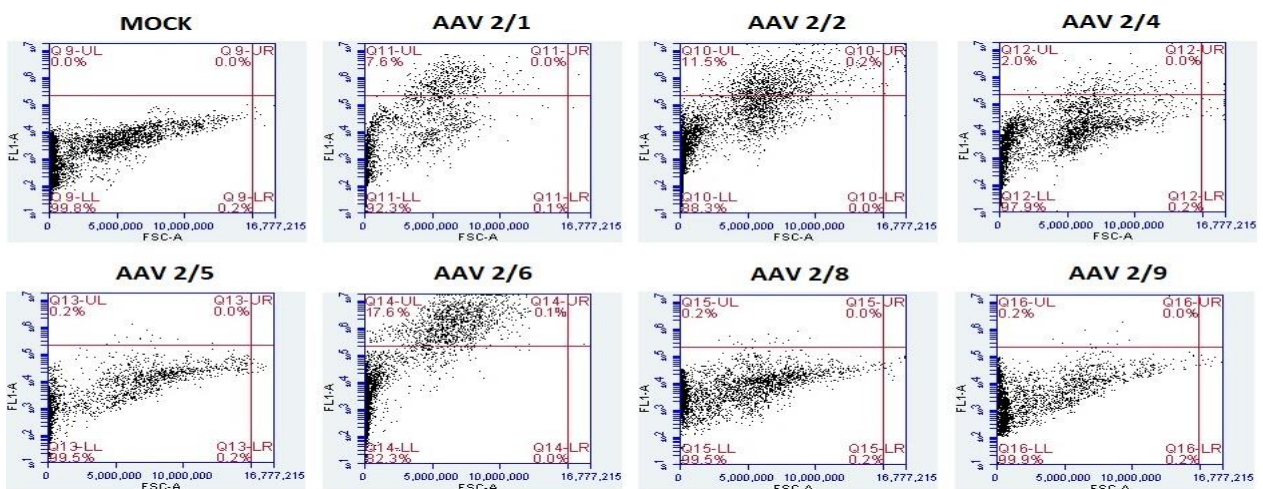
and rAAV2/6 serotypes. In particular, the transduction efficiency in cells infected with the AAV2/2 serotype was maximal at 67.1%, while the transduction efficiency in cells infected with rAAV2/1 and rAAV2/6 was 52.3% and 65.1%, according to FACS analysis. As in fluorescent microscope analysis, PC-3 cells were found resistant to rAAV2/4, rAAV2/5, rAAV2/8, and rAAV2/9 infection, and the number of infection-related GFP-positive cells was found to be 3.5%, 8.1%, 0.2%, and 0.2%, respectively. Also, no GFP-expressed cells are detected in mock-infected cells, according to FACS analysis.



**Figure 3.** FACS analysis of GFP positive PC-3 cells infected with different serotypes of rAAV.

From the results shown in Figure 4, the DU-145 cell groups infected with rAAV2/1, rAAV2/2, and rAAV2/6 serotypes had a significantly higher transduction efficiency compared to all other rAAV serotypes. In particular, the transduction efficiency

in cells infected with the rAAV2/2 serotype was maximal at 11.5%, while the transduction efficiency in cells infected with rAAV2/1 and rAAV2/6 was 7.6% and 17.6%, according to FACS analysis.



**Figure 4.** FACS analysis of GFP positive DU-145 cells infected with different serotypes of rAAV.

As in fluorescent microscope analysis, DU-145 cells were found resistant to rAAV2/4, rAAV2/5, rAAV2/8, and rAAV2/9 infection, and the number of infection-related GFP-positive cells was found to be 2.0%, 0.2%, 0.2%, and 0.0%, respectively (Figure 4).

Also, no GFP-expressing cells were detected in mock-infected cells, according to FACS analysis. In particular, according to the results obtained from the FACS analysis, it was observed that the transduction efficiency does not exceed 20% in DU-145 cells in contrast with the PC-3 cells. However, the number of viable cells passing through the device for analysis was less than that of PC-3 cells.

### 3.3. Cell Viability Assay of PC-3 and DU-145 Prostate Cancer Cell Lines Infected with Seven Serotypes rAAV Vectors

Cell viability was measured by flow cytometry after transduction of the GFP gene in PC-3 and DU-145 cell lines with rAAV vectors (Figure 5 and 6). Our results showed that infection of PC-3 cells with rAAV2/1, rAAV2/2, rAAV2/4, rAAV2/5, rAAV2/6, and rAAV2/8 at MOI of 10000 did not create a significant difference ( $p > 0.05$ ) between the groups. The cell viability rate exceeded 95% in all groups infected with different rAAV serotypes (Figure 5). Mean value of the cell viability rate for mock, rAAV2/1, rAAV2/2, rAAV2/4, rAAV2/5, rAAV2/6, rAAV2/8, and rAAV2/9 serotype infected cells were, 97.4%, 97.0%, 97.6%, 98.4%, 97.8%, 98.0%, 98.0% and 98.6%, respectively.

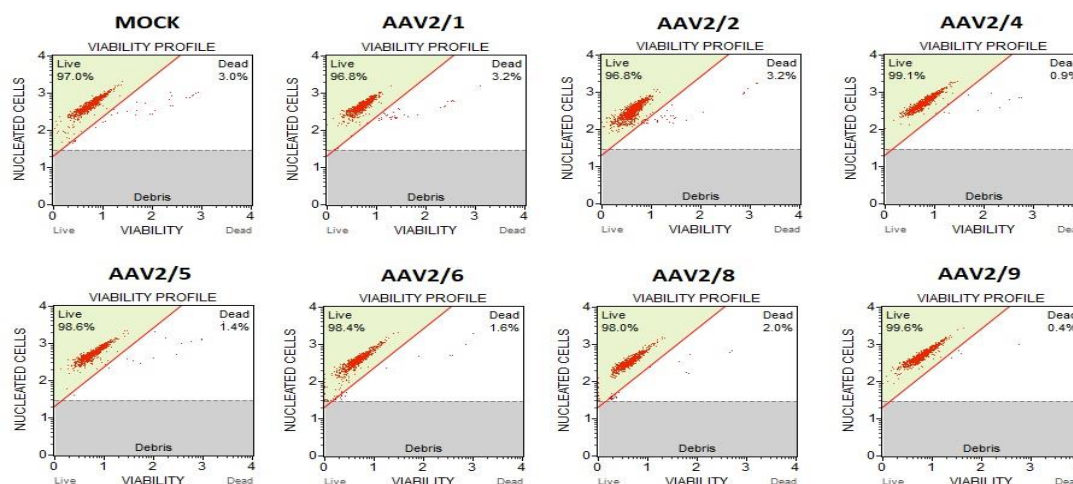


Figure 5. Cell viability of PC-3 cells infected with different serotypes of rAAV.

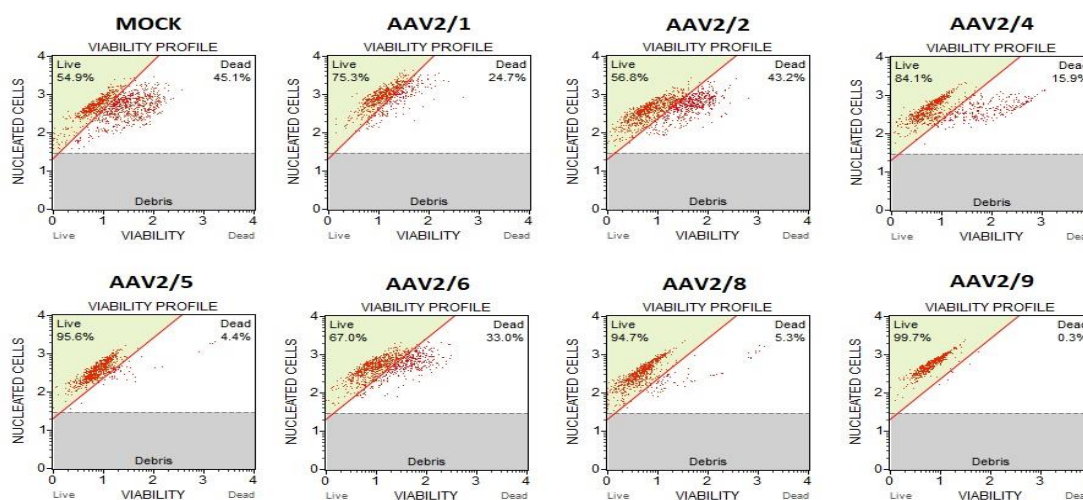


Figure 6. Cell viability assay of DU-145 cells infected with different serotypes of rAAV.

Unlike PC-3 cells, DU-145 cells infected with rAAV2/1, rAAV2/2, rAAV2/4, rAAV2/5, rAAV2/6, and rAAV2/8 serotypes at MOI of 10000 showed decreases in cell viability for some groups. These decreases in cell viability were determined for rAAV2/1, rAAV2/2, and rAAV2/6 serotypes, where the infection rates were the highest. Mean value of the cell viability rate for mock, rAAV2/1, rAAV2/2, rAAV2/4, rAAV2/5, rAAV2/6, rAAV2/8, and rAAV2/9 serotype infected cells were, 55.2%, 76.6%, 57.4%, 84.6%, 97.2, 68.0%, 94.2% and 99.0%, respectively.

#### 4. Discussion

Gene therapy is a recent and alternative remedy for cancer types that cannot be quickly cleared by surgery and are invulnerable to radiotherapy and chemotherapy. For this reason, researchers take advantage of the viral vector-mediated gene transfer for cancer therapy. Hence, the increased transduction efficiency of the preferred viral vector recreates a crucial function in the maximum success rate of gene therapy [29]. Adeno-associated viruses are now vastly investigated and utilized for gene therapy. Also, AAV vectors are fast evolving as one of the prevailing gene delivery approaches due to their prolonged-expression and numerous serotypes qualified for transducing different cell classes. Considerable AAV serotypes have been defined, and individual serotypes have specific cell tropisms based on distinct capsid proteins [30,31].

Another possible use of transduction with AAV vectors is in ex vivo investigations. Here, via rAAV vectors, the genome of mutant cells of the patient can be corrected by homologous recombination to be altered. This modification directly induces a site-specific DNA double-strand break [32-34]. These site-specific double-strand breaks can be produced by nucleases found in the rAAV genome, such as a zinc finger nuclease, transcription activator-like effector nuclease (TALEN), or clustered Regularly Interspaced Short Palindromic Repeats/Cas9 (CRISPR/Cas9) [35-38]. Hence, a wild-type gene can be added instead of the mutant gene in the patient's cell. The most crucial point in using rAAV vectors in this way is to determine the tissue type-specific serotype.

In this study, we revealed the transduction efficiency of seven different rAAV serotypes (rAAV2/1, rAAV2/2, rAAV2/4, rAAV2/5, rAAV2/6, rAAV2/8, rAAV2/9) which induce cell expression of the fluorescent gene GFP in PC-3 and DU-145 prostate cancer cells. In other studies, it has been shown that the rAAV2/2 and rAAV2/5 serotypes' transduction efficiency is higher in respiratory epithelial cells and lung cancer cell lines [40]. In addition, the serotype of

rAAV2/4 in muscle tissue, rAAV2/5 serotype in brain tissue, rAAV2/2 serotype in kidney tissue, rAAV2/8 serotype in pancreatic tissue, and rAAV2/1, rAAV2/8 and rAAV2/9 serotypes in heart tissue were reported to have higher transduction efficiencies [40-44]. Our study revealed that the transduction efficiency of especially rAAV2/2 and rAAV2/6 viruses was superior to other virus serotypes tested. Transduction efficiency in cell line PC-3 was found as rAAV2/2>rAAV2/6>rAAV2/1>rAAV2/5>rAAV2/4>rAAV2/9>rAAV2/8 and in cell line DU-145 was found as rAAV2/6>rAAV2/2>rAAV2/1>rAAV2/4>rAAV2/5=rAAV2/8=rAAV2/9. In particular, the increased MOI rates were effective in the transduction of rAAV serotypes; the increase in the number of GFP-positive cells with the increase in the number of viruses entering the cell medium is evident in fluorescence microscopy analyses. For this reason, starting with a high MOI rate in future studies will increase the effectiveness of gene therapy.

Differences in cell viability were found after infection in PC-3 cells and DU-145 cells infected with rAAV serotypes. Cell viability below 95% was not observed in any group of PC-3 cells after infection with seven different rAAV serotypes, whereas cell viability decreased below 80% in cell groups infected with rAAV2/2, rAAV2/6, and rAAV2/1 serotypes. Furthermore, it was observed that the viability decreased below 60%, especially in DU-145 cells infected with rAAV2/2. Indeed, this result indicates that the serotypes with the highest tropism towards DU-145 cells are rAAV2/2, rAAV2/6, and rAAV2/1. Several reasons can explain this situation. Differently from PC-3 cells, a different receptor mediating the binding of viruses on the membrane surface of DU-145 cells may cause the cells to enter the apoptotic process, or there may be differences in the expression of genes associated with the DNA repair mechanism or apoptosis pathway in the cells. Our knowledge of the cellular receptors utilized by different serotypes of rAAV for cell attachment is limited [40]. In order to understand the difference in cell viability, we may reveal the gene expression levels by performing transcriptome analyses of PC-3 and DU-145 cells after rAAV infections and thus elucidate the mechanism of action of AAV-triggered differential cell death.

All taken together, these results indicate helpful knowledge on selecting the most relevant rAAV serotype for forthcoming viral vector-mediated analyses, which target human prostate cancer cells. rAAV2/2 and rAAV2/6 can readily infect a human prostate cancer cell. Therefore, using serotypes rAAV2/2 and rAAV2/6 equipped with a restorative gene would be the most appropriate option for administering different kinds of human prostate cancer as an alternative therapy choice.



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## Author's Contributions

**Muhammet Burak Batır:** Conceptualized the related study, performed the experiments and analyses of the result, drafted and wrote the manuscript.

## Ethics

There are no ethical issues after the publication of the related manuscript.

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