RESEARCH ARTICLE

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Adenovirus Serotype 5 Vectors with Tyr3-Octreotate Modified as a Virulytic Oncotherapy Agent in AGS Cells

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ABSTRACT

Background**:** Oncolytic viral therapy is known as a new promising strategy to treat cancer. Oncolytic viruses (OVs) can replicate in cancer cells, and besides this primary effect, OVs can also stimulate the immune system. Moreover, Adenovirus serotype 5 (Ad5) is widely used as an oncolytic agent for cancer therapy. The present study aimed to make a change in the adenovirus fiber area.

Methods**:** The tyr3-octreotate sequence was inserted into the HI-loop in the adenovirus fiber knob. In addition, the recombinant virus was transferred to the HEK-293 packaging cells using the calcium phosphate method; therefore, the new virus was prepared. Accordingly, the virus titer was calculated using the TCID50 method. Afterward, recombinant adenovirus and wild-type 5 strain were transferred to perform against the human adenocarcinoma gastric cell line (AGS). Subsequently, they were evaluated for cytopathic effects, and the manipulated virus was then confirmed Using PCR and sequencing.

Results and conclusions**:** In this regard, the obtained results showed that the recombinant adenovirus has a greater ability in infecting AGS cells compared to the wild-type adenovirus. Our data suggest that modification of Ad5 with tyr3-octreotate expands its usage as an oncolytic agent against the AGS cells.

fruits. The results are important for increasing mutation efficiency in watermelon breeding.

Key words: Adenovirus, Oncolytic viruses, tyr3-octreotate, AGS

Introduction

Oncolytic virotherapy (OV) is the use of a replication-competent virus for the treatment of cancer (Russell et al., 2012). There are more than 3,000 species of viruses; however, all of them are not suitable as oncolytic agents. The OV must be non-pathogenic, must have intrinsic cancer-selective killing activity, or can be engineered to express the attenuating genes or arming genes (Maroun et al., 2017). Notably, Tumor selectivity could be performed at the level of receptor-mediated cell entry, intracellular antiviral responses and/or restriction factors that determine how susceptible the infected cell is to support the viral gene's expression and replication (Kaufman et al., 2015; Seymour & Fisher, 2016). Historically, there have been anecdotal reports on temporary tumor regression and cancer remission after the patient contracted with a natural viral infection including the responses of lymphoma after wild type measles virus infection (Lin & Nemunaitis, 2004; Kelly & Russell, 2007). In the 1950s–1970s, live viruses were deliberately injected into the patients with cancer, which showed promising activity, and particularly notable were the followings: Egypt 101 West Nile virus (4/34 transient regressions), adenovirus lysates (26/40 showing localized tumor necrosis), and Urabe strain mumps virus [37/90 complete remission or partial responses (PR)] (Kelly & Russell, 2007). However, toxicity was also noted in previous studies using several viral isolates that were not engineered for tumor selectivity, especially in the immune-suppressed patients with leukemia or lymphoma whereby 5 out of 8 patients experienced severe encephalitis after receiving the Egypt 101 isolate of West Nile virus (Southam & Moore, 1952; Kelly & Russell, 2007).

By genetic engineering, we can currently design live replicating viruses to not only be highly tumor-selective through cell entry and transcription targeting but also armed with the reporter genes for noninvasive monitoring of the pharmacokinetics of virotherapy as well as for enhancing cytotoxic activity, immunogenic cell death or immune modulators. To date, three OVs are commercially available for cancer treatment. Accordingly, these include

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Rigvir approved in Latvia, Georgia, and Armenia; Oncorine (H101) approved in China; and talimogene laherparepvec (T-VEC) approved in the USA (Russell & Peng, 2018). Moreover, different types of viruses have been tested for OV including the RNA viruses such as human rheumatoid arthritis, Newcastle disease virus (NDV), influenza virus, poliovirus, myxoma virus, vesicular stomatitis virus (VSV), Senecavirus, sindbis virus (SINV), and DNA viruses such as adenoviruses and herpes simplex virus (HSV) (Mathis et al., 2005; Cervantes-García et al., 2008).

Adenoviruses are non-enveloped, icosahedral, double-stranded DNA viruses with long fiber knobs protruding from each capsid vertex (Vellinga et al., 2005; Ahi & Mittal, 2016). At least, 70 serotypes of human adenovirus exist, with serotype 5 as the most commonly used one. Accordingly, 6 out of 7 oncolytic adenoviruses in clinical testing use a serotype 5 backbone. Moreover, clinical data has been published on telomelysin in solid tumors (Nemunaitis et al., 2010), CG0070 in bladder cancer (Packiam et al., 2018), and DNX-2401 in malignant brain tumors (Lang et al., 2018).

Adenovirus receptor binding occurs at both of the RGD motifs of penton base proteins and at fiber knobs that are extended from them (Cao et al., 2012). Receptor specificity is dependent on the virus subgroup and serotype, and to date, at least 11 receptors have been shown to facilitate adenovirus binding (Zhang & Bergelson, 2005). Moreover, many adenoviruses bind integrins via penton RGD motifs, facilitating entry, and infection of the permissive cells (Mathias et al., 1994). Ad5, as a subgroup of C virus, can also infect the cells through coxsackie adenovirus receptor (CAR); heparan sulfate glycosaminoglycans (HS-GAG); and other receptors such as MHC-I, VCAM-1, and DPPC; while Ad3, as a subgroup of B virus, binds CD46, CD80, and CD86 (Zhang & Bergelson, 2005). Notably, efforts to modify tissue tropism include the modifications to the penton base RGD binding domain and serotype switching or modifications of fiber knob proteins (Arnberg, 2012). Accordingly, Ad5 enters the cells using a CAR receptor, which is highly expressed at the surface of normal epithelial cells, astrocytes, and neurons. While the expression of this receptor at the surface of cancer cells is very variable. Therefore, to tackle this problem, various strategies have been used such as the genetic manipulation of the capsid, especially the end of C-terminal, and the HI loop or modification of the fiber molecule by replacing the fiber with another serotype fiber. Therefore, the corrected tropism allows the virus to infect cancer cells with a system that is independent of the CAR receptor (Coughlan et al., 2010; Aurelian, 2013; Garcia-Moure et al., 2017). Moreover, several oncolytic adenoviruses incorporate payloads to enhance antitumor activity. Accordingly, these payloads include GM-CSF, expressed by CG0070 and ONCOS-102, which activate the antigen-presenting cells (APCs) and may also enhance the uptake and the presentation of both viral- and tumor-associated antigens following the oncolysis (Kuryk et al., 2016). Notably, LOAd703 expresses CD40 and 4–1BB ligands, which activate APCs and T-cells through co-stimulation, respectively (Eriksson et al., 2017). In addition, VCN-01 expresses soluble hyaluronidase, which degrades extracellular M hyaluronic acid and may also enhance the virus spread in solid tumors (Guedan et al., 2010).

The aim of this study was to make a change in the adenovirus fiber area. Therefore, a new adenovirus would be created that has a high compositional tendency to bind to the tumor cells selectively.

Materials and Methods

Cell Culture

In this study, HEK-293 cell line was used for the recombinant virus culture. Accordingly, HEK-293 originates from the human embryonic kidney and is a type of epithelial cell. In this regard, HEK-293 cell, as a part of Ad5 DNA, is able to produce E1A and E1B proteins to meet the need for an incomplete virus during the reproduction process. In this study, HEK-293 (ATCC® CRL-1573™, human embryonic kidney cell line) and AGS (ATCC® CRL-1739™, human gastric cell line) were purchased from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Afterward, the cells were cultured firstly in DMEM and then in RPMI-1640 medium, with 10% of the heat-inactivated fetal bovine serum (FBS) (Gipco, MD, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Thermo Fisher Scientific) at 37ºC in a humidified atmosphere containing 5% CO_2.

Production of recombinant virus

E-*coli* strain SW102 was applied using bacmid pAdZ5-CV5-E3+ (kindly provided by Richard Stanton, Cardiff University, Cardiff, United Kingdom). Accordingly, this bacmid contains the Ad5 genome, with the E1 region that was replaced by a selection/counterselection cassette (*als* cassette) consisting of the *bla* (ampicillin resistance), *lacZ* (β-galactosidase), and *sac*B (sucrose resistance) genes. In this study, the PCR product was generated by the desired modification with the flanking sequences of 50 bp with homology to the virus genome on each side of the site of the modification.

The tyr3-octreotate sequence was placed in the HI-loop of the Ad5. Afterward, the TATE sequence was cloned at the BstB site of pfiber, which was a gift from Dr. PJ Bosma (University of Amsterdam, Amsterdam, The Netherlands), to create the pfiber-TATE. Accordingly, the pfiber included the adenovirus fiber protein with the BstBI site located in the sequence encoding the HI-loop.

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To generate pAd5 (GFP), the cytomegalovirus promoter-green fluorescent protein (CMV-GFP) cassette was PCR amplified from Ad5 (GFP) using the primers CMV-GFP-F and CMV-GFP-R, which was then purified by gel extraction (Fermentase). The heat-activated and freshly made competent *E. coli* SW102 cells containing pAdZ5-CV5-E3+ were transferred by 50 ng of PCR product using chemical methods. Also, the selection was performed on LB-sucrose plates, containing LB with no NaCl, 6% sucrose, 200 µM IPTG (isopropyl-β-d-thiogalactopyranoside; Sigma-Aldrich, St. Louis, MO), and 40 µg of X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside; Invitrogen)/ml. Moreover, the positive colonies have designated pAd5 (GFP).

To change the HI-loop area of the adenovirus fiber, the *als* cassette was PCR amplified using the primers F-als and R-als, which was then knocked into the HVR5 site in pAd5 (GFP). The selection was performed on LB agar plates containing 50 µg of ampicillin/ml, 200 µM IPTG, and 40 µg of X-Gal/ml. Subsequently, the *als* cassette was replaced by the PCR product of the Oct F/R primers from the fiber area containing the tyr3-octreotate sequence in the pFiber-TATE plasmid. Afterward, the selection was performed on LB-sucrose plates. All the primers used are shown in Table 1.

Recombinant virus production and titration

Because this bacmid has an I-SceI sequence, it has no need to be linearized prior to the transfection process to eukaryotic cells. The recombinant viruses were transferred to HEK-219 cells using the calcium phosphate transfection method. After 15 days of viral transduction, viral particles were released from the infected cells by 4 times freezing, thawing, and vortexing. Afterward, all the viruses were stored in aliquots at −80 °C.

Notably, we have used the TCID50 titration method as a reference for the validation of our rapid titration technique. Accordingly, TCID50 was performed based on the end-point dilution of the virus at which a cytopathic effect (CPE) that is detected in 50% of the cell culture replicates infected by a given amount of virus suspension.

Infection of human AGS cells with recombinant structures

The AGS cells were plated in a 6-well plate at $2*10^5$ cells/well in the RPMI-1640 medium with 10% FBS (Gipco, MD, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Thermo Fisher Scientific) at 37ºC in a humidified atmosphere containing 5% CO_2. In passing that time, the AGS cells were treated by Multiplicity of infection (MOI) coefficient equal to 10^7 of the recombinant virus for 12 hours. In this regard, after changing the medium with the fresh one, the cells were incubated in at 37ºC with 5% CO_2.

In this study, Wild-type Ad5 with the same GFP gene (Ad-GFP) as the recombinant virus, was used as a negative control. In passing 3 to 4 days from the inoculation, the cells were examined for the cytopathic effects, and expression of the GFP gene was also assessed under a fluorescence microscope.

Confirmation of the presence of recombinant virus in AGS cells

In order to confirm the presence of the recombinant virus, the infected cells were firstly collected from the wells using a trypsin enzyme. Moreover, to purify the viral DNA from the final samples, the High Pure Viral nucleic acid kit (Roche, Germany) was used in terms of the manufacture instructions.

In addition, the resulting DNA was used as a template in the PCR reaction to replicate and also to reveal the tyr3-octreotate sequence using the specific primers. In this study, all tests were performed in terms of the principles of class II bioavailability for working with the viruses.

Results

In this study, after the cloning of the Tyr TATE sequence in the fiber adenovirus HI Loop area, the presence of a sequence in the plasmid was confirmed using the OCT F/R primers (Figure 1).

Moreover, the CMV-GFP sequence was extracted from the gel and its sequence was then determined (Figure 2A). Accordingly, this sequence was transferred to the bacterium. After the homologous recombination and replacement of the cassette als sequence by CMV GFP, the bacteria containing CMV-GFP sequence formed ampicillin sensitive and white colonies (Figures 2B, C). Moreover, performing the Cloning PCR also confirmed the presence of the sequence in the

Table 1. *Sequences of primers used in this study.*

Primer's name	Sequences	Annealing temp (ºC)	Product length (bp)
Oct-F	5'- GCAGCGGATAAGGCAATT-3'	55	209
Oct-R	5'- CGATCTACTCTACAGTGTAATT-3'		
*Als-F	5'ACTAAACCTGTAACACTAACCATTACACTAAACGGTACACCCTGTGACGGAAGATCACTTCG -3'	70	4.290
*Als-R	5'GGATGTGGCAAATATTTCATTAATGTAGTTGTGGCCTGAGGTTCTTATGGCTCTTG-3'		
CMV-GFP-F	5'-GATTTGGCCATTTTCGCGGG-3'	71	3052
CMV-GFP-R	5'-GGCGGCTGCTGCAAAACAGAT-3'		

*Sequences with homologous regions are underlined.

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bacmid.

The 4200 bp als sequence was extracted from the gel, which was then placed in the HI Loop fiber area of Ad. Afterward, the positive blue colonies (ampicillin resistance) were selected (Figure 3A). Using the cloning PCR and als F2/R2 primers, the presence of the als gene was confirmed in the bacmid (Figure 3B).

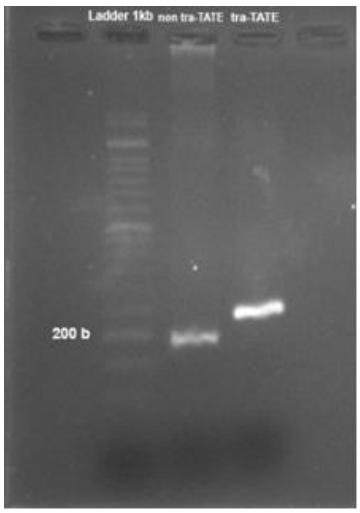

Figure 1. *Identification of Tyr TATE sequence using PCR amplification by using the OCT F/R primers.*

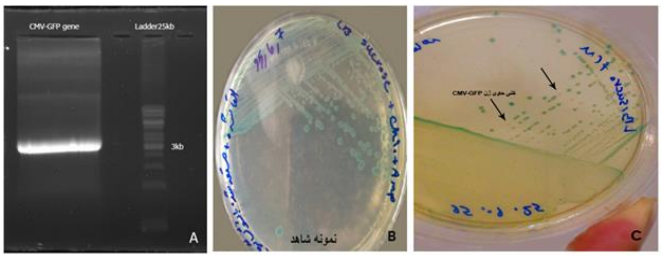

Figure 2. *A) Extraction of CMV-GFP from the gel. B) Bacterial culture of control samples. C) Bacterial culture of recombinant bacmid.*

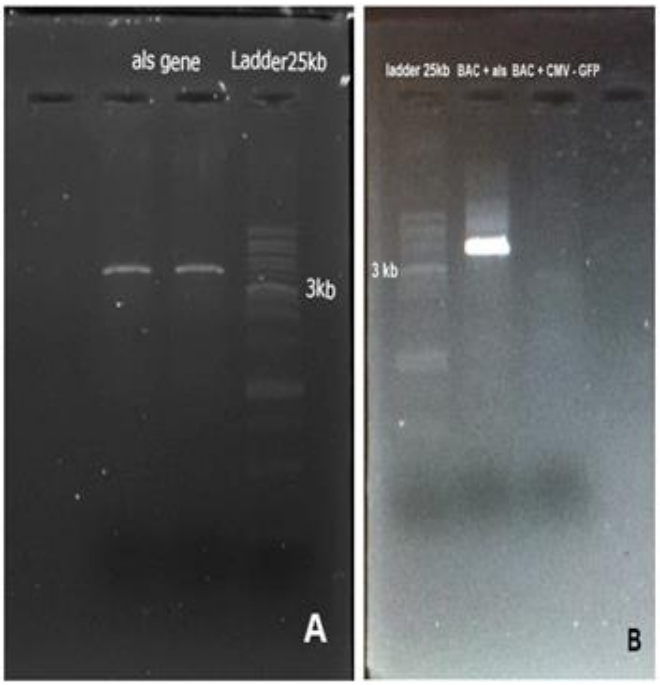

Figure 3. *A) Extraction of als sequence. B) Cloning PCR for blue colonies.*

In the last step, the Tyr-TATE sequence was extracted from the gel (Figure 4A), which was then transferred into SW102 cells. Eventually, the sequence replaced the als cassette in the HI Loop fiber area, and white colonies were then selected (Figure 4B).

Moreover, the bacmid containing Tyr-TATE was extracted from a bacterium, which was then transferred to HEK 293 cells using the calcium phosphate method. After 15 days, to isolate the recombinant virus, the recombinant cells were collected by observing the cytopathic effects, (Figure 5).

After proliferation, the recombinant virus combined with MOI 2 was added to AGS cell culture, and after 2 to 3 days, cytopathic effects were observed in cell culture (Figures 6A, C). During the cell culture, in which the wild virus was inoculated, no cytopathic effects were observed after 10 days (Figure 6B).

Discussion

Oncolytic viral therapy is a way to treat cancer, which is performed due to the virus's ability to infect, multiply, and lysis the host cells. Accordingly, various types of DNA and

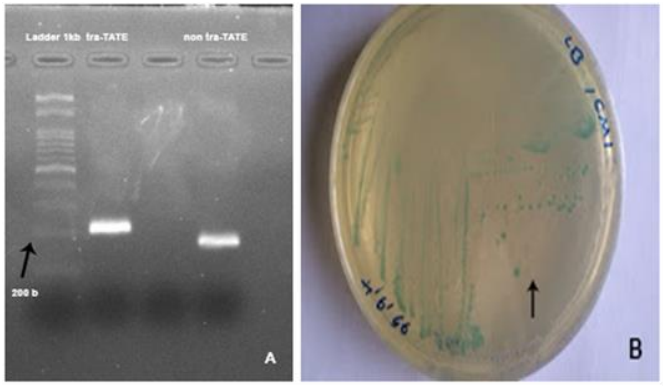

Figure 4. *A) Extraction of Tyr-TATE sequence. B) White colonies containing Tyr-TATE.*

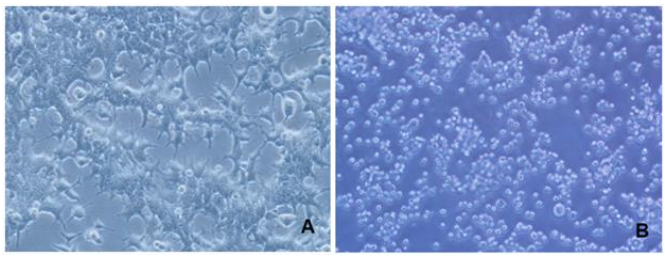

Figure 5. *A) Culture of HEK cells before inoculation of the virus. B) Symptomatic effects 15 days after inoculation.*

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RNA viruses, as oncolytic viral agents, have been used for this purpose (Aurelian, 2013). Moreover, adenoviruses, especially adenovirus type 5 (Ad5), have been used in many cases as an oncolytic agent to treat cancer. Notably, type 5 adenoviruses naturally enter the cells by expressing the coxsackievirus and adenovirus receptor (CAR). One of the limitations of the use of Ad5 for oncotherapy is that the CAR receptor is poorly expressed on the surface of tumor cells (Wohlfahrt et al., 2007). However, it is highly expressed in epithelial cells, neurons, and steroids. Therefore, to solve this problem, the designed mutants have been modified in the virus fiber to infect cancer cells through a mechanism that is independent of the CAR receptor (Aurelian, 2013).

Tyr3-octreotate is one of the analogs of somatostatin that has the ability of binding to the somatostatin receptors, especially SSTR2 and SSTR5 receptors. Accordingly, these receptors are expressed on the surface of different cancer cells like neuroendocrine tumors. In this regard, various studies have shown that these receptors cause cell death and apoptosis in the AGS cells (de Herder & Lamberts, 2002; Kumar & Grant, 2010; Barbieri et al., 2013).

In this study, the area of the adenovirus binding to the CAR receptor was modified to be able to bind to the somatostatin receptors at the surface of the AGS cancer cells.

So, to fulfill this, the sequence of tyr3-octreotate was located in the pFiber plasmid by cloning in the HI loop area of the fiber. In addition, the HI loop fiber area was amplified with this sequence, and then it was eventually replaced by the bacmid HI loop fiber area, using homologous recombination methods in the SW102 bacterium (Stanton et al., 2008). The results show that the use of Ad5 along with the Tyr-TATE sequence is able to infect cancer cells with somatostatin receptors like AGS cells, which can also lead these cells to death.

In conclusion, the recombinant virus, which has a sequence of tyr3-octreotate compared to the wild-type virus, has also the ability to infect the gastrointestinal adenocarcinoma cells. Notably, because the high levels of somatostatin receptors are expressed at the surface of many cancer cells, the recombinant viruses can be used for transferring genes into these cells.

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