

Design and Engineering of Recombinant Oncolytic Adenoviruses Expressing an Anti-EpCAM/Anti-CD3 BiTE for Targeted Immunotherapy of A549 Lung Cancer Cells

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Background: Oncolytic virotherapy is a novel therapeutic approach in oncology that uses viruses to target and eradicate cancer cells specifically. To improve therapeutic effectiveness, we investigated the application of genetically modified oncolytic adenoviruses (OVs) engineered to express bispecific T-cell engager (BiTE) antibodies.

Methods: In particular, we engineered Ad5-delta24 to express an EpCAM-targeting BiTE under the major late promoter, yielding the recombinant virus Ad5D24-Anti-EpCAM-Anti-CD3-scFv (rOAd5-BiTE). Following infection of A549 lung cancer cells, rOAd5-BiTE promoted the expression and secretion of BiTE antibodies targeting EpCAM and CD3.

Results: In co-culture experiments, rOAd5-BiTE elicited significant T-cell activation and proliferation, characterized by increased secretion of IFN- γ and IL-2, as well as augmented cytotoxic activity from neighboring bystander cells. Moreover, the combination of rOAd5-BiTE with peripheral blood mononuclear cells (PBMCs) markedly enhanced the antitumor activity against A549 cells.

Conclusion: The results indicate that equipping OVs with BiTE molecules may address critical challenges in virotherapy by enhancing and redirecting T-cell activity within the tumor microenvironment. This strategy offers a promising avenue for developing targeted immunovirotherapy approaches for the treatment of solid tumors.

Keywords: recombinant adenovirus, EpCAM, bispecific T-cell engager, oncolytic virotherapy, A549, lung cancer

Introduction

With the world's population growing older, cancer has become a significant cause of death and a considerable obstacle to improving the overall health of numerous countries. According to the latest GLOBOCAN reports in 2022, lung cancer (LC) ranked as the most prevalent cancer in men and the third most prevalent cancer in women. In 2022, approximately 2.5 million new instances of lung cancer were reported globally, accounting for 15.3% (1.5 million) and 9.4% (0.9 million) of all new cancer diagnoses in men and women, respectively.¹ In recent years, the occurrence of lung cancer has shown a consistent decrease among men in many high-income countries, including those in Europe and Oceania. At the same time, there has been a notable rise in incidence rates among women.²⁻⁴ Roughly 70% of individuals are diagnosed with advanced-stage lung cancer, and the survival rate at 5 years post-diagnosis is only 15%.⁵ In addition to the prompt detection of lung cancers, there is a need for an appropriate treatment strategy to handle these tumors effectively.

A range of treatment options, including surgery, radiation therapy, radiosurgery, chemotherapy, and immunotherapy, are frequently used to treat lung cancer. In cases where lung tumors are inoperable due to local tissue involvement or

when surgical intervention is not indicated, the conventional treatment approach has been a combination of radiation and chemotherapy.⁶ Nevertheless, the incorporation of targeted and immunotherapy alongside these modalities has recently altered the treatment landscape for these tumors.⁷ Viral therapy offers a hopeful method for treating solid tumors. Oncolytic viruses (OVs) can selectively infect and replicate within tumor cells, ultimately destroying them.^{8–12} Furthermore, OVs can elicit potent, widespread, and durable antitumor immune responses in addition to their direct, localized antitumor effects.^{13–16}

The release of diverse viral particles from dying tumor cells can stimulate antitumor immunity and elicit therapeutic responses at distant tumor sites.^{17–20} OVs produce multiple effects that support their anti-cancer properties, such as: (i) releasing tumor antigens, (ii) emitting PAMP-like molecules that activate the innate immune system to identify and destroy infected cells, including cancerous ones, (iii) enhancing innate immune responses, and (iv) secreting proinflammatory cytokines.²¹ Additionally, oncolytic viruses influence particular cell death mechanisms, including apoptosis, autophagic cell death, necrosis, and necroptosis; many of these pathways are inherently immunogenic.²² However, oncolytic viruses have demonstrated limited efficacy as standalone treatments for solid tumors despite extensive investigation. Adenovirus serotype 5 (Ad5) was chosen as the viral vector backbone owing to its extensively characterized genome, superior transduction efficiency, and the relative simplicity of genetic modification, which enables the incorporation of therapeutic transgenes such as bispecific T-cell engagers (BiTEs).²³ Nevertheless, the high seroprevalence of Ad5 and the presence of pre-existing neutralizing antibodies in a substantial proportion of the population may limit its efficacy upon systemic administration and warrant careful consideration in clinical application.²⁴ Consequently, there is a need to develop new and more efficient oncolytic vectors. Hence, in this research project, we employed a dual approach using an oncolytic virus and a bispecific antibody (BiTE) to target T-cell activity. BiTEs comprised two single-chain variable fragments (scFv) of antibodies, with one scFv targeting CD3 and the other targeting a tumor-associated antigen (TAA).^{25,26} The initial investigation demonstrating the efficacy of an oncolytic virus encoding a BiTE was conducted by Yu et al in 2014, who examined an EphA2-targeted T-cell in conjunction with a vaccinia virus, referred to as EphA2-TEA-VV. In the A549 tumor model, treatment with EphA2-TEA-VV significantly reduced tumor growth.²⁷

We hypothesize that oncolytic adenoviruses are promising vectors for delivering bispecific T-cell engagers (BiTEs) to direct immune responses against cancer cells selectively. In the present study, we have engineered an oncolytic adenovirus to secrete an EpCAM-targeting BiTE upon viral replication within cancer cells. Extensive tissue microarray analyses encompassing over 14,000 human tumor samples revealed EpCAM expression in the majority of epithelial tumor types, with at least 90% positivity observed in 60 of 78 epithelial tumor categories. These findings highlight the potential of EpCAM as a broadly applicable target for therapies targeting solid tumors.²⁸ Our findings demonstrate that the BiTE-expressing adenovirus elicits potent and specific T-cell activation and proliferation following infection of cancer cell lines *in vitro*. This targeted T-cell activation results in redirected cytotoxicity against cancer cells, thereby enhancing the virus's antitumor efficacy in the A549 lung cancer cell line. Taken together, these results underscore the potential of EpCAM-targeted BiTE-expressing oncolytic adenoviruses as a viable immunovirotherapy approach with clinical applicability. This strategy offers a promising avenue for integration with current lung cancer therapies to enhance antitumor efficacy.

Accordingly, the present study was undertaken to design and construct a recombinant oncolytic adenovirus (rOAd5-BiTE) encoding a bispecific T-cell engager (BiTE) targeting EpCAM and CD3. We sought to characterize its replication efficiency and BiTE expression *in vitro*, as well as to assess its oncolytic efficacy and capacity to redirect and activate T cells against A549 lung carcinoma cells.

Materials and Methods

Cell Lines and Cell Culture Techniques

The HEK 293 (Human Embryonic Kidney) cell line used for adenoviral packaging was obtained from the Iranian Biological Resource Center (IBRC), Tehran, Iran. The A549 cells, a human lung adenocarcinoma cell line, were obtained from the Pasteur Institute of Iran. These cell lines were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS, Gibco, UK) and 1% (v/v) penicillin/streptomycin (10 mg/mL, Sigma-

Aldrich) at 37 °C in a 5% CO₂ and 95% humidity environment. Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers after obtaining informed consent, in accordance with protocols approved by the Ethics Committee of Golestan University of Medical Sciences, Gorgan, Iran (approval code: IR.GOUMS.REC.1401.366). PBMC experiments were conducted utilizing samples obtained from three distinct donors to provide biological replicates and enhance the statistical robustness of the findings. PBMCs were initially cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). For viral infection experiments or plasmid transfection, cells were transferred to DMEM supplemented with 2% FBS.

Design and Generation of Recombinant Adenovirus Plasmids

In this project, recombinant viruses were generated utilizing the AdEasy vector system (Agilent Technologies, Inc).²⁶ The AdEasy system facilitates the production of recombinant adenoviruses using *Escherichia coli* (*E. coli*) and homologous recombination. Specifically, recombination occurs between a shuttle plasmid harboring the target gene and a large adenoviral backbone plasmid. This bacterial recombination event results in the substitution of a non-essential segment of the adenoviral genome with the gene of interest (GOI), thereby generating a recombinant adenoviral plasmid. The GOI in this study, anti-EpCAM/anti-CD3_E1AΔ24, was developed and optimized by the international biotechnology firm Biomagic Gene (BMG Biotech Company). The EpCAM bispecific T-cell engager (BiTE) comprised two single-chain variable fragments (scFvs) linked by a flexible glycine-serine (GS) linker. It was specifically designed to target the human epithelial cell adhesion molecule (EpCAM) and the CD3 molecules. (Figure 1). The GOI was inserted into the pAdTrack shuttle vector (Plasmid #16404), which harbors the homologous sequences required to facilitate recombination. Additionally, the pAdEasy-1 plasmid was used as the backbone vector to construct the virus. This plasmid contains a significant part of the human adenovirus serotype 5 (Ad5) genome and is devoid of the E1 and E3 genes. To produce bacterial homologous recombination, the shuttle plasmid carrying the target gene and the adenoviral backbone plasmid (pAdEasy-1) are co-transformed into the specialized *E. coli* strain BJ5183. Within bacterial cells, the RecA protein complex facilitates homologous recombination, enabling the gene of interest carried by the shuttle plasmid to replace a corresponding segment of the adenoviral genome present in the backbone plasmid. This process resulted in the formation of the recombinant adenoviral plasmid as recAd5D24-Anti-EpCAM-Anti-CD3-scFv. The recombinant plasmid was isolated from the bacterial cells and subsequently linearized using the restriction enzyme Pac I to excise the viral inverted terminal repeats (ITRs), which are critical for viral packaging. In the subsequent sections of the study, this recombinant oncolytic virus will be designated by the acronym rOAd5-BiTE.

Detection of Recombinant Clones Through Colony PCR Followed by PacI Restriction Enzyme Analysis

The PCR reaction mixture was initially assembled by combining 10 μL of 2× Taq Plus PCR Master Mix (Ampliqon, Denmark), 1 μL each of CMV forward and sV40 reverse primers (both at 10 μM), and 8 μL of double-distilled water (ddH₂O). A sterile needle was employed to transfer ten of the smallest colonies from an independent LB agar plate into

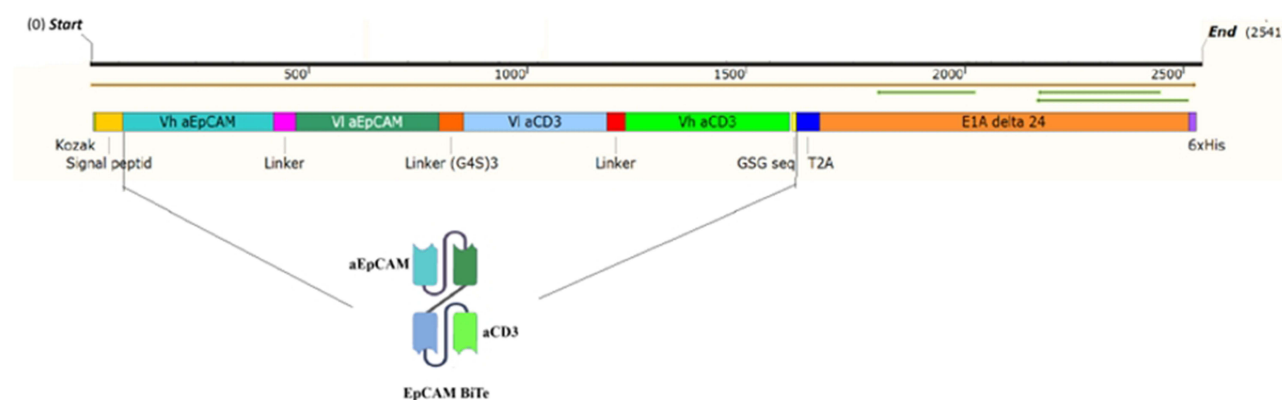


Figure 1 Schematic representation of aEpCAM-aCD3_E1A-delta 24.

the PCR reaction mixture. These colonies were subsequently screened using colony PCR. Concurrently, each of the ten colonies was separately cultivated in 5 mL of LB medium supplemented with 50 µg/mL kanamycin and incubated for 10 hours on an orbital shaker at 37°C. The polymerase chain reaction (PCR) was conducted under the following thermal cycling parameters: an initial denaturation at 95°C for 3 minutes, followed by 25 cycles consisting of denaturation at 55°C for 45 seconds and annealing/extension at 72°C for 2 minutes, with a final extension step at 72°C for 5 minutes. After a 10-hour incubation period, cells were harvested, and plasmid DNA was extracted using a Plasmid DNA Purification Kit (Bio Basic Inc., Canada) according to the manufacturer's protocol. Recombinant clones that tested negative during clonal PCR screening were retained for additional verification.

Purified recombinant plasmids were introduced into highly competent *Escherichia coli* DH5α cells via transformation. From each transformed LB agar plate, a single colony was selected and cultured overnight in 5 mL of LB medium supplemented with 50 µg/mL kanamycin, maintained at 37°C with orbital shaking. Subsequently, a small quantity of the amplified recombinant plasmid, extracted from DH5α cells, was subjected to PacI restriction enzyme digestion for final verification.

Rescuing the Recombinant Adenovirus Particles

To rescue the recombinant viruses, adenovirus genomes were initially digested with the restriction enzyme PacI to excise the kanamycin resistance gene. Subsequently, the linearized genomes were purified through ethanol precipitation for further use. Approximately 1.5×10^6 Hek293T cells were seeded in 25 cm² flasks 16 hours before transfection, at which point they reached 50% confluence. A total of four micrograms of recombinant adenoviral vector DNA, which had been digested with PacI, was utilized for the transfection of each 25 cm² flask. A transfection mixture was prepared by incubating 20 µL of Lipofectamine 3000 DNA transfection reagent (Invitrogen) with linearized plasmid DNA in 500 µL of Opti-MEM medium, in accordance with the manufacturer's guidelines. Following a 30-minute incubation period at room temperature, the transfection mixture was introduced to the HeK293T cells. Transfected cells were assessed using a Nikon ECLIPSE Ti-S microscope for green fluorescent protein (GFP) expression, and subsequently harvested 14 days post-transfection by scraping cells from culture flasks and pelleting them along with any suspended cells. Subsequently, the sample underwent four freeze-thaw-vortex cycles, followed by centrifugation at $600 \times g$ for 10 minutes to remove cellular debris. The resulting supernatant was then stored at -80°C and utilized as the seed virus. Subsequent rounds of amplification were conducted similarly, utilizing 75 cm² flasks. The final amplification step was performed in 10 T-175 cell culture flasks.

BiTe Expression Analysis with Western Blotting (WB)

Monolayers of HEK-293T cells were infected with the recombinant adenovirus rOAd5-BiTE at a multiplicity of infection (MOI) of 10 for a duration of 72 hours. Following the infection period, the cells were lysed using RIPA buffer supplemented with 1 mM PMSF protease inhibitor (Thermo Fisher Scientific Inc., Germany). The lysates were centrifuged at 16,000 rpm for 10 minutes to remove precipitates. Protein samples were loaded onto a 12% SDS-PAGE gel and subsequently transferred onto a nitrocellulose membrane. A blocking buffer consisting of 3% skim milk in Tris-buffered saline (TBS) was utilized, and the membrane was incubated for 1 h at room temperature on a shaker set to 60 rpm. Afterward, the membrane was treated with a mouse anti-Histidine tag antibody (BioLegend) at 1:1000 in blocking buffer and incubated overnight at 4°C on a laboratory rotor. Following incubation, the membrane underwent three 10-minute washes in Tris-buffered saline containing Tween 20 (TBST). Finally, the membrane was incubated with a 3,3'-diaminobenzidine (DAB) solution until a reaction product with optimal coloration developed.

Viral Oncolysis and Replication Studies

Determination of Multiplicity of Infection (MOI)

Multiplicity of Infection (MOI) refers to the ratio of viral particles to the number of host cells.²⁹ Consequently, to quantify the number of viral particles in the crude extracts, we implemented the multiplicity of infection (MOI) calculation protocol as detailed in the reference.²⁶ In a 6-well plate containing 1×10^6 HEK-293 cells per well, the existing medium was entirely discarded, and 500 µL of fresh medium was subsequently introduced. Volumes of 0, 5, 10,

15, 25, and 50 μL of the viral supernatant were introduced into the wells, with one well designated as a negative control. The flask was subsequently incubated at 37°C in an atmosphere containing 5% carbon dioxide for 3 hours. A volume of 1.5 mL of fresh culture medium was added to each well, and the plate was incubated at 37°C in an atmosphere containing 5% carbon dioxide for 72 h. The cytopathic effects observed in each well were assessed daily using an inverted microscope. For each infection factor, three wells were maintained under identical conditions. Subsequently, the rate of cellular destruction attributable to viral replication was assessed at multiple time points to determine the optimal infection factor.

Determination of the Titer of Recombinant Viruses Through the TCID₅₀ Assay

The TCID₅₀, or Median Tissue Culture Infectious Dose, denotes the volume of virus (measured in milliliters) that is sufficient to induce cytopathic effects in 50% of the cells that have been inoculated.^{29–32} In other terms, this methodology quantifies the viral load required to induce mortality in 50% of the infected cellular population. The Spearman–Kärber method was employed to determine the TCID₅₀ of recombinant viruses.^{33,34} Hek293T cells were cultured at a density of 5×10^3 cells per well in a 96-well plate. Serial dilutions ranging from 10–1 to 10–10 were conducted for the recombinant virus. The plates were incubated in an incubator at 37°C with a 5% carbon dioxide atmosphere for 10 days. The cells were monitored daily for cytopathic effects (CPE) and subsequently stained using a solution composed of 20% methanol and 2% crystal violet. Cells treated solely with DMEM were utilized as the negative control group (NC). Based on CPE, the biological titers of the recombinant adenovirus vectors were quantified by the TCID₅₀ method. The overall quantity of viruses present in the stock sample was quantified using the Karber formula:

$\text{Log (TCID}_{50}) = \text{log (lowest dilution at which 100\% CPE is observed)} + I \times [0.5 - (\text{total number of wells displaying CPE} / \text{total number of replicate wells per dilution})]$.

Replication and Cell Lysis Assay

Quantitative assessment of cell viability was performed by measuring the conversion of MTT to formazan over a specified period. A549 cells were plated in a 96-well plate at a density of 1×10^4 cells per well and incubated for 24 h. The media were replaced the following day, and recombinant oncolytic viruses were inoculated at MOIs of 1, 5, 25, 50, and 100. Following a 72-hour incubation period, 20 μL of MTT solution (5 mg/mL; Cat# IM0280, Solarbio, Beijing, China) was added to each well, and the cells were incubated for 4 hours. Subsequently, the supernatants were aspirated, and 150 μL of dimethyl sulfoxide (DMSO) was added to each well. The absorbance was measured at a wavelength of 570 nm using a microplate reader (ChroMate 4300 ELISA Reader-Awareness Technology, USA). Cells that were not exposed to the virus served as control samples. The experiments were conducted in triplicate.

Quantification of Viral Titer Utilizing qPCR

Quantitative real-time polymerase chain reaction (qPCR) was conducted to quantify the DNA copies of recombinant human adenovirus type 5 (hAd5) contaminants. DNA was isolated from infected cells utilizing the Invitrogen PureLink Viral RNA/DNA Mini Kit (Thermo Fisher Scientific, USA), in accordance with the manufacturer's instructions. The purified plasmid, at a concentration of 100 ng/ μL , served as a standard for the analysis, and the quantification of virus particles was based on the total number of base pairs comprising the plasmid. The quantification of virus particles in the purified plasmid was performed using the genome copy number formula, in conjunction with resources from the Technology Networks bioinformatics platform (<https://www.technologynetworks.com>).^{29–32} Quantitative polymerase chain reaction (qPCR) was performed using the StepOnePlus™ Real-Time PCR System, employing RealQ Plus 2x Master Mix Green High ROX™ (Ampliqon, Denmark) with custom-designed primers, 5'-CAGCGTAGCCCCGATGTAA-3' and 5'-TTTTTGTAGCAGCACCTTGCA-3'. These primers were designed to amplify a nucleotide sequence within the Ad5 packaging domain, which is frequently found in Ad5-derived adenoviral vectors and in oncolytic viruses. The total reaction volume was set to 20 μL , comprising 2 μL of DNA, 0.20 μL of forward primer, 0.20 μL of reverse primer, 10 μL of master mix, and 7.60 μL of sterile double-distilled water. Quantitative polymerase chain reaction (qPCR) was performed using the following cycling parameters: an initial denaturation step at 95°C for 15 minutes, followed by 40 cycles comprising denaturation at 95°C for 10 seconds, annealing at 60°C for 40 seconds, and extension at 72°C for 20 seconds. The fluorescence signal was recorded at the conclusion of each cycle. The

mean of the resulting values was used as the amplification factor (quantity) for that specific sample, and the findings are expressed as 2^{-Ct} values.

Co-Culture Assays

The cytotoxic effects of recombinant adenoviruses on the A549 target cell line were evaluated using a lactate dehydrogenase (LDH) release assay kit (LDH-Glo™ Cytotoxicity Assay, Promega, USA) in accordance with the manufacturer's guidelines. Initially, 2×10^4 lung cancer cells were seeded into each well of a 96-well plate containing 200 μ L of assay medium. Peripheral blood mononuclear cells (PBMCs) were isolated from 10 mL of blood utilizing Ficoll density gradient centrifugation. Subsequently, the isolated PBMCs were cultured in 48-well plates and incubated for 24 hours. After counting, the PBMCs were added at an effector-to-target (E: T) ratio of 5:1. Following a 4-hour incubation, the cells were infected with the virus at a multiplicity of infection (MOI) of 10 or 100. Culture media were collected from the cells at 24-, 48-, and 72 hours post-infection and used to conduct lactate dehydrogenase (LDH) assays. Lactate dehydrogenase (LDH) levels were assessed utilizing the specified assay kit, and the percentage of cytotoxicity was determined using the following formula: Percent cytotoxicity = ("experimental" – "effector plus target spontaneous") / ("target maximum" – "target spontaneous") $\times$ 100%.

Likewise, the capacity of the recombinant oncolytic adenovirus to stimulate T-cell activity was evaluated through the measurement of interleukin-2 (IL-2) and interferon-gamma (IFN- γ) expression. After 24 hours of co-culture, Cell supernatants were collected for the quantification of cytokines (IL-2 and IFN- γ) using enzyme-linked immunosorbent assay (ELISA) kits provided by R&D Systems, USA, in accordance with the manufacturer's instructions.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). Data were expressed as means $\pm$ standard deviation (SD) or standard error of the mean (SEM). Student's *t*-test and two-way analysis of variance (ANOVA) were employed to assess significant differences in cytokine release and killing efficacy. The experiments were replicated at least three times. Statistical significance was defined as P-values ($p < 0.0001$).

Results

The Development and Analysis of Oncolytic Recombinant Viruses

Recombinant viruses encoding the BiTEs were electroporated through homologous recombination between the pAdTrack-anti-EpCAM/anti-CD3 vector and the pAdeasy-1 backbone in the BJ5183 competent cells, as detailed in the preceding section. To evaluate the acquired clones for the presence or absence of mutant clones, 10–20 of the smallest colonies derived from electroporation were selected (Figure 2a). Each colony was subsequently cultured in 2 mL of LB medium supplemented with 25 mg/mL kanamycin for 16 hours on an orbital shaker at 37°C. DNA was isolated from miniprep cultures utilizing a standard alkaline lysis technique. The precision of transgene integration within the gene construct was evaluated by polymerase chain reaction (PCR) with universal primers, which yielded a 2,900-bp amplification fragment. The dimensions of the supercoiled plasmids were assessed by subjecting each miniprep to electrophoresis on a 0.8% agarose gel (Figure 2b). Subsequently, restriction digestion was conducted on the selected clones. The correct recombinant constructs generally yielded a single predominant fragment of approximately 30 kb, along with a smaller fragment of 3.0 or 4.5 kb (Figure 2c).

Generation of rOAd5-BiTE in the Hek-293 Cell Line

Following the successful generation of adenoviruses expressing anti-EpCAM/anti-CD3 bispecific T-cell engagers (rOAd5-BiTE), the viral genomes were linearized using the PacI restriction enzyme. Subsequently, the linearized DNA was transfected into HEK293T cells. Transfected cells were analyzed using an inverted microscope. Viral plaques were observed on the cell monolayer seven days following transfection, indicating the successful rescue of recombinant viruses. Fourteen days following transfection, most cells displayed cytopathic effects (CPE), characterized by a rounded morphology and detachment from the culture flask surface (Figure 3).

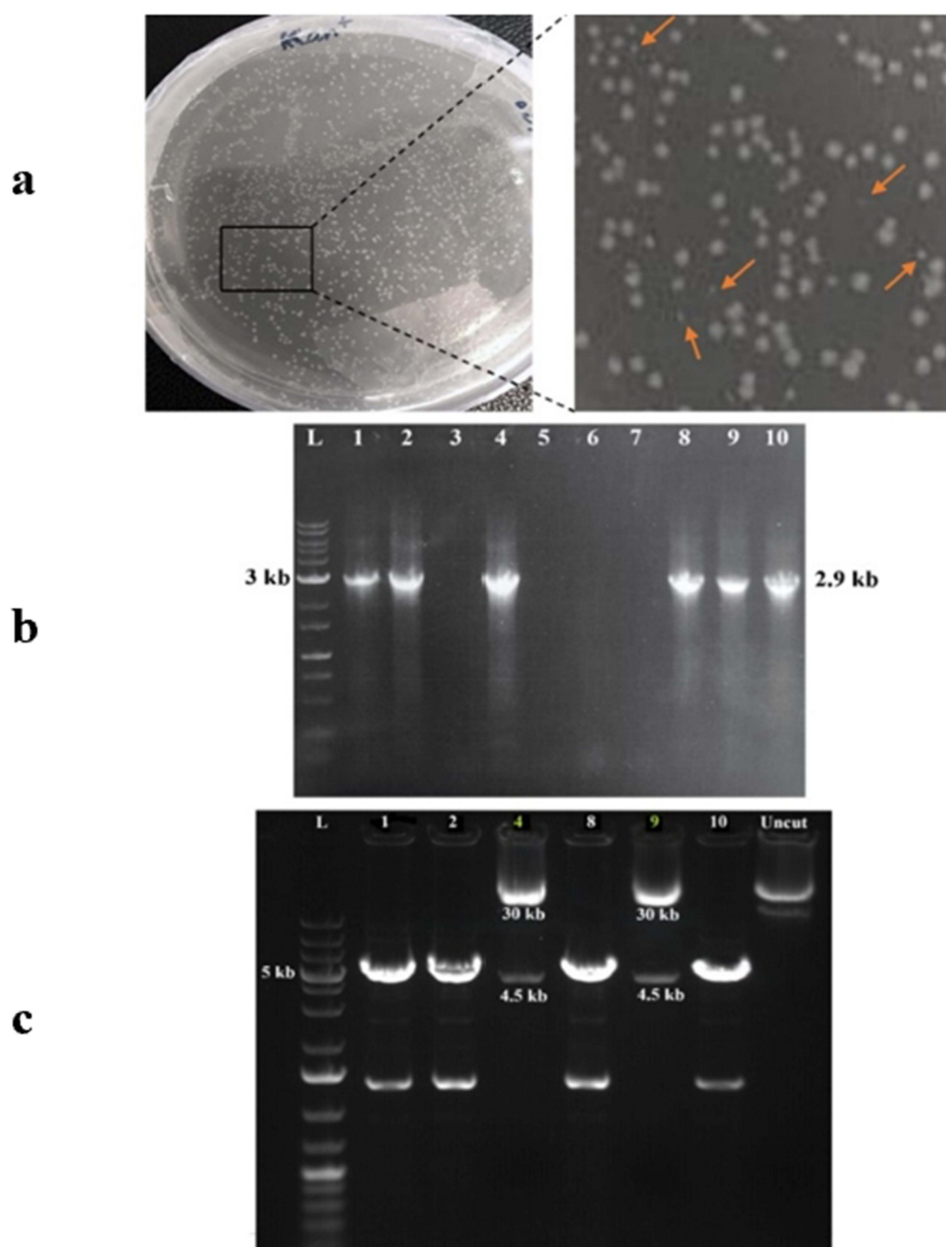


Figure 2 The process of selecting and characterizing recombinant adenoviral plasmids. (a) The identification of potential adenovirus recombinants following homologous recombination in AdEasy cells. The small colonies that are most likely to harbor recombinants are denoted by arrows. (b) A screening of supercoiled plasmids was conducted on ten randomly selected clones. Among these, six clones (numbers 1, 2, 4, 8, 9, and 10) exhibited characteristics indicative of potential recombinants, as evidenced by their migration at positions exceeding that of the 2-kb marker. (c) The digestion of candidate recombinant clones using *PacI* restriction endonuclease was conducted. Validation of all six tested clones, as depicted in (b), was achieved through this analysis. Notably, only two of the six clones yielded ≈4.5-kb and 30-kb fragments following *PacI* digestion, indicating the presence of the desired recombination. The GeneRuler 1-Kb Plus served as a DNA size marker in this experiment.

aEpCAM-aCD3_E1A-Δ24 Expression Through Recombinant Oncolytic Adenovirus Infection

To ascertain the expression of the EpCAM BiTE recombinant protein by the oncolytic adenovirus (OAd), HEK-293T cells were infected with rOAd5-BiTE. Following a 72-hour incubation period at a multiplicity of infection of 10, the cell lysates were subjected to Western blot analysis. Assessment of EpCAM BiTE expression in HEK293T cells revealed elevated BiTE levels. Western blot analysis confirmed the presence of the EpCAM BiTE gene, with a predicted

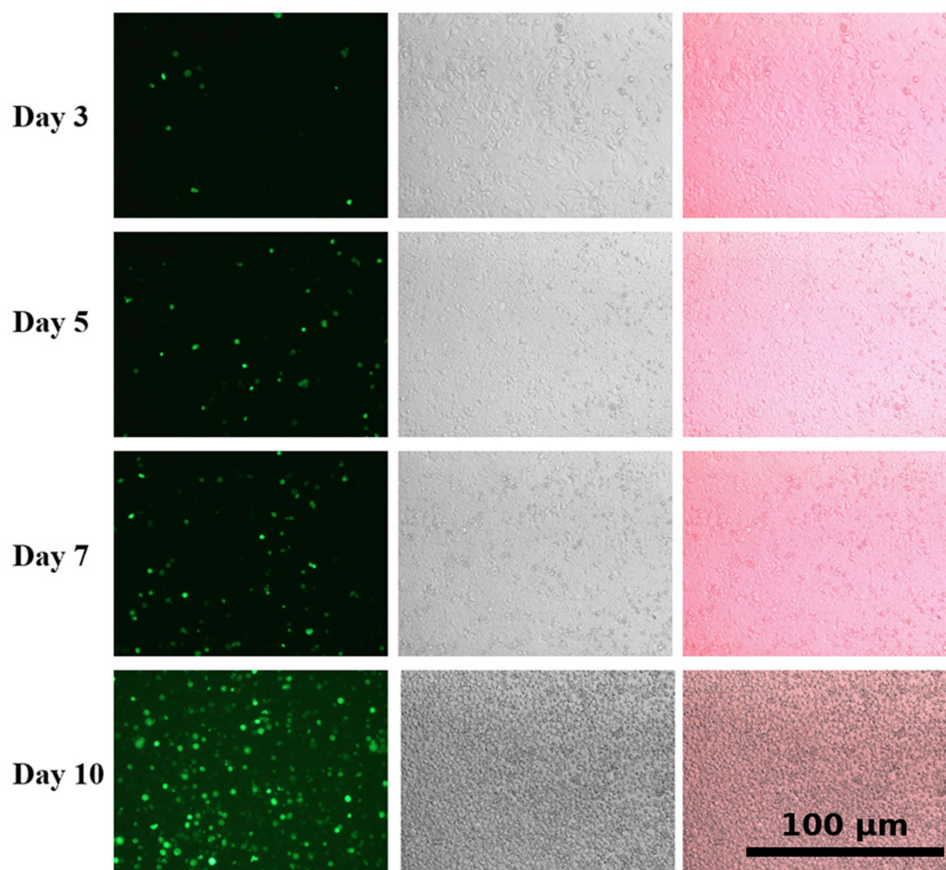


Figure 3 Generation of recombinant adenoviruses following the transfection of 293T cells. Adenoviral recombinants generated from the pAdTrack shuttle vector, which encodes monomeric GFP, were introduced into HEK-293T cells via transfection. The “scattered stars” phenomenon associated with recombinant adenoviruses became evident between three and four days. The transfected cells were subsequently analyzed using bright field (BF) and fluorescence field (FF) microscopy at 3, 5, 7, and 10 days post-transfection, with a magnification of 100x.

molecular weight of 55 kDa (Figure 4). The relatively small size of the EpCAM BiTE is advantageous for its penetration and distribution within tumor tissues.

Outcomes of Quantifying Viral Titer Through the TCID50 Methodology

The TCID50 of rOAd5-BiTE was determined by infecting HEK-293T cells with varying viral concentrations ranging from 10^{-1} to 10^{-12} . Cytopathic effects were observed to initiate within 18 hours post-infection and reached completion by 72 hours. The initial titer of the recombinant viruses was determined by the Spearman–Kärber method, yielding 107 TCID50/mL in 100 μ L of the viral solution, corresponding to 10^8 TCID50/mL (Figure 5).

Quantification of rOAd5-BiTE Derived from qPCR Analysis

The concentration of purified plasmid was estimated at 3.24×10^{10} virus particles per microliter. Subsequently, logarithmic dilutions ranging from 10^{-1} to 10^{-6} were employed to generate the amplification plot for the plasmid standard samples. Based on the findings, the highest viral copy number recorded was 2.316×10^8 , which corresponded to the lowest CT1 (CT) value. This observation suggests a significant viral titer. An examination of the melting curve in the 83°C range reveals favorable primer characteristics and confirms the absence of mispriming and primer dimer formation. The virus titer in the lysate sample was estimated per unit volume (milliliter) using the following formula: Virus titer = (dilution factor $\times 10 \times 70 \times$ virus copy number)/5.

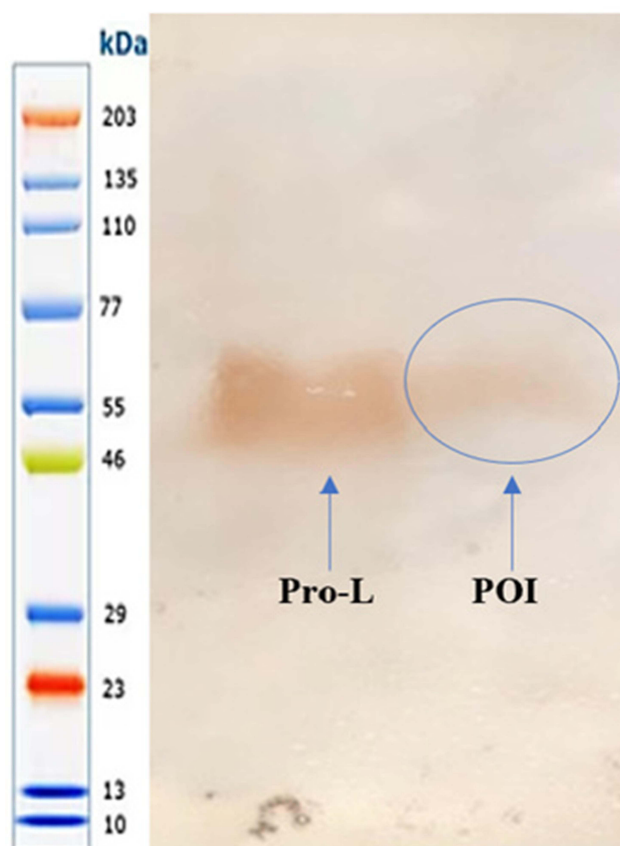


Figure 4 Verification of aEpCAM-aCD3 protein expression in HEK-293T cells within a viral context using DAB substrate. 55 kilodalton band corresponding to the protein of interest (POI) was observed at an MOI of 10. Protein L (pro-L) is used as a positive control. Protein Marker band sizes are indicated on the left.

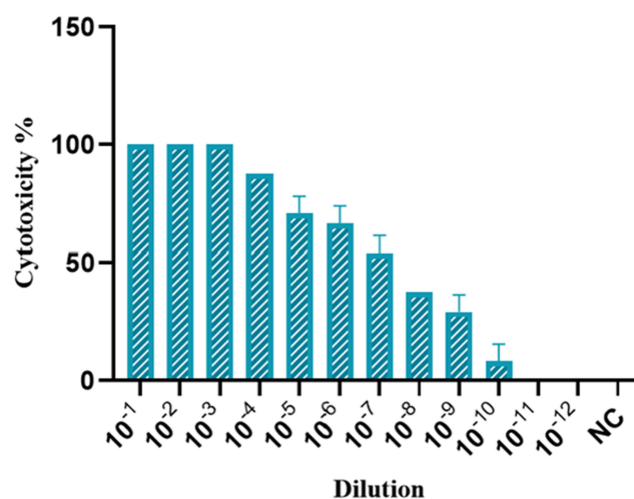


Figure 5 Calculation of TCID₅₀ for rOAd5-BiTE in HEK293T cells (n = 3, mean + SD).

Abbreviation: NC, Negative Control.

MTT Cytotoxicity Assay

A549 cells were exposed to varying MOI (1, 5, 25, 50, and 100) of rOAd5-BiTE, peripheral blood mononuclear cells (PBMCs), a combination of rOAd5-BiTE and PBMCs, as well as a control group without treatment, to assess cytotoxic effects. An increase in cellular cytotoxicity correlated positively with the administered dose. The most potent cytotoxic

effect was observed at a multiplicity of infection (MOI) of 100, whereas the lowest cytotoxicity was observed at an MOI of 1. Furthermore, cytotoxic effects were observed to begin at 24 hours and peak at 72 hours. The oncolytic efficacy of the combined treatment with rOAd5-BiTE and PBMC on A549 cells was markedly greater than that observed in the other experimental groups, as illustrated in Figure 6.

Cytotoxicity Effects of rOAd5-BiTE

To stimulate a therapeutic immune response, a novel oncolytic recombinant adenovirus has been engineered to express anti-EpCAM/anti-CD3. This construct is intended to inhibit the RB/E2F signaling pathway while simultaneously delivering a costimulatory signal to effector immune cells. In this study, we employed an engineered recombinant oncolytic adenovirus that expresses the anti-EpCAM/anti-CD3 effector gene in target lung cancer cells. This approach was implemented to assess T cell activation and the cytotoxic effects on cancer cells. A549 cells were subjected to treatment with recombinant oncolytic adenovirus (rOAd5-BiTE) at two multiplicities of infection (MOI), specifically 10 and 100, alongside peripheral blood mononuclear cells (PBMC) at an effector-to-target (E: T) ratio of 5:1. The cytotoxic effects induced by the virus were assessed by quantifying the release of lactate dehydrogenase (LDH) in the conditioned media. At elevated MOI and time period ratios, EpCAM BiTE cells demonstrate a statistically significant increase in the cytotoxic effect against lung cancer cells (p -value < 0.0001) (Figure 7). Therefore, the cytotoxicity of rOAd5-BiTE increased in a time- and dose-dependent manner. The results are expressed as means $\pm$ standard deviation (SD) derived from a minimum of three independent experiments.

T Cell Activation

The secretion of cytokines by rOAd5-BiTE indicates T-cell activation and targeted cytotoxicity. Consequently, we assessed the concentrations of the pro-inflammatory cytokines interferon-gamma (IFN- γ) and interleukin-2 (IL-2) following the incubation of rOAd5-BiTE with the A549 lung cancer cell line. The findings of our study indicate that

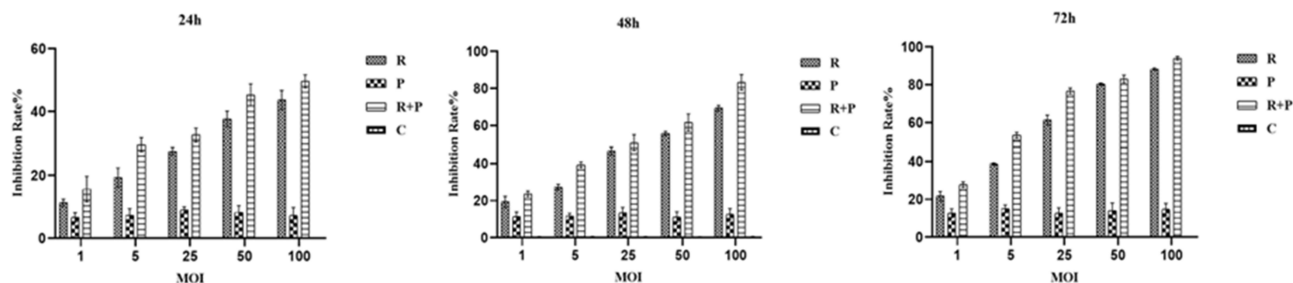


Figure 6 Cell viability analysis using the MTT assay at 24, 48, and 72 hours following the treatment of A549 cells with R, P, R+P, and C. The data are analyzed using two-way ANOVA followed by Tukey's post hoc test, with a significance level set at $p < 0.0001$. The bars represent the standard deviation ($\pm$ SD).

Abbreviations: R, Recombinant adenovirus; P, PBMC; C, control.

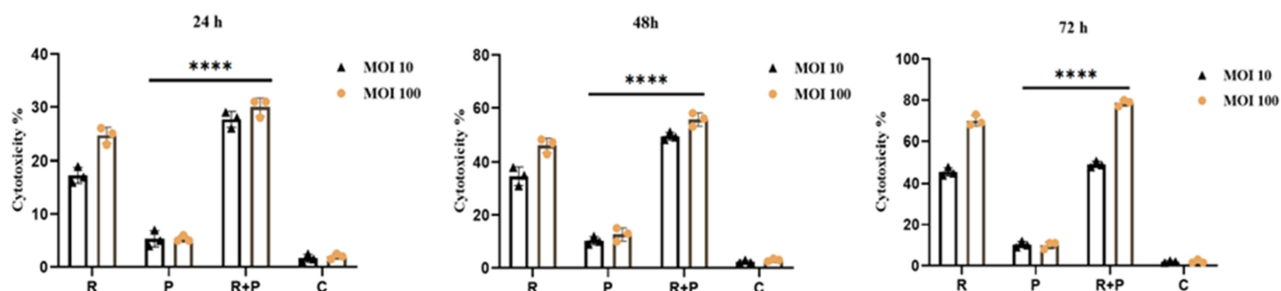


Figure 7 Cytotoxic effect of rOAd5-BiTE on lung cancer cell line in groups R, P, R+P, and C. The data are analyzed using two-way ANOVA followed by Tukey's post hoc test, with a significance level set at $p < 0.0001$. The bars represent the standard deviation ($\pm$ SD). **** $p < 0.0001$.

Abbreviations: R, Recombinant adenovirus; P, PBMC; C, control.

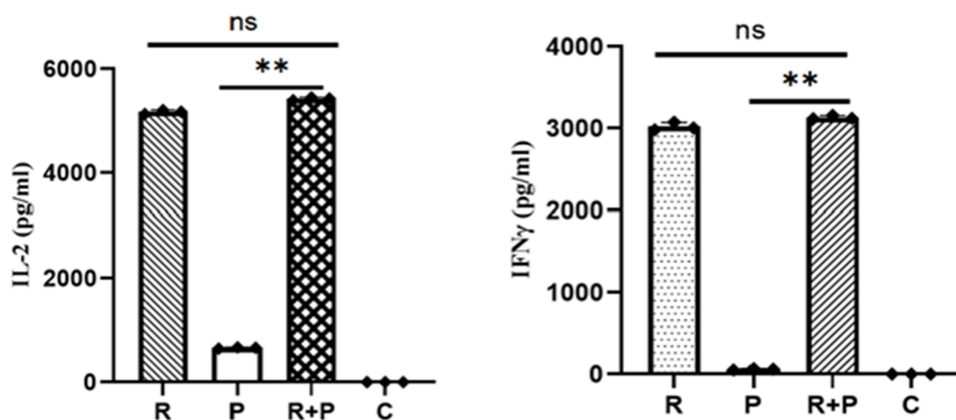


Figure 8 EpCAM-BiTE antibodies produced by cells infected with rOAd5-BiTE enhance the functionality of T-cells. Peripheral blood mononuclear cells (PBMC) were co-cultured with the A549 cell line in the presence of recombinant oncolytic adenovirus (oAd). Forty-eight hours post-culture, the expression levels of interleukin-2 (IL-2) and interferon- γ (IFN- γ) were evaluated using an enzyme-linked immunosorbent assay (ELISA) detection kit. Data are shown as the Row means with $\pm$ SD of killing efficiency in three replicates. ** $p < 0.01$.

Abbreviations: R, Recombinant adenovirus; P, PBMC; C, control.

EpCAM BiTE T cells exhibit markedly higher IL-2 and IFN- γ secretion than untransduced T cells when co-cultured with A549 cells (Figure 8).

Discussion

This research investigates the feasibility of developing a modified oncolytic adenovirus (rOAd5-BiTE) that expresses and secretes a bispecific T-cell receptor (BiTE) targeting EpCAM-expressing tumor cells. The successful execution of homologous recombination and the accurate integration of transgenes into the adenoviral backbone were validated through polymerase chain reaction (PCR) and restriction enzyme digestion, thereby affirming the integrity of the recombinant virus. Additionally, the synthesis and secretion of EpCAM BiTE from HEK-293 cells infected with the virus were corroborated by Western blot analysis, which showed the anticipated protein at 55 kDa.

The cytotoxicity assessment using A549 tumor cells and peripheral blood mononuclear cells (PBMCs) demonstrated a significant oncolytic effect, particularly when the adenovirus expressing bispecific T-cell engagers (BiTEs) was administered in conjunction with immune cells. Results from the MTT assay revealed a time- and dose-dependent reduction in A549 cell viability, thereby supporting the hypothesis that BiTE secretion promotes tumor-specific immune activation. This finding is consistent with prior research demonstrating the therapeutic efficacy of T-cell engagers in cancer immunotherapy.

Fajardo et al developed an oncolytic adenovirus, designated ICOVIR-15K, engineered to express a bispecific T-cell engager (BiTE) targeting the epidermal growth factor receptor (EGFR). The EGFR-BiTE-ICOVIR-15K demonstrated the capability to replicate and induce lysis of tumor cells *in vitro*.³⁵ Additionally, research by Feng Yu et al in 2014 showed that EphA2-TEA-VVs activated human T cells and directed them toward EphA2-positive A549 lung cancer cells.²⁷ Likewise, in their investigation of an oncolytic vaccinia virus engineered to express BiTE, Min Wei et al provided evidence that the VV-EpCAM BiTE significantly augmented the infiltration of immune cells, activated tumor-infiltrating effector T cells, and mitigated the exhaustion of CD8⁺ T cells. Their findings indicated that an oncolytic vaccinia virus expressing a bispecific T-cell receptor enhanced immune responses in EpCAM-positive solid tumors, consistent with the results obtained in the present study.³⁶

In this investigation, quantification of viral titer using TCID₅₀ and qPCR methods provided critical information on viral replication efficiency. The functional titer analysis corroborated a substantial viral load, thereby affirming the stability of the modified adenovirus. Furthermore, profiling of cytokine secretion indicated elevated levels of IL-2 and IFN- γ following BiTE exposure, further illustrating immune activation and targeted cytotoxic effects against EpCAM-positive lung cancer cells.

Notwithstanding the encouraging results obtained, numerous challenges persist. Although in vitro studies demonstrate practical BiTE expression and the ability to eliminate tumor cells, additional in vivo investigations are essential to assess therapeutic efficacy within the intricate tumor microenvironments. Furthermore, optimizing linker design and viral stability may increase BiTE persistence and enhance T-cell activation. Future research endeavors should prioritize refining viral delivery systems, reducing potential immunogenicity, and evaluating therapeutic responses in preclinical tumor models.

Additionally, incorporating a non-BiTE control virus, along with evaluating additional EpCAM-positive tumor models, would enhance understanding of the respective roles of oncolysis and BiTE-mediated T-cell activation. Previous research has demonstrated that comparisons between armed and unarmed adenoviral vectors facilitate more precise mechanistic insights.^{37,38} Furthermore, EpCAM-targeting BiTEs exhibit variable efficacy across epithelial cancer types, underscoring the need for validation across diverse tumors.³⁹ Although these investigations extend beyond the scope of the current study, their significance is recognized, and they are identified as critical avenues for future research.

We emphasize that targeting EpCAM/CD3 provides broader coverage of epithelial tumors than previous constructs targeting more limited antigens, such as EGFR or HER2. This design strategy may augment the translational applicability of BiTE-expressing oncolytic viruses by utilizing a broadly expressed epithelial marker while maintaining effective T-cell redirection. Collectively, these characteristics highlight the prospective clinical significance of our engineered platform.

Conclusion

The current investigation offers definitive evidence of potent oncolytic activity and BiTE-facilitated T-cell activation in vitro; however, additional in vivo studies are necessary to confirm therapeutic efficacy within a physiological tumor microenvironment. Owing to its capacity to redirect T cells toward EpCAM-expressing malignancies, this platform demonstrates translational potential, either in combination with established immunotherapeutic modalities or through further refinement of targeted viral delivery systems. Collectively, these results support the ongoing development of EpCAM/CD3-armed oncolytic adenoviruses as a promising avenue in immunovirotherapy.

Data Sharing Statement

All data generated or analyzed during this study are included in this publication.

Ethical Approval and Informed Consent Statement

Informed consent was obtained from all participants in the study (Blood sample). The study protocol was approved by the Ethics Committee of Golestan University of Medical Sciences, Gorgan, Iran (approval code: IR.GOUMS.REC.1401.366). All procedures were carried out in strict adherence to the ethical principles outlined in the Declaration of Helsinki and its subsequent amendments.

Acknowledgment

We would like to acknowledge the Arya Tina Gene (ATG) Company for its support of this work, which made this research possible.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This work was supported by a grant from Arya Tina Gene (ATG) Biopharmaceutical Company, Gorgan, Iran Grant number: ATG/1400-104.

Disclosure

The authors report no conflicts of interest in this work.

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