Gene therapy belongs to contemporary methods of the treatment of various diseases. The first gene therapy clinical trial is well described (1) and the most of others are still in progress (2). The preliminary outcomes are very encouraging and the cardio-vascular and cancer gene therapy clinical reports are promising (3). A great effort is still put into the understanding the mechanisms of cellular gene uptake and to design a genetic tools for gene delivery. Many studies have already described the usefulness of a virus derived vectors for gene therapy applications because their unique properties, such as capability to infect both proliferating and non proliferating cells of broad host range, and possibilities of long-term expression and site-specific integration. rAAV are also described as vectors neither toxic nor pathogenic to the cells. rAAV vectors are also thought to be attractive for cancer gene therapy. Here, we used rAAV2 vectors encoding reporter genes, rAAV/GFP and rAAV/LacZ to transduce cancer cells. The rAAV preparations were produced by a transient triple AAV plasmid transfection of AAV-293 packaging cells and isolated / purified by iodixanol-gradient method. We report a different rAAV transduction efficiency of the two cancer cell lines cells – ovarian carcinoma (OVP 10) and hepatocellular carcinoma (C3A) cells. The expression of the reporter genes due to rAAV uptake was about two fold higher for ovarian cells than for hepatocellular cells. Our studies have also revealed the long-term expression of GFP gene in hepatocellular (C3A) rAAV/GFP transduced cells. These findings indicate that adeno-associated virus derived vectors could be very useful for cancer gene therapy applications, however, further investigations of the mechanisms of rAAV gene delivery are still needed.

Keywords: gene therapy; viral gene preparations; rAAV2; cancer

Gene therapy belongs to contemporary methods of the treatment of various diseases. The first gene therapy clinical trial is well described (1) and the most of others are still in progress (2). The preliminary outcomes are very encouraging and the cardio-vascular and cancer gene therapy clinical reports are promising (3). A great effort is still put into the understanding the mechanisms of cellular gene uptake and to design a genetic tools for gene delivery. Many studies have already described the usefulness of a virus derived vectors for gene therapy applications, mainly because of their high transfection rates in vivo (2, 4). So far, the adenoviral and retroviral vectors are the most frequently used in the clinic. Nevertheless, very often, the safety reasons seriously limit their clinical application (5, 6). Therefore, the new viral vectors are continually designed and cloned by many gene therapy research groups (7-9).

Among viral vectors, initial clinical applications used adeno-associated virus derived vectors (Table 1) (7, 9). Recombinant AAV (rAAV) gene vehicles are derived from discovered in the 1960s adeno-associated viruses (10, 11). AAV belong to Parvoviridae family, genus Dependovirus and represent small, about 20 nm diameter, icosahedral, non-enveloped particles containing single-stranded 4.7 kb DNA genome (11, 12). The first papers based on electron microscopic studies of adenovirus preparations have originally described the adeno-associated viruses as a small adenovirus subunits or adenovirus contaminating viruses (10, 11). Finally, it was evaluated that AAV represent a small defective, DNA – containing viruses which require a helper adenovirus or herpesvirus to replicate in host cells (10-12).

Recombinant AAV vectors are now considered to be an interesting gene therapy vehicles because of their ability to infect both dividing and non-dividing cells of large host range and the potency for stable transfection of the cells; in addition, a lack of immunogenicity and pathogenicity.
of rAAV are also noted, therefore they seem to be safer than retroviruses and adenoviruses (6, 12, 13). rAAV have been shown to infect the normal organ tissues such as muscle, nervous system, liver, lung, skin (8, 14), but the application of them for cancer gene therapy is also discussed (9, 12, 15). As the vectors are integrated to the host genome, the rAAV are not lost upon cell division and they can infect a broad range of tumors of different origin (12, 15). The expression cassette of rAAV is framing by two inverted terminal repeats (ITR) of 145 base pairs each. rAAV can effectively transduce a various type of cancer cell lines cells and tumors in vivo, and the observed transgene expression time is long (9, 15).

In this study we used the recombinant adenovirus vectors serotype 2 (rAAV2) produced in our laboratory. The work shows the studies based on rAAV2 vectors encoding reporter genes, green fluorescent protein (GFP) and β-galactosidase (LacZ). The main aim of the experiments was the evaluation of rAAV serotype 2 transduction efficiency on two cancer cell line cells – ovarian carcinoma cell line (OVP 10) and hepatocellular carcinoma cell line (C3A). These two cancer cell lines can be potentially thought as a target for therapeutic gene based preparations. The obtained results seem to be directly useful for further experiments closely related to gene therapy clinical trials. In the near future the clinical trial of ovary carcinoma patients will be organized in Warsaw Centre of Oncology.

**EXPERIMENTAL**

**Plasmids**

Four AAV plasmid expression vectors were used in the experiments. The vectors pRC, pHelper, pGFP and pLacZ (Stratagene) supply most of the AAV and adenovirus genes that are required for the production of AAV particles. The pRC vector contains rep and cap genes encoding AAV replication and capsid proteins, respectively. The pHelper vector supplies the adenovirus E2A, E4, VA genes. The pGFP or pLacZ vectors contain the GFP and β-GAL reporters genes, respectively, and represent the ITR-containing plasmids with cmv promoters. The AAV plasmid vectors were amplified in bacteria *Escherichia coli* and isolated by a conventional alkaline lysis method using Qiagen Endofree columns. The quality of the isolated plasmids was confirmed spectrophotometrically and by restriction digestion mapping.

**Cell cultures**

The AAV-293 cell line cells (Stratagene) were used to production of recombinant infectious AAV particles. AAV-293 cells are human embryonic kidney cells that have been originally stable transformed by adenovirus type 5 DNA and therefore contain adenovirus E1 gene required for rAAV production in vitro. The AAV-293 cells were maintained in Dulbecco modified Eagle medium (DMEM) with 10% fetal bowine serum, 4 mM L-glutamine and 4,5 g/L glucose at 37°C in a humid-

### Table 1. rAAV vectors based gene therapy clinical trails in the world (2).

<table>
<thead>
<tr>
<th>Clinical category</th>
<th>Gene delivered</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer diseases (prostate cancer)</td>
<td>Herpes simplex thymidine kinase (HSV-TK)</td>
<td>Suicide gene therapy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAV/HSV-TK it</td>
</tr>
<tr>
<td>Cancer diseases (prostate cancer)</td>
<td>Granulocyte-macrophage colony stimulating factor (GM-CSF)</td>
<td>immunomodulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rAAV/GM-CSF id</td>
</tr>
<tr>
<td>Cancer diseases (melanoma)</td>
<td>Granulocyte-macrophage colony stimulating factor (GM-CSF) B7.2</td>
<td>immunomodulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rAAV/GM-CSF it</td>
</tr>
<tr>
<td>Cancer diseases (EBV-positive naso-pharyngeal carcinoma)</td>
<td>Latent Membrane Protein 2A and 1 (LMP2A and LMP1)</td>
<td>immunomodulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rAAV/LMP2A, rAAV/LMP1 iv</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>Cystic fibrosis transmembrane conductance regulator (CFTR)</td>
<td>gene deficiency</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rAAV/CFTR in</td>
</tr>
<tr>
<td>Lipoprotein lipase deficiency</td>
<td>Lipoprotein Lipase (LPL)</td>
<td>gene deficiency</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAV/LPL im</td>
</tr>
</tbody>
</table>
fied atmosphere of 5% CO2. The cells were cultured to 60-70% of confluence and then the triple cotransfection procedure with AAV plasmids was performed.

To determine the rAAV transduction activity the human ovarian carcinoma (OVP 10) and human hepatocellular carcinoma (C3A) cell lines were used (16, 17). The cells were cultured in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum and 0.1 mg/mL penicillin + streptomycin at 37°C in a humidified atmosphere of 5% CO2. The cells were cultured to 70-80% of confluence and then subjected to the further experiments.

**rAAV vectors isolation and purification**

rAAV vectors were produced in helper-free system, without using a helper adenovirus or herpes virus for productive infection. To produce the recombinant infectious AAV vectors, AAV-293 packaging cells were triple transient cotransfected with a rAAV plasmid vector pRC, pHelper and pGFP / pLacZ at a molar ratio of 1 : 1 : 1 using polyethyleneimine 25 kDa (PEI25 kDa) cationic polymer as a DNA carrier agent (18). The transfected cells after 48-72 h were harvested and lysed by freeze-thaw method to release rAAV virions. The isolation and purification procedure was based on the observation that AAV vectors can bind to cell surface heparan sulfate proteoglycan and was performed according to Zolotukhin et al. protocol (19). Briefly, after the temperature dependent cell lysis, the virus–containing supernatant (crude lysate) was purified by iodixanol gradient ultracentrifugation followed by heparin agarose affinity chromatography.

![Figure 1](image1.png)

**Figure 1.** Restriction digestion mapping of pGFP, pRC, pLacZ and pHelper AAV plasmids. The studied vectors were digested with the appropriate restrictases; the size, as base pairs, of digestion fragments are in brackets. 1, 5, 9, 13 – non digested vectors; the digestions are as follows: 2 – NotI (2438, 3703), 3 – EcoRI (6141), 4 – PstI (406, 949, 1365, 3421), 6 – EcoRI (218, 7109), 7 – BamHI (807, 6520), 8 – Xhol (186, 7141), 10 – NotI (2887, 4383), 11 – Ndel (3482, 3788), 12 – PstI (2605, 4665), 14 – BamHI (2613, 9022), 15 – EcoRI (1739, 9896), 16 – EcoRV (2052, 2319, 2617, 4647); M1 – size marker (l/HindIII), M2 – size marker (pK03/Hinfl).

![Figure 2](image2.png)

**Figure 2.** PCR analysis of pGFP, pRC, pLacZ and pHelper AAV plasmids. The studied vectors were analyzed by amplification of the 675 base pairs fragment of ampicillin resistance gene by standard PCR method. The procedure was carried out according to M&M with 1 mg (1), 2 mg (2) and 4 mg (4) of template plasmid DNA. M2 – size marker (pK03/Hinfl); 0 – negative control (without DNA).

**Table 2.** rAAV/GFP transduction of OVP 10 cells. The cells were transduced by 0.6 E+8 and 1.2E+8 of viral particles. The GFP positive cells were counted manually under inverted fluorescence microscope. Results represent the mean ± SD.

<table>
<thead>
<tr>
<th>Cells</th>
<th>OVP 10 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transduction conditions</td>
<td>-rAAV/GFP</td>
</tr>
<tr>
<td>Number of GFP positive cells</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>n = 12</td>
</tr>
</tbody>
</table>

rAAV vectors were produced in helper-free system, without using a helper adenovirus or herpes virus for productive infection. To produce the recombinant infectious AAV vectors, AAV-293 packaging cells were triple transient cotransfected with a rAAV plasmid vector pRC, pHelper and pGFP / pLacZ at a molar ratio of 1 : 1 : 1 using polyethyleneimine 25 kDa (PEI25 kDa) cationic polymer as a DNA carrier agent (18). The transfected cells after 48-72 h were harvested and lysed by freeze-thaw method to release rAAV virions. The isolation and purification procedure was based on the observation that AAV vectors can bind to cell surface heparan sulfate proteoglycan and was performed according to Zolotukhin et al. protocol (19). Briefly, after the temperature dependent cell lysis, the virus–containing supernatant (crude lysate) was purified by iodixanol gradient ultracentrifugation followed by heparin agarose affinity chromatography.
stock was desalted and concentrated by centrifugation through the 100 MVCO filter columns (Millipore) and stored at −80°C for further studies. AAV2 was the only serotype produced and used in the studies.

Quantitative analysis of the rAAV vector stocks

To determine the number of genome particles in rAAV vector stocks quantitative real time PCR (SYBR® Green PCR Master Mix, Applied Biosystems) was performed using primers within cmv promoter sequence (forward primer: CAC-CAAAATCAACGGGACTT, reverse primer: GAGGTCAAAACAGCGTGGAT; 156 base pairs product; T<sub>m</sub> = 50°C). The qPCR curve of each AAV stock sample was related to standard curve prepared using a 6 log spanning serial dilution of pLacZ plasmid vector containing one cmv promoter sequence per molecule. Each dilution step was measured in duplicate per run. Standard curve was calculated by the ABI Prism 7000 SDS Software (Version 1.1, Applied Biosystems) by regression of the crossing points of the PCR curves from the dilution series of

Figure 3. PCR analysis of the rAAV vectors. The obtained rAAV/GFP and rAAV/LacZ viral vectors were analyzed by amplification of the 675 base pairs fragment of ampicillin resistance gene by standard PCR method. The pLacZ and pGFP plasmids served as positive controls. 0 – negative control (without DNA).

Figure 4. Transduction of OVP 10 cells by rAAV/GFP vectors. The cells were transduced by 0.6 E+8 and 1.2E+8 of viral particles. The GFP positive cells were counted manually under inverted fluorescence microscope (see Table 2).

Figure 5. Transduction of C3A cells by rAAV/GFP vectors. The preliminary long-term studies revealed the GFP expression for over forty five days.
Rercombinant adeno-associated virus derived... 97

In addition, the studied AAV plasmid vectors as well as recombinant AAV viral vectors were analyzed by amplification of the 675 base pairs fragment of ampicillin resistance gene by standard PCR method (forward primer: ATAGTTGCCT-GACTCC, reverse primer: GTTACATCGAACTG-GA; 675 base pairs product; \( T_m = 37^\circ \text{C} \)).

**Transduction**

The OVP 10 and C3A cancer cells were grown to 70-80% confluence in six well plates. The growth medium was removed, and rAAV preparations (rAAV/GFP, rAAV/LacZ) at different dilutions (1.2 \( \times 10^6 \) to 4.2 \( \times 10^6 \) gc/mL; MOI of 6-10) were added to each plate in 3 mL of serum-free Eagle’s medium. The cells were then incubated 24 h, after which the culture medium was replaced with the appropriate growth medium supplemented with FBS. The cells were cultured for additional 24-48 h or longer, even up to 46 days as for C3A rAAV/GFP transduced cells. After the incubation time the cells were harvested and the presence of virus DNA sequences as well as the expression of reporter genes in the studied cells were evaluated. The GFP positive cells were also observed and counted under inverted fluorescence microscope (Olympus IX51).

**β-Galactosidase test**

To determine the transduction efficiency of cancer cells with rAAV/LacZ vector the β-galactosidase test was performed. The β-galactosidase enzyme assay was a method for study β-galactosidase activity in OVP 10 and C3A cell lysates prepared from the cells transduced with rAAV/LacZ vector. The test was performed according to Promega β-Galactosidase Assay System protocol with ONPG (o-nitrophenyl-β-D-galactopyranoside, Sigma) as a substrate. β-Galactosidase derived from rAAV/LacZ transduced cells hydrolyzes the colorless ONPG substrate to yellow o-nitrophenol, which content is defined with a spectrophotometer as an absorbance at 420 nm. Additionally, the total protein content was determined by the method of Lowry (the absorbance was read at 750 nm) (20). The relative β-galactosidase activity was calibrated as the ratio of β-gal 420 nm absorbance to total protein 750 nm absorbance [ABS 420/750 nm].

**RESULTS**

The quality of the AAV plasmid vectors was confirmed electrophoretically and by restriction digestion mapping (Figure 1). In addition, the presence of appropriate plasmid sequences was also confirmed by PCR analysis (Figures 2 and 3).

The rAAV vectors were produced in adenovirus-free conditions by a transient transfection of AAV-293 cells with AAV plasmids. As seen in Figure 3 standard PCR analysis showed the presence of rAAV particles in the final fraction stock of AAV preparation. The determination of physical particles was performed using quantitative real time PCR method. According to this method it was estimated that obtained virus stocks contain 0.3 – 0.5 \( \times 10^8 \) genome copies per mL [gc/mL].

The experiments evaluating the infectious activity of obtained recombinant adeno-associated virus vectors encoding reporter genes (GFP and β-GAL) were performed on two different cell lines – ovarian carcinoma cell line and hepatocellular-
lar carcinoma cell line cells. The cells were transduced for 24 h with rAAV preparations at the dose ranging from $1.2 \times 10^5$ to $4.2 \times 10^6$ gc/mL. Figures 4-7 and Table 2 illustrate the results of the cell experiments. As shown in Figure 6, the tested cells revealed an ability to uptake the rAAV vectors and to express the reporter $\beta$-GAL gene. The OVP 10 cells displayed a higher rAAV transduction sensitivity than C3A cells. As seen in Figures 4-7 and Table 2 the transduction efficiency were closely dependent on the titration of rAAV preparations. As expected, increasing the quantities of rAAV vectors resulted in an increase in the rAAV uptake by the studied cells. The observation is also in accord with the rAAV/GFP transduction studies. Table 2 and Figure 4 show the increased number of GFP positive cells closely related to quantity of used rAAV/GFP preparations. Expression of the reporter gene, GFP, was demonstrated with 48-72 h post transduction. Nevertheless, in the preliminary long-term studies performed on C3A cells, the GFP expression was also documented for over forty five days (Figure 5).

**DISCUSSION AND CONCLUSION**

Gene therapy preparations responsible for gene transfer to the cells of interest are currently being dynamically evaluated both in experimental and clinical studies. Recently, a great effort has been put on the engineering gene transfer vectors based on adeno-associated viruses. AAV are a defective viruses closely dependent on the presence of helper viruses for replication. The most commonly described helpers for AAV are adenoviruses and herpesviruses, although the genotoxic agents can also stimulate AAV replication in some cases (21, 22). It is also well described that wild-type AAV2 may integrate in a site-specific manner into a region of human chromosome 19 (AAVS1) (23). Recombinant AAV are though to be maintain in transduced cells as extrachromosomal episomes or as integrated forms (24). The rAAV used in our experiments are based on a recombinant genome flanked by the ITR sequences of AAV serotype 2 (AAV2). These viral vectors were produced in adeno-free system by a transient transfection of AAV-293 packaging cells with the AAV expression plasmids. The plasmids contained all of the genes required for recombinant AAV production in vitro (adenoviral E2A, E4, VA and adeno-associated rep, cap genes).

It is known that the quantitative analysis based on a DNA hybridization or quantitative real time PCR methods, can assess the rAAV production efficiency due to above protocol (25, 26). In this work the obtained viral stocks were estimated by qPCR at 0.3 – 0.5 $\times 10^6$ gc per mL. To determine the rAAV capabilities to gene delivery, the OVP 10 and C3A cell line cells were transduced with the rAAV/GFP and rAAV/LacZ vectors at the dose of $1.2 \times 10^5$ to $4.2 \times 10^6$ gc per mL of cell medium. As seen in Figures 4-7 rAAV were uptaken by the cells at the dose dependent manner. It was also clearly readable that OVP 10 were transduced better and therefore the estimated $\beta$-gal (LacZ) expression was observed at a higher level. It is described that the level of rAAV cell transduction may vary between different cell types (12). The mechanism of this phenomenon is rather poorly understood, but several factors are known to be involved in rAAV transfer. These include expression of a cell membrane receptor for AAV, endosomal processing and some nuclear events as a viral genome conversion from a single to a double-stranded genome (9, 12). Nevertheless, rAAV vectors have efficiently delivered genes into a wide type of cells including in the liver, lung, heart, nervous system and muscle (9). Our results show that rAAV vectors have capability to transduce cancer cells of different origin. The ovarian carcinoma cell line (OVP 10) was originally established from the cells of peritoneal fluid (16), and the C3A cell line represent the population of hepatocellular carcinoma cells (17). The differences between the transduction efficiency may be related to AAV receptor expression, but further experiments are needed to discuss these results. It is also worth mentioning that our outcomes confirm the results of others concerning the long-term expression following to rAAV transduction. In the preliminary long-term studies (Figure 5) the reporter GFP gene expression was documented for over forty five days.

The rAAV represent one of the safest gene delivery system used in gene therapy approaches. Many gene therapy groups have used recombinant adeno-associated virus vectors with considerable success (2, 27, 28). Table 1 shortly describes the examples of AAV gene therapy clinical trials performed in the world. The most of them are still in progress. The clinical success of rAAV based gene therapy protocols seems to be closely dependent on the basic laboratory studies. The better understanding of the AAV gene delivery and integration mechanisms is needed.

**Acknowledgments**

This work was supported by a grant from Polish Committee for Scientific Research (KBN 2P05E03328).
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Received: 02. 06. 2008