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ORIGINAL ARTICLE

Development of novel AAV serotype 6 based vectors with selective tropism for human cancer cells

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Viral vectors-based gene therapy is an attractive alternative to common anti-cancer treatments. In the present studies, AAV serotype 6 vectors were identified to be particularly effective in the transduction of human prostate (PC3), breast (T47D) and liver (Huh7) cancer cells. Next, we developed chimeric AAV vectors with Arg-Gly-Asp (RGD) peptide incorporated into the viral capsid to enable specific targeting of integrin-overexpressing malignant cells. These AAV6-RGD vectors improved transduction efficiency approximately 3-fold compared with wild-type AAV6 vectors by enhancing the viral entry into the cells. We also observed that transduction efficiency significantly improved, up to approximately 5-fold, by the mutagenesis of surface-exposed tyrosine and threonine residues involved in the intracellular trafficking of AAV vectors. Therefore, in our study, the AAV6-Y705-731F+T492V vector was identified as the most efficient. The combination of RGD peptide, tyrosine and threonine mutations on the same AAV6 capsid further increased the transduction efficiency, approximately 8-fold *in vitro*. In addition, we mutated lysine (K531E) to impair the affinity of AAV6 vectors to heparan sulfate proteoglycan. Finally, we showed a significant increase in both specificity and efficiency of AAV6-RGD-Y705-731F+T492V+K531E vectors in a xenograft animal model *in vivo*. In summary, the approach described here can lead to the development of AAV vectors with selective tropism to human cancer cells.

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INTRODUCTION

Despite the recent advances in common methods of treatment including surgery, radiation and chemotherapy, cancer still is a leading cause of human death worldwide.[1] The possibility of manipulating viral genomes and the natural ability of viruses to efficiently infect various cell types has led to increased interest in using a virus-based delivery system for cancer gene therapy.[2–5] Recombinant adeno-associated virus-based vectors have shown advantages as delivery vehicles for gene replacement therapy in order to treat a variety of genetic disorders. Adeno-associated virus (AAV) based vectors have been used successfully in many animal models and have recently shown efficacy in several clinical trials.[6–8] However, the significant challenge in using such a delivery system is general promiscuity of AAV, which limits the possibility of systemic administration of the vectors, especially for peripheral organs and solid tumors where local delivery is not feasible. In addition, a number of steps in the life cycle of AAV, such as viral entry into the cell and intracellular trafficking, limit the effectiveness of these vectors in cancer gene therapy.[9]

The simple organization and natural plasticity of AAV structural elements provide an opportunity to manipulate the viral capsid and to develop customized vectors with unique features.[10,11] Several studies on AAV capsid organization have identified specific regions of the capsid proteins, which are surface exposed and tolerant to insertion of specific peptides.[12,13] It was shown that incorporation of such peptides, which can serve as a ligand to a particular cellular receptor, result in receptor-directed targeting of AAV vectors.[14–16] Recent studies on AAV capsid structure, in combination with information gained from mutagenesis experiments on the capsid genes, have led to the identification of particular regions that have a critical role in AAV vectors tissue-tropism and intracellular trafficking.[17–21] These studies resulted in the development of novel AAV vectors containing one or more mutations on the capsid that transduce various cell types and tissues more efficiently compared with wild-type (WT) AAV vectors.[20,22–27]

In current studies, we show that AAV6 vectors can effectively transduce several distinct human cancer cell lines, such as prostate adenocarcinoma (PC3), mammary gland ductal carcinoma (T47D) and hepatocarcinoma (Huh7). We modified the capsid of AAV6-WT through a number of approaches described above and developed an AAV-based vector for specific and efficient targeting of these human malignant cells. These optimization of the AAV6 capsid included: providing the vector with alternative receptor binding, improvement of nuclear translocation and de-targeting from its primary receptor. We documented the following significant observations: (i) Arg–Gly–Asp (RGD) peptide when incorporated in the AAV6 capsid facilitates viral entry into the integrin-overexpressing cancer cells and improves transduction efficiency approximately 3-fold compared with AAV6-WT vectors; (ii) site-direct mutagenesis of surface-exposed residues involved in intracellular trafficking increases transduction efficiency of AAV6-Y705-731F+T492V vectors up to approximately 5-fold compared with AAV6-WT; (iii) the combination of the RGD peptide and mutations of surface exposed residues on the AAV6 capsid further increases

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transduction efficiency up to approximately 8-fold; (iv) finally, we showed that chimeric AAV6-RGD-Y705-731F+T492V+K531E vectors can specifically target human cancer cells in a xenograft animal model *in vivo* unlike AAV6-WT vectors that efficiently transduce mice hepatocytes. Taken together, these studies suggest that three independent strategies can be combined to optimize the capsid of AAV6, which leads to the development of vectors with selective tropism to a variety human cancer cells, which may prove to be a promising tool for cancer gene therapy.

RESULTS

Insertion of RGD peptide into AAV6 capsid improves vector-mediated transgene expression in human cancer cells *in vitro*

The RGD motif works as a targeting peptide owing to its ability to bind to particular integrin molecules, such as αVβ5″. These integrin molecules are expressed in a variety of cell types and especially at a high level in malignant cells. This RGD motif was identified from a phage-display library selected on cancer cells and later proven to possess targeting abilities *in vivo* when inserted into the viral capsids of adenovirus and AAV2 vectors.[15] We used a similar approach and introduced the RGD motif into AAV6 capsid. The ability of this chimeric vector to transduce cancer cells was evaluated on three different cancer cell types: breast (T47D), liver (Huh7) and prostate (PC3). Our data indicate that AAV6-RGD vectors increase transduction efficiency of these cell lines approximately 3–4-fold (Figures 1–3). The packaging ability of chimeric AAV6-RGD vectors was impaired compared with AAV6-WT and titers of these vectors were approximately 5-10 fold lower. Interestingly, the integrity of AAV6-RGD capsid and ration of structural viral proteins (VP) was intact; however, mobility of viral capsid proteins VP1 and VP2 was slower compare with that from the AAV6-WT (Figure 4a).

Site-directed mutagenesis of surface-exposed tyrosine (Y), serine (S) and threonine (T) residues on AAV6 capsid improves vector-mediated transgene expression in cancer cells

Our recent studies on the AAV capsid crystal structure, combined with data from point-mutagenesis experiments on the capsid genes have outlined the critical role of several specific tyrosine (Y), serine (S) and threonine (T) residues involved in the interaction of these vectors with cellular kinases during infection.[17,28,29] Genetic modifications of these regions increases the efficiency of transduction of AAV vectors in different tissues and cells types, most likely by preventing phosphorylation of these residues, and thus inhibiting subsequent degradation of the vectors by host proteasome machinery. These studies have led to the development of several AAV vectors with higher activity in a variety of cell and animal models.[17,20,22–26,28,30] The capsid of AAV serotype 6 contains 5 tyrosine (445, 674, 701, 705, 731), 17 serine (264, 268, 277, 385, 453, 455, 467, 472, 499, 547, 551, 587, 588, 663, 669, 703 708) and 18 threonine (246, 251, 265, 326, 331, 332, 492, 494, 502, 504, 553, 589, 593, 665, 672, 702, 714 and 722) residues that are surface exposed. Priority was given to the positions that are conserved among various serotypes, and have previously shown a better performance for other AAV vectors[17,22,23,28,30] (Table 1). Indeed, site-directed mutant AAV6-Y705-731F+T492V was identified as the most efficient and it increases the transduction efficiency of several human cancer cell lines approximately 3–5-fold (Figures 1–3).

Combination of RGD peptide and mutagenesis of surface exposed residues on AAV6 capsid further improves vector-mediated transgene expression

Next, we evaluated whether the combination of the RGD motif along with several site-directed mutations can further increase the transduction efficiency of chimeric AAV6 vectors compared with AAV6-WT. These optimizations of the capsid target different steps in the AAV life cycle and presumably might have an additive effect on ability of the vectors to transduce cancer cells. Insertion of the RGD motif improves AAV entry to the cancer cells because it provides binding to an alternative receptor. Alternatively, mutagenesis of surface-exposed residues also improves intracellular trafficking of the AAV vectors. Several single and multiple combinations were tested (Table 1) and the best performing vector was identified as AAV6-RGD-Y705-731F+T492V. These combined modifications increased infectivity of these vectors in the three different cancer cell lines used above: breast (T47D), liver

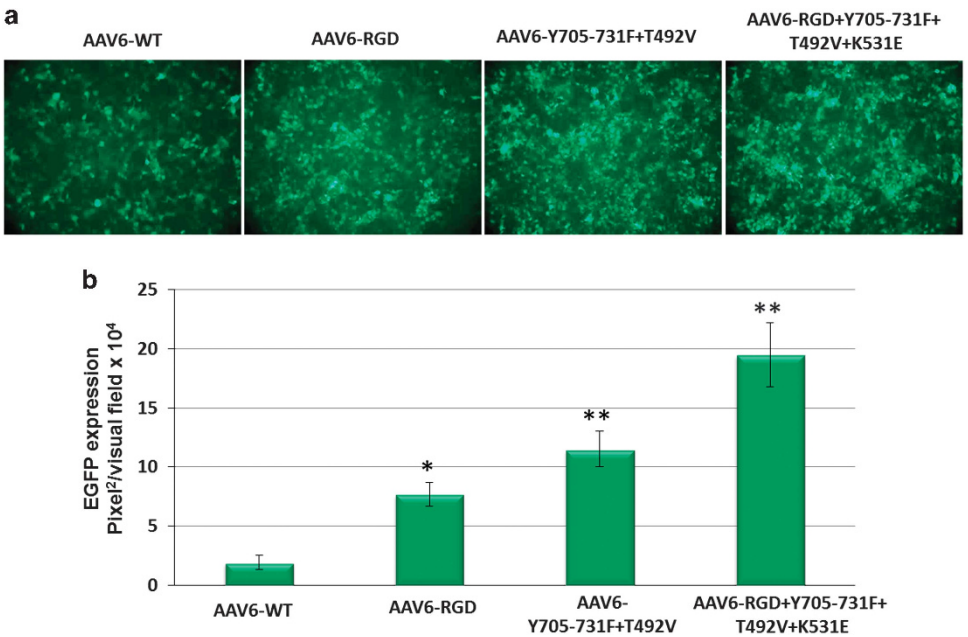

Figure 1. Analysis of EGFP expression in mammary gland ductal carcinoma cell line (T47D) with capsid-optimized scAAV6 vectors. (**a**) EGFP expression analysis at 48 h post infection at an MOI of 2000 vgs per cell. (**b**) Quantitation of transduction efficiency of each of the AAV6 vectors. Infection was performed in triplicates, $*P < 0.005$, $**P < 0.001$ vs WT- AAV. Representative AVV6 vectors from Table 1 are shown.

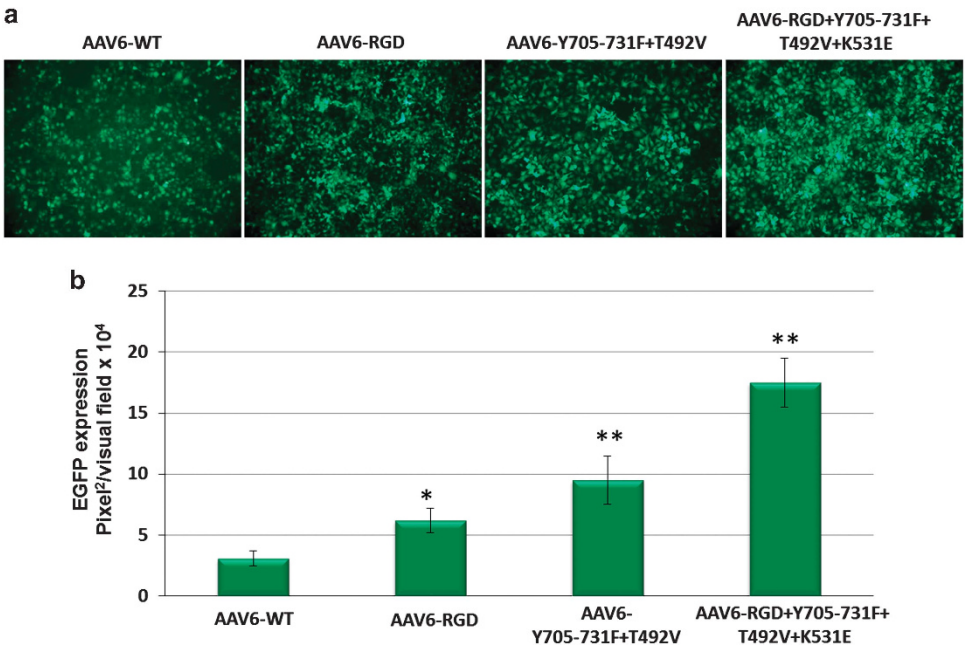

Figure 2. Analysis of EGFP expression in prostate adenocarcinoma cell line (PC3) with capsid-optimized scAAV6 vectors. (**a**) EGFP expression analysis at 48 h post infection at an MOI of 2000 vgs per cell. (**b**) Quantitation of transduction efficiency of each of the AAV6 vectors. Infection was performed in triplicates, $^{*}P < 0.005$, $^{**}P < 0.001$ vs WT- AAV. Representative AVV6 vectors from Table 1 are shown.

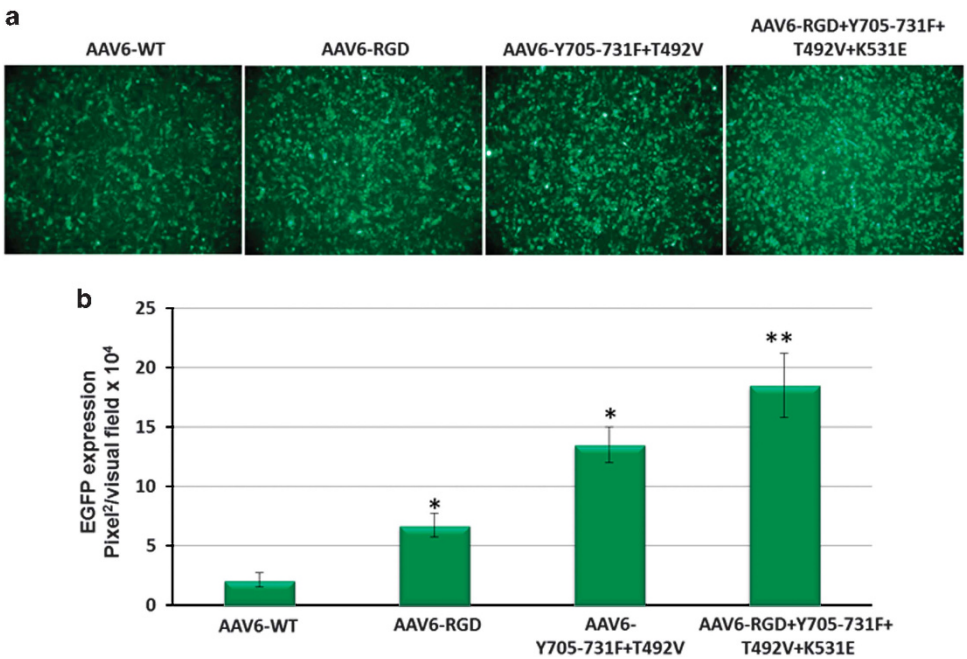

Figure 3. Analysis of EGFP expression in hepatocarcinoma (Huh7) cell line with capsid-optimized scAAV6 vectors. (**a**) EGFP expression analysis at 48 h post infection at an MOI of 2000 vgs per cell. (**b**) Quantitation of transduction efficiency of each of the AAV6 vectors. Infection was performed in triplicates, $^{*}P < 0.005$, $^{**}P < 0.001$ vs WT- AAV. Representative AVV6 vectors from Table 1 are shown.

(Huh7) and prostate (PC3). Our data indicated that the AAV6-RGD-Y705-731F+T492V vector transduced all types of cells ~8-10 fold more efficiently than its AAV6-WT counterpart and approximately 2–3-fold higher than AAV6-RGD or AAV6-Y705-731F+T492V vectors (data not shown). Additional mutation of lysine (K531E), which was previously shown to impair the affinity of AAV6 vectors to heparan sulfate proteoglycan,[19] does not change the efficiency of AAV6-RGD-Y705-731F+T492V+K531E vectors to transduce cancer cells (Figures 1–3). We also performed flow cytometry analysis on integrin-$\alpha_V\beta_5$ expression levels on different cancer cell lines. The level of integrin expression is roughly correlated with the activity of AAV6-RGD-Y705-731F+T492V+K531E vectors compared with AAV6-WT vectors (Figures 4b–d). Similar to AAV6-RGD, the packaging ability of all RGD vectors was lower than AAV6-WT and mobility of structural components was slower (data not shown).

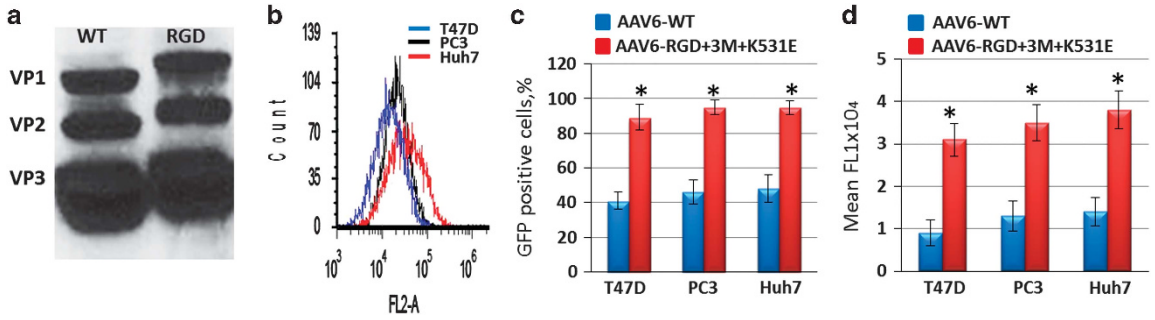

Figure 4. Analysis of capsid-optimized AAV6 vectors integrity and infectivity. (**a**) WB on AAV6 capsid structural proteins VP1, VP2 and VP3 ration and mobility. (**b**) Flow cytometry analysis on integrin-$\alpha_V\beta_5$ expression level in three different cancer cell lines. Flow cytometry analysis of EGFP positive cell number (**c**) and expression level (**d**) of three different cancer cell lines transduced with AAV6-RGD-3M (Y705-731F+T491V) +K531E and AAV6-WT at MOI 2000 vg per cell. Infection was performed in triplicates, $^*P < 0.005$, considered significant.

Table 1. Mutations of surface-exposed tyrosine (Y), serine (S), threonine (T) and Lysine (K) residues in combination with RGD peptide insertion on the AAV6 capsid

AAV6 with RGD and Mutations
S663V
K531E
T492V+S663V
S663V+K531E
Y705-731F+K531E
T492V+S663V+K531E
Y705-731F+T492V (3M)
Y705-731F+T492V+K531E
RGD
RGD+S663V
RGD+K531E
RGD+S663V+K531E
RGD+T492V+S663V
RGD+T492V+S663V+K531E
RGD+Y705-731F+T492V
RGD+Y705-731F+T492V+K531E

The first letter corresponds to the amino acid in the wild-type AAV6 capsid, the number is the VP3 amino acid position that was mutated, and the last letter is the mutant amino acid.

AAV6-RGD-Y705-731F+T492V+K531E vectors were used in all the subsequent studies described below.

Combined optimizations of AAV6 capsid enhance both viral entry into the cell and nuclear translocation

Next, we examined how transduction efficiency of chimeric AAV vectors correlates with viral particles entry into cells and nuclear transport. Our data obtained from quantitative (qPCR) analysis on of three different cell lines at 2 h post infection indicated that AAV6 containing RGD peptide increased viral entry (Figure 5a) to each cell type tested. The efficiency of entry roughly correlates with the expression of integrin-$\alpha_V\beta_5$ on these cells (Figure 4b). Viral genome distribution in the cytoplasm and nucleus 48 h after infection was further analyzed. It is evident that AAV6 vectors containing combined modifications translocate to the nucleus more efficiently than it's AAV6-WT counterpart vectors for each cell line tested, consistent with our previous observation using AAV6 vectors containing tyrosine and threonine mutations (Figure 5b). Approximately 50% of the genomes from the WT vectors were detected in the nuclear fraction 48 h post infection whereas ~80–90% of the genomes from mutant AAV6 vectors were detected in the nuclear fraction at the same time point (Figure 5b). Our data presented as ratio of vector copy number between cytoplasmic and nuclear fractions, suggest that additional mechanisms might be involved in the differences of nuclear trafficking of AAV6-mutant vectors, as that ratio was different for each cell line tested.

Optimized AAV6 vectors efficiently target human cancer cells in a xenograft model *in vivo*

As stated above, we performed several optimizations on the AAV6 capsid aiming to develop vectors with preferable tropism and high transduction efficiency on human malignant cells. These optimizations included: insertion of the RGD peptide that provided vector with alternative affinity for integrin-$\alpha_V\beta_5$, mutations of Y705, Y731 and T492 that improved nuclear translocation, and mutation of K531 that de-targeted vector from heparan sulfate proteoglycan. Therefore, it was of interest to evaluate the efficacy of AAV6-WT, AAV6-RGD, AAV6-Y705-731F+T492V and AAV6-RGD-Y705-731F+T492V+K531E vectors, expressing firefly luciferase (Fluc), in xenograft animal model.

Thus, human liver cancer cells were engrafted into immune-compromised mice and viral vectors were administrated systemically as described in Methods. As can be seen, the Fluc activity from the AAV6-WT and AAV6-Y705-731F+T492V vectors was restricted to mouse hepatocytes, and the level of transgene expression from the mutant vector was approximately 4-fold higher than that from the WT vector (Figure 6). In contrast, the AAV6-RGD and AAV6-RGD-Y705-731F+T492V+K531E vector-mediated transgene expression was localized within the tumor. The expression level was approximately 5-fold higher for vector containing both RGD and point mutations, compared with that for vector containing RGD only (Figure 6). Thus, this data suggests that chimeric AAV6-RGD-Y705-731F+T492V+K531E vectors can specifically discriminate between human cancer cells and animal tissues and express a transgene within tumor at high level.

DISCUSSION

The development of novel oncolytic viruses has several advantages for their clinical applications such as enhanced selectivity for tumor cells and effective control of tumor growth with limited side effects.[31,32] There are several common mechanisms by which oncolytic viruses can destroy malignant cells.[31–34] First, viruses, such as the adenovirus or herpes virus, destroy tumor cells by replicating. This cycle will repeat by infection of adjacent cells with a similar mechanism. The second type of mechanism, employed by viruses, such as adenoviruses, synthesizes cytotoxic proteins during replication that kill the cancer cells. A third mechanism, used by the adenovirus or vaccinia virus, involves initiating specific or nonspecific anti-tumor immune responses which results in a long-term defense against cancer recurrence.

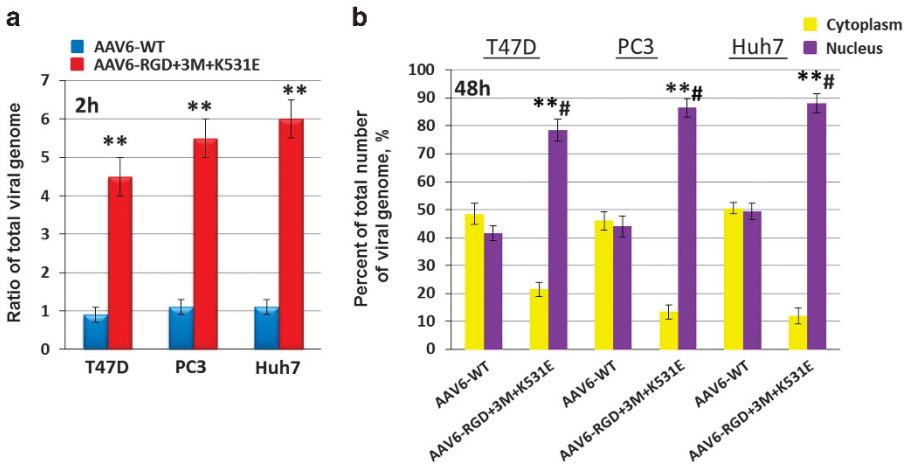

Figure 5. Analysis of intracellular trafficking of AAV vectors to the nucleus. Ratio of total viral genomes in cytoplasm 2 h post infection: (**a**) capsid-optimized AAV6 & AAV6-WT vectors for three human cancer cell lines; (**b**) capsid-optimized AAV6 and WT-AAV6 vectors viral genome distribution at 48 h post infection in three different human cancer cell lines. 3M is Y705-731F+T492V. Cytoplasm – purple, nucleus – yellow. $^{**}P < 0.001$ between nucleus & cytoplasm, $^{\#}P < 0.005$ between WT and capsid-optimized AAV, considered significant.

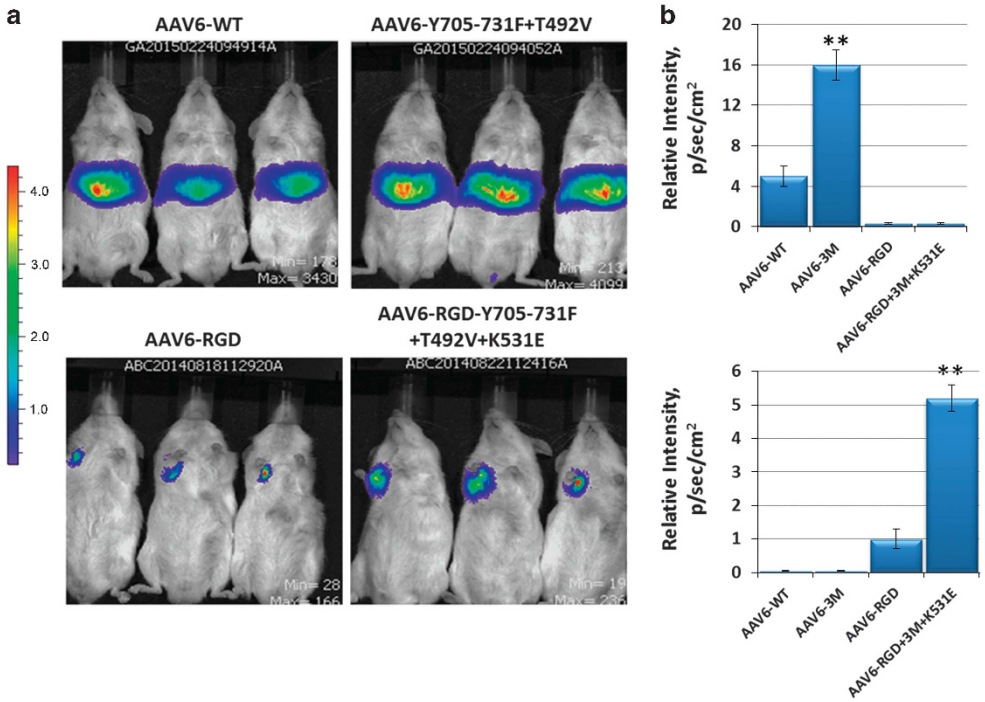

Figure 6. *In vivo* imaging of luciferase after tail vein injection of capsid-optimized AAV6 vectors distribution between human cancer cell and mice hepatocytes. Huh7 cell were xenograft into NSG mice and animals were injected with 5 × 10e10 vg per animal of several most efficient mutants ssAAV6 vectors from Table 1, carrying firefly luciferase gene. Live images were taken to analyze difference in luciferase activity. The visual output represents the number of photons emitted per second per cm^2 as a false color image where the maximum is red and the minimum is blue (**a**) and relative signal intensity (**b**). $^{*}P < 0.005$ was considered as significant.

In addition, these strategies can be combined with cytotoxic or apoptotic gene expression to enhance their therapeutic effect.[35–37] Although many gene therapy clinical trials address cancer used viral vectors, only few were based on AAV vectors.[38] Interestingly, the non-structural element of the WT-AAV2 replication proteins (Rep) show killing efficacy in multiple breast cancer lines but not in normal mammary epithelial cells by activation of caspase dependent or independent apoptosis.[39] Although AAV vectors are proven to be potent delivery systems to a wide range of cells, including malignant cells, lack of specificity and low efficiency *in vivo* are considered major challenges for use of these vectors for potential treatment of cancer. To date, only one member of the family, AAV serotype 3, has been found to have specificity for human hepatocytes and human liver cancer cells. AAV3 vectors have been shown to be able to discriminate between human liver cancer cells and mouse hepatocytes in xenograft animal model *in vivo*.[25,30,40]

However, the natural plasticity of AAV warrants discovering or developing variants capable of specifically target particular type of cells.[19] An even greater possibility to identify a superior AAV vector for the manipulation of host immune response was offered by bioengineering of the viral capsid using recombinant libraries

or by inserting an specific ligand on the capsid surface.[4,5,11] It was shown previously that AAV vectors can be modified by incorporation of specific peptides into capsid protein and these chimeric vectors have prevalent binding to particular receptors. Although cancer cells are derived from different tissues, specific markers can be identified that are common for most, if not all, malignancies regardless of origin. The integrin molecules are wildly expressed in different types of cell. However, they are highly overexpressed in cancer cells and contribute to cancer progression via adhesion-dependent and -independent pathways. They are considered as stem cell markers and contribute to multiple steps of the metastatic cascade, and importantly, they can drive therapeutic resistance of the tumor.[41] Thus, integrins represent a good target to develop viral vectors with high specificity to cancer cells. The ligand for integrins (α5β1, αvβ3, αvβ5 and αIIbβ3) was pull-down from a phage-display library[42] and was used previously to improve the specificity of adenovirus and AAV2 vectors.[15] As we showed that AAV6 vectors transduce human cancer cells originated from different organs, we generated AAV6-RGD vectors and proved its increased transduction efficiency and specificity *in vitro* and *in vivo*. This increase is roughly correlated with the expression of integrin-$\alpha_V\beta_5$ on each cell type (Figures 4b–d) and associated with improved viral entry to the cells, which was confirmed with qPCR analysis of viral genome copies entered cell in 2 h after infection (Figure 5a). It also was previously shown that heparin do not block efficient binding and entry of the AAV-RGD vectors.[15]

We also previously showed that the efficacy of naturally occurring AAV serotypes can be significantly enhanced by substitution of the surface-exposed tyrosine, serine or threonine residues on their capsids. These surface-exposed residues can be recognized and phosphorylated by cellular kinases such as epidermal growth factor receptor protein tyrosine kinase and mitogen-activated protein kinase (p38 MAPK).[20,28,43] Consequent ubiquitination of the capsid has a negative impact on the AAV-mediated gene transfer by directing the vectors for proteasome-mediated degradation. In our recently published data, we analyzed over one hundred of single tyrosine (Y), serine (S) and threonine (T) residues and multiple AAV2 capsid-mutants and identified several combinations with beneficial phenotype.[17] Improvement of the infectivity of AAV vectors was initially done on the serotype 2 capsid whereas these vectors were the most extensively studied and used for several clinical trials. Considering the similarity of capsid structures between AAV2 and AAV6 serotypes,[44,45] we hypothesized and experimentally proved that mutations of these important conserved residues could lead to the improvement of transduction efficiency of AAV6 vectors in several different cell types.[23,27,46,47]

In current studies, we combined these three independent approaches and generated AAV serotype 6 based vectors with alternative tropism and improved intracellular trafficking. These vectors contain two types of mutation: RGD peptide insertion and substitution of critical surface-exposed residues which are involved in recognition of heparan sulfate proteoglycan (K531)[19] and intracellular trafficking (Y705, Y731, S663 and T492) of these vectors. We developed a panel of vectors (Table 1) that contained combination of all three modifications. We showed that combinations, such as AAV6-RGD-Y705-731F+T492V+K531E, greatly improved transduction of several human cancer cells originated from different organs, specifically the breast, prostate and liver. Particularly interesting results were obtained in an animal model *in vivo*. The modified RGD-containing AAV6 vectors display a significantly different profile of gene transfer compared with AAV6-WT vectors. Although, AAV6-WT vectors were predominantly expressing a reporter gene such as Fluc in mouse liver, AAV6-RDG vectors were expressing in human liver cancer cells (Figure 6). Mutations of tyrosine and threonine residues did not change the specificity of the expression, but significantly increased the activity of the vectors up to 3-fold. Considering the fact that integrins are also expressed in other cells, it is possible that limited number of the viral genome can be found by more sensitive methods then luciferase assay, for example qPCR analysis. Thus, our data suggest that detectible expression appears in human cancer cells only, whereas itergrins are overexpressed in malignant cells. However, the use of cell specific promoters, such as alpha-fetoprotein (AFP) promoter for liver cancer[48] or prostate specific antigen (PSA) promoter for prostate cancer, will be necessary owing to safety consideration in case AAV6-RGD vectors will be expressing pro-apoptotic or cytotoxic genes.

The studies have resulted in the development of a novel optimized mutant AAV6-RGD-Y705-731F+T491V+K531E vector which is capable of mediating specific and high-efficiency transduction of human cancer cells. These results suggest that this could be an effective system for the potential delivering of cytotoxic genes to cancer cells. As integrin expression increased in many other types of cancer, similar strategies can be used as a general approach for targeting human malignancies.

METHODS

Cells and antibodies

Packaging cell line HEK293 cell, human prostate (PC3), breast (T47D) and liver (Huh7) cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and maintained as monolayer cultures in DMEM medium (Invitrogen, Madison, WI, USA) supplemented with 10% fetal bovine serum (Sigma, St Louis, MO, USA) and antibiotics (Lonza, Allendale, NJ, USA). Human Integrin alpha V beta 5 expression level was analyzed with mouse monoclonal PE-conjugated antibody (R&D System, Minneapolis, MN, USA; Clone P5H9), murine IgG1 (Invitrogen). Anti-AAV-cap B1 monoclonal antibodies was generously provided by Dr N. Muzyczka, University of Florida.

Constriction of AAV packaging plasmids

Site-directed mutagenesis was performed as a two-stage PCR with plasmids pACGr2c6 as described previously using Turbo Pfu Polymerase (Stratagen, Santa Clara, CA, USA).[17] Primers were designed to introduce changes from tyrosine (TAT) to phenylalanine (TTT or TTC) or threonine (AGC) to valine (GTA or GTC) for each of the residues mutated. Fragment sequence – 5′-accggctgcgattgccgcggcgattgcttttgcgcgctgagc-3′ corresponding to the amino acid sequence TGSDSRGDSFSALS[15] was inserted into AAV6 capsid gene at position 585 counted from initiation codon. Insertion of the fragment was confirmed by unique SacII digestion.

Production of recombinant AAV vectors

Recombinant AAV vectors were generated as described previously[17,49] by triple transfection of HEK293 cells using Polyethelenimine (PEI, linear, MW 25,000, Polysciences, Inc., Warrington, PA, USA). Cells were harvested 72 h posttransfection, and vectors were purified by iodixanol (Sigma, St Louis) gradient centrifugation followed by ion exchange column chromatography (HiTrap Q HP 5 ml, GE Healthcare, Piscataway, NJ, USA). The virus was then concentrated into lactated Ringer's using centrifugal spin concentrators (Apollo, 150-kDa cutoff, 20-ml capacity, CLP; Orbital Biosciences, Topsfield, MA, USA). DNAse I-resistant AAV particle titers were determined by qPCR with the following primer-pairs specific for the CBA promoter: F-5′-TCCCATAGTAACGCCAATAGG-3′, R-5′-CTTGGCATATGATACACTTGATG-3′ and SYBR GreenER PCR Master Mix (Invitrogen).

In vitro transduction assays

Human cancer cells were transduced with AAV vectors with 2000 vgs per cell and incubated for 48 h. Transgene expression was assessed by flow cytometry analysis or as the total area of green fluorescence (pixel^2) per visual field (mean ± s.d.) by ImageJ software (Bethesda, MD, USA) as described previously.[17,20] Images were obtained by fluorescent microscope Leica CTR4000 (Wetzlar, Germany) with × 20 objective. Analysis of variance was used to compare test results and the control, which were determined to be statistically significant. In separate experiments GFP expression was analyzed by flow cytometry on Accuri C6 instrument (Franklin Lakes, NJ, USA).

Analysis of vector genome distribution in cytoplasm and nuclear fractions

Cancer cells (~2×10^5) were infected by various AAV vectors with MOI 10,000 vgs per cell. Cells were collected at different time points, treated with trypsin to remove adsorbed viral particles and then washed extensively with phosphate buffer saline. Nuclear and cytoplasmic fractions were separated with Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific, Waltham, MA, USA) according to manufacturer instructions. Viral genomes were extracted by proteinase K digestion followed by phenol/chloroform purification and DNA precipitation by 100% EtOH. Viral DNA was detected by qPCR analysis with the BGH specific primers as described above. The difference in amount of viral genome between cytoplasmic and nuclear fractions was determined by the following rule: C_T values for each sample from cells treated with virus were normalized to corresponding C_T from mock treated cells (ΔC_T). For each pair wise set of samples, the fold change in packaged genome presence was calculated as fold change = $2^{-(\Delta CT\text{-cytoplasm} - \Delta CT\text{-nucleus})}$. ref. 17,23) Data from three independent experiments were presented as a percentage of the total amount of viral genome in the nuclear and cytoplasmic fractions (Figures 2d and e) or as a ratio between two samples infected with different vectors (Figures 2a–c).

Western blot analysis

Western blot analysis was performed as described previously.[50] Samples for pure viral stock normalized by protein concentration were separated on 10% polyacrylamide/SDS gels, followed by transfer to a nitrocellulose membrane. Primary antibodies, anti-cap B1 monoclonal antibodies (1:3000), followed by secondary horseradish peroxidase-linked rabbit-anti-mouse antibodies (1:3000, BoiRad, Hercules, CA, USA) were used to visualize protein expression.

In vivo bioluminescence imaging

All animal experiments were approved by the University of Florida Institutional Animal Care and Use Committee. All procedures were done in accordance with the principles of the National Research Council's Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize suffering. Ten-week-old non-obese diabetic/severe-combined immunodeficient, interleukin 2-gamma-deficient male mice (Jackson Laboratory, Bar Harbor, ME, USA) were injected subcutaneously with 5×10^6 per mouse human prostate cancer cells, PC3. Two weeks later animals were injected intravenously with 5×10^{10} vgs per animal of AAV6 vectors expressing firefly luciferase (Fluc) gene. Luciferase activity was analyzed 72 h post AAV vectors injection using a Xenogen IVIS Lumina System (Caliper Life Sciences, Waltham, MA, USA). Briefly, mice were anesthetized with 2% isofluorane and injected intraperitoneally with luciferin substrate (Beetle luciferin, Caliper Life Sciences) at a dose of 150 mg g^{-1} of body weight. Mice were placed in a light-tight chamber and images were collected at 5 min after the substrate injection. Images were analyzed by the Living Image 3.2 software (Caliper Life Sciences) to determine relative signal intensity.[17,30]

Statistical analysis

Results are presented as mean ± s.d. Differences between groups were identified using a grouped-unpaired two-tailed distribution of Student's *t*-test. *P*-values < 0.05 were considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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