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ORIGINAL ARTICLE

Antitumor effect of adenoviruses expressing mutant non-oncogenic E7 versions from HPV-16 fused to calreticulin

Olivia Abigail Robles-Rodríguez[1], José Juan Pérez-Trujillo[1], José Alberto Barrón-Cantú[1], Abigail Torres-Cerda[2], Yolanda Gutiérrez-Puente[2], Aracely García-García[1], Humberto Rodríguez-Rocha[1], Arnulfo Villanueva-Olivo[1], Laura M. Zavala-Flores[3], Odila Saucedo-Cárdenas[1,3], Roberto Montes-de-Oca-Luna[1], María de Jesús Loera-Arias[1]

[1]Universidad Autonoma de Nuevo Leon (UANL), Histology Department, Faculty of Medicine, Monterrey, Nuevo Leon, México; [2]Universidad Autonoma de Nuevo Leon (UANL), Department of Chemistry, Faculty of Biological Sciences, Monterrey, Nuevo Leon, México; [3]Department of Molecular Genetics, Northeast Biomedical Research Center (CIBIN) of IMSS, Nuevo Leon Delegation, Monterrey, Nuevo Leon, México.

Summary

Purpose: *To compare the antitumor effect of adenoviruses that express mutant variants of the protein E7 from HPV-16 fused to calreticulin.*

Methods: *Recombinant adenoviruses were generated to express calreticulin fused to mutant versions of E7 (CRT/E7m and CRT/E7dm). Western blot and immunofluorescence assays were made to demonstrate protein expression. Antitumor assays were performed in C57BL6 mice injected with TC-1 cell line.*

Results: *When HEK293 cells were infected with these adenoviruses, we detected that all the recombinant proteins were expressed at endoplasmic reticulum, as expected. Next, the antitumor effect was tested on a murine tumor model established by inoculation of TC-1 cell line. We detected that both Ad CRT/E7m and Ad CRT/E7dm were capable of reducing the antitumor volume when compared to Ad LacZ, which was used as negative control. No significant difference was observed when compared to Ad CRT/E7, a positive control.*

Conclusions: *Here we demonstrated that the mutant versions of E7 HPV-16 fused to calreticulin generate similar antitumor effect than the wild type version.*

Key words: *adenovirus, calnexin, calreticulin, E7, HPV-16, TC-1*

Introduction

Cervical cancer is one of the most common types of gynecological malignancies, affecting women worldwide with variations in incidence and mortality rates. Its incidence is estimated to be 527,624 every year, with 265,672 deaths from the disease [1]. High-risk human papillomavirus infection is associated with the development of cervical cancer. In 80% of the cases, HPV types 16, 18, 45 or 31 are involved, HPV-16 being the most common with 54.6% of the cases [2]. HPV-16 E7 protein is crucial for cellular malignancy by interfering retinoblastoma protein (pRB) half-life, besides it has a constant expression in infected cells [3]. Hence, E7 has been noticed as an ideal antigen target for cervical cancer vaccines research that is capable of inducing a $CD4^+$ and $CD8^+$ specific immune response [4,5].

Due to E7 oncoprotein activity, several mutant variants of E7 protein have been developed to reduce its transforming ability and increase the antitumor activity. Substitutions of Cys-58 and Cys-91 to glycine produce changes in the struc-

Corresponding author: María de Jesús Loera Arias, PhD. Universidad Autonoma de Nuevo León, Department of Histology, Faculty of Medicine. 66460 Monterrey, Nuevo León, México.
Tel: +52(81)83294195, Email: loera.arias@gmail.com
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tural zinc-binding motif located in the CR3 region of E7. The generated protein is highly unstable and, consequently, degraded by the proteasome, producing antigenic peptides to be presented in the MHCI pathway [6]. Mutations in the pRB binding motif LXCXE, aminoacids (aa) 21-26 located in the CR2 region reduce pRb affinity and the transforming ability of E7. These mutations also produce a stronger antitumor effect [7].

One of the strategies to produce a tumor-specific immune response is the fusion of E7 to calreticulin. Calreticulin (CRT) is a chaperon protein that is in the endoplasmic reticulum (ER) and leads antigen presentation through a series of interactions with TAP [8]. When E7 is fused with CRT, E7 is transported to the ER, facilitating peptide presentation through the MHCI pathway [9]. Immunization with a DNA vaccine encoding E7 linked with calreticulin has a demonstrated increase in $CD8^+$ T-cell activity and a better antitumor effect than vaccination with wild-type E7 DNA [10].

In the present study, an adenoviral vector encoding a double mutant E7 (E7dm) fused to CRT was developed (Ad E7dm/CRT). The mutations of E7 were the substitution of Cys-58 and Cys-91 to glycine and the deletion of the pRB binding motif to enhance the antitumor effect.

Methods

Generation of recombinant adenovirus vectors

Replication-deficit recombinant adenovirus expressing LacZ (Ad LacZ) was purchased from Cell Biolabs, Inc (San Diego, CA, USA) and used as a negative control vector. Ad CRT/E7 virus was kindly provided by Dr. Gomez-Gutierrez from the Division of Surgical Oncology at the University of Louisville (Louisville, KY) [9], and was used in all experiments as a reference control. For the generation of Ad CRT/E7m, E7 mutant (E7m) gene was amplified from pBC219-E7 mutant vector (kindly provided by MD. Liang Qiao from the Department of Microbiology and Immunology, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois) [6] using the following primers: E7FECORI: 5´ CGGAATTCCTGCATGGAGATACACCTAC and E7EVKIRw: 5' CGGGTACCGATATCTTATGGTTTCTGAGAACA. PCR product was excised with *EcoRI* and *KpnI* enzymes (New England Biolabs, Ipswich, MA, USA) and subcloned into PCDNA3.1-CRT-E7 (without E7 gene excised with same restriction enzymes). After that, CRT/E7m gene was amplified with primers: CalXhoFw: 5'CCGGCTCG AGATGCTGCTCCCTGTGCCGCT and E7EVKIRw: 5' CGGGTACCGATATCTTATGGTTTCTGAGAACA and amplicon was cloned into a pShuttle-CMV vector (Quantum Biotechnologies, Montreal, QC, Canada). To generate Ad CRT/E7dm, the CRT/E7dm gene was gene-optimized *in silico* and then synthesized on pGA4 vector by GenArt (Regensburg, Germany). CRT/E7dm was excised with *Bgl*II and *Not*I restriction enzymes (New England Biolabs, Ipswich, MA, USA) and subcloned into a pShuttle-CMV vector (Qbiogene). Homologous recombination was carried out between pShuttle and pAdEasy-1 plasmids following AdEasy Vector System guidelines (Qbiogene, Carlsbad, CA, USA). All adenoviral constructs were propagated in HEK293 cell line, and the resultant viral particles (VPs) were purified with the ViraBind Adenovirus Purification kit (Cell Biolabs, San Diego, CA, USA) and quantified with QuickTiter Adenovirus Titer Immunoassay Kit (Cell Biolabs, San Diego, CA, USA). The complete sequences of CRT/E7m and CRT/E7dm were deposited in GeneBank under accession number MK628597 and MK628598, respectively.

Cell lines

HEK293 (QBI-293A) cell line, derived from human embryonic kidney cells, was maintained in Advanced DMEM supplemented with 4% heat-inactivated fetal bovine serum (FBS), 1mM L-glutamine, and 100 U/mL penicillin/streptomycin (Cellgro, Manassas, VA, USA). TC-1 (CRL-2785) cell line derived from primary lung epithelial cells of C57BL/6 mice, was co-transformed with HPV-16 E6 and E7 oncoproteins, and the c-Ha-ras oncogenes. TC-1 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1 mM sodium pyruvate, 100 U/mL penicillin/streptomycin (Cellgro, Manassas, VA, USA), and G418 at 0.5 mg/mL (A1720; Sigma-Aldrich, St.Luis, MO, USA). Both cell lines were purchased from America Type Culture Collection (ATTC) and were maintained at 37°C in a 5% CO_2 atmosphere.

Detection of recombinant proteins

To determine the expression of recombinant proteins, HEK293 (5×10^5) cells were seeded in 6-well plates and infected the next day with different adenoviruses at various multiplicity of infection (MOI). After 24 h, the infected cells were harvested and lysed with RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS). Next, the protein extracts were quantified with the Bradford dye-binding method (Bio-Rad) before heat-denaturalization in the presence of 1 mM dithiothreitol (DTT; Sigma-Aldrich, St.Luis, MO, USA). 30 μg total cell proteins were loaded on a 10% SDS-PAGE and transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was incubated with mouse monoclonal antibody anti-HPV16 E7 (1:2500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by an anti-mouse IgG conjugated with horseradish peroxidase (1:10,000; Sigma Aldrich, St.Luis, MO, USA). The membrane was revealed with Super Signal West Pico Chemiluminescent Substrate (Pierce, Biotechnology Inc, Rockford, IL, USA) and registered on a X-OMAT Scientific Imaging Film (Eastman Kodak Co., Ruchester, NY, USA).

Immunofluorescence

HEK293 (5×10^4) cells were seeded over glass coverslips placed in 24 well plates, and the next day cells were infected with Ad LacZ, Ad CRT/E7, Ad CRT/E7m or Ad CRT/E7dm. After 24 h, cells were washed with PBS

followed by fixation with cold methanol/acetone solution and incubation with 1% bovine serum albumin (BSA). Next, cells were incubated with mouse anti-HPV16 E7 monoclonal antibody and rabbit anti-calnexin polyclonal antibody mix (1:200 each; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 12 h. The slides were washed with ice-cold phosphate-buffered saline (PBS) and incubated with anti-mouse CF594 and anti-rabbit CF488 mix (1:300 each; Biotum, Freemont, CA, USA) for 2 h. The slides were mounted with Vectashield mounting medium with DAPI (Vector Laboratories Inc, Burlingame, CA, USA) observed with a Leica Fluorescence Microscope (Leica) and photo-documented with the QImage Pro 7 program.

Mice

Female C57BL/6 mice aged from 6-8 weeks were purchased from Harlan Laboratories (México City, México) and housed in the animal facilities of the Histology Department of the School of Medicine (UANL) at 12 h dark/light cycles with *ad libitum* access to food and water. Mice care and handling were according to the Guide for the Care and Use of Laboratory Animals of the United States National Institutes of Health and NOM-062-ZOO-1999. The protocol was approved by the Ethics Committee of the School of Medicine, Universidad Autónoma de Nuevo León (Monterrey, México) (protocol HT13 002).

Tumor protection assay

Four groups of 5 mice each were vaccinated by intraperitoneal (i.p.) injection with 5×10^{10} PV diluted in 100 µl of PBS (Gibco, ThermoFisher Scientific, Lafayette, CO, USA) with adenovirus: Ad LacZ, Ad CRT/E7, Ad CRT/E7m or Ad CRT/E7dm. Two weeks later mice were subcutaneously (s.c.) injected in the right thigh with 5×10^{4} TC-1 cells in 100 µl Hank's Balanced Salt Solution (HBSS; Sigma-Aldrich, St.Louis, MO, USA). Tumor growth was measured with a digital Vernier caliper and was calculated using the following formula: Tumor volume = (tumor minor diameter2) (tumor major diameter)/2.

Therapeutic assay

Four groups of 6 mice were each injected s.c. in the right thigh with 5×10^{4} TC-1 cells in 100 µl HBSS. Mice were intraperitoneally injected on days 3, 21 and 25 after tumor induction with 5×10^{10} PV in 100 µl of PBS with adenovirus Ad LacZ, Ad CRT/E7, Ad CRT/E7m or Ad CRT/E7dm. Tumor growth was measured with a digital Vernier caliper and was calculated using the following formula: Tumor volume = (tumor minor diameter2) (tumor major diameter)/2.

Statistics

Two-way analysis of variance (ANOVA) and *post-hoc* Tukey's test were performed using Prism software V. 6 (GraphPad Software, Inc, San Diego, CA, USA). $P<0.05$ was considered as a statistically significant difference. Experiments were performed independently at least three times.

Results

Design of mutant E7 versions

Fusion of mutant E7 to CRT was performed using mutant E7 reported before [6]. This mutant version possesses substitutions of Cys-58 and Cys-91 to glycine (Figure 1), and these substitutions are in two zinc-binding motifs and have been reported to enhance its antitumor activity. Double mutant E7 was designed *in silico*, using the mutant E7 version and adding a deletion in pRB binding-motif (aa 21-26). The sequence was codon-optimized for its production in human cells. We used the complete open reading frame of rabbit calreticulin, which had been reported to induce a potent antitumoral effect when fused to wild-type E7 [9,10].

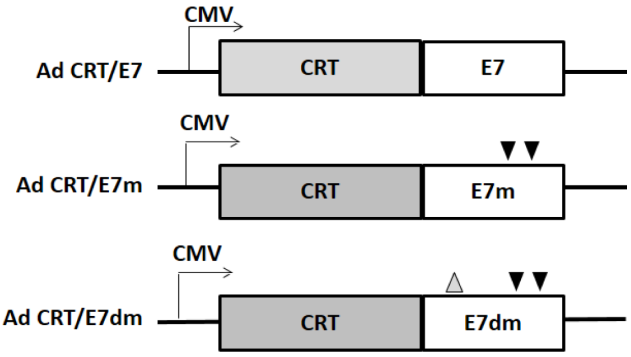

Figure 1. DNA constructs. Construction of adenovirus vectors expressing CRT/E7, CRT/E7m, CRT/E7dm. CRT/E7, CRT/E7m and CRT/E7dm fusion constructs with cytomegalovirus (CMV) promoter. CRT: calreticulin; E7m: mutant E7; E7dm: double mutant E7. Black inverted triangles: mutations in Cys-58 and Cys-91; gray triangle: deletion in pRB binding-motif (aa 21-26).

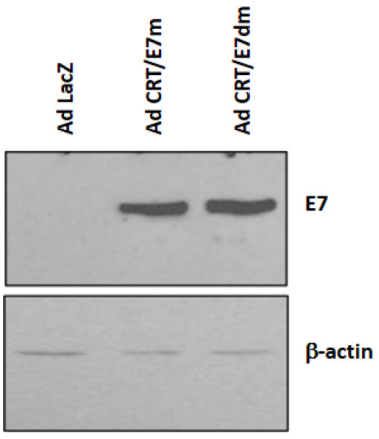

Figure 2. Recombinant protein expression. Western blots showing the expression of mutant variants of E7 fused to calreticulin mediated by Ad CRT/E7m and Ad CRT/E7dm. HEK293 cells were infected and harvested 48 h later. Ad LacZ construct was used as negative control. E7 expression was detected using a mouse anti-HPV-16 E7. CRT/E7m and CRT/E7dm were detected as 75 kDa bands.

Cells infected with Ad CRT/E7m or Ad CRT/E7dm express recombinant antigens

Once the adenoviruses were constructed, we analyzed if they were able to induce the production of the recombinant proteins. For this, HEK293 cells were infected with Ad LacZ (negative control), Ad CRT/E7m or Ad CRT/E7dm. After 48 h postinfection, cell extracts were analyzed by Western blot, detecting protein signals at 75 kDa in cells infected with Ad CRT/E7m and Ad CRT/E7dm (Figure 2), which corresponds to the size of calreticulin (60 kDa) and E7 (15 kDa) fusion. Cells infected with Ad LacZ did not express E7. We demonstrated that the adenoviruses constructed were able to direct the expression of the recombinant antigens.

Mutant E7 versions fused to calreticulin are expressed within the endoplasmic reticulum

When wild-type E7 antigen is expressed, it resides in the nucleus and the cytoplasm. Previously, it had been reported that by fusing the E7 antigen to the protein calreticulin, the antigen was directed to the ER [10], where calreticulin usually resides. Therefore, we wanted to determine the cellular location of the mutant proteins fused to calreticulin. When HEK293 cells were infected with Ad CRT/E7, Ad CRT/E7m and Ad CRT/E7dm, a positive signal was observed around the nucleus (Figure 3) and merged with the calnexin signal, which is an ER-marker. These results demonstrate that E7 mutant versions fused to calreticulin are located in the ER.

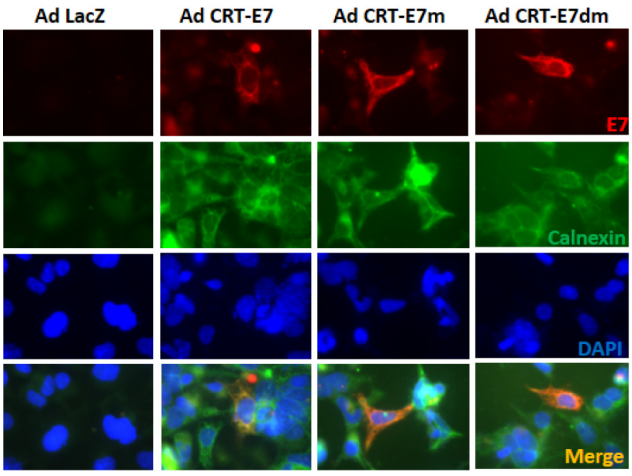

Figure 3. Subcellular localization of recombinant proteins. Fluorescent microscopy demonstrating the expression and distribution of CRT/E7, CRT/E7m and CRT/E7dm proteins in HEK293 cells. HEK293 cells were infected with Ad CRT/E7, Ad CRT/E7m or Ad CRT/E7dm and analyzed for the detection of endogenous calnexin (red fluorescence) and CRT/E7, CRT/E7m or CRT/E7dm (green fluorescence) 24 h after infection.

Mutant E7 versions fused to calreticulin induce a potent antitumor effect

To compare the antitumor effect of mutant versions of E7 fused to calreticulin, we evaluated the prophylactic and therapeutic effect of our adenoviral constructs *in vivo*. First, C57BL/6 female mice

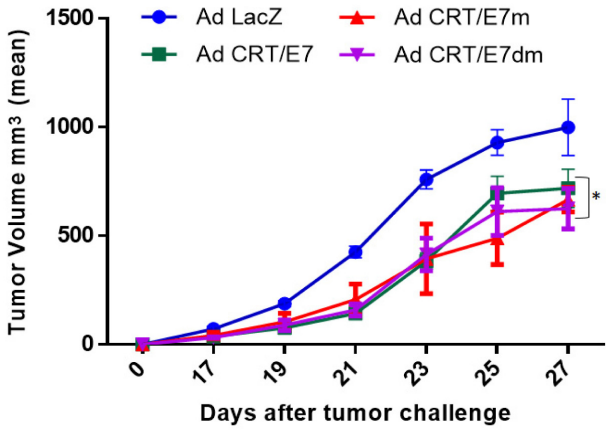

Figure 4. Prophylactic assay. Mice were vaccinated by intraperitoneal (i.p.) injection with Ad LacZ (circle), Ad CRT/E7 (square), Ad CRT/E7m (triangle), and Ad CRT/E7dm (inverted triangle) at a concentration of 5×10^{10} viral particles (VPs)/mouse. Two weeks later, mice were inoculated subcutaneously (s.c.) with live TC-1 cells at a concentration of 5×10^{4} TC-1 cells in the right thigh. Tumor growth was monitored three times a week for over a period of 27 days, and tumor size was expressed as the mean of two perpendicular diameters. *p<0.05, Ad CRT/E7, Ad CRT/E7m and CRT/E7dm compared to Ad LacZ.

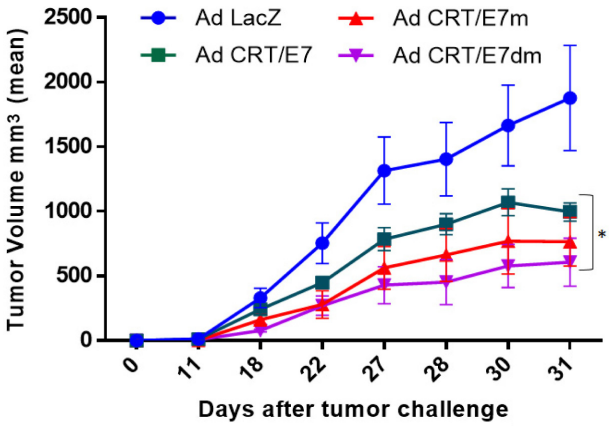

Figure 5. Therapeutic assay. Mice were inoculated s.c. with live TC-1 cells at a concentration of 5×10^{4} TC-1 in the right thigh. Three days later, mice were vaccinated (i.p.) with Ad LacZ (circle), Ad CRT/E7 (square), Ad CRT/E7m (triangle), or Ad CRT/E7dm (inverted triangle) at a concentration of 5×10^{10} VPs/mouse. The therapeutic effect was boosted with adenoviruses administration at days 21 and 25 after the inoculation with TC-1 cells. Tumor growth was monitored three times a week for over a period of 31 days, and tumor size was expressed as the mean of two perpendicular diameters. *p<0.05, Ad CRT/E7, Ad CRT/E7m and CRT/E7dm compared to Ad LacZ.

were treated with different adenoviruses, and then two weeks later TC-1 cells were injected into the right thigh to test the prophylactic effect. Tumor volume was monitored, and we observed a significant difference in mice treated with Ad CRT/E7, Ad CRT/E7m, and Ad CRT/E7dm, compared to mice treated with Ad LacZ as from day 21 (Figure 4), although, there was no significant difference between Ad CRT/E7, Ad CRT/E7m, and Ad CRT/E7dm.

Next, we tested the therapeutic effect of the adenoviruses. Mice were injected with TC-1 cells, and after 3, 21 and 25 days after tumor induction, they were treated with different adenoviruses. There was a significant antitumor effect in mice treated with Ad CRT/E7, Ad CRT/E7m and Ad CRT/E7dm compared to mice treated with Ad LacZ, as from day 22 (Figure 5). However, there was no significant difference between Ad CRT/E7, Ad CRT/E7m, and Ad CRT/E7dm. With these results, we concluded that mutant E7 versions from HPV-16 fused to calreticulin conserve their ability to induce a potent antitumo effect when tested in a murine cancer model.

Discussion

Nowadays, a significant part of scientific research focuses on the study of cancer and the development of new therapies that could eradicate this disease. Thus, we designed and constructed vaccines based on gene therapy using an adenoviral vector.

Cervical cancer is associated with HPV-16 infection. The cancer cells express viral proteins, which are a target for an antitumor immune response. Currently, two prophylactic vaccines approved by the FDA, based on HPV-16 L1 and L2 virus-like particles, provide high-level protection against persistent HPV-16 infection. However, therapeutic vaccines against cervical cancer are not available [11].

Most of the HPV vaccines research is based on the use of the HPV-16 E7 antigen because it is highly expressed in advanced stages of cervical cancer. The antigen E7 binds to the tumor suppressor protein pRb, which makes it capable of transforming cells [12]. Therefore, the use of E7-wild type on humans is not recommended. Hence, it is essential to develop mutant versions.

Adenovirus-mediated delivery of therapeutic transgenic genes has been widely reported as a useful tool tested on different cancer models [13]. Based on a previous work, where the immunization with an adenovirus expressing E7 fused with calreticulin generated a powerful antitumor response against TC-1 cells expressing E7 [9], we used E7 variants that lack the cell-transforming capability. One of the E7 versions had mutations in Cys-58 and Cys-91, which caused the loss of the zinc-binding conserved region 3 (CR3) domain of E7, resulting in a highly unstable protein that can be quickly degraded, and generating more epitopes to be presented in the MHCI pathway [6]. Although mutations in these cysteine residues drastically decrease the transforming capacity of the protein, E7 preserves its transformative ability because the pRb binding site locates in the LXCXE motif (21-26 aa) of the CR2 domain [14,15]. Therefore, we created a double mutant variant with mutations in amino acids 58 and 91, and the deletion of amino acids 24-26, which makes E7 safer for clinical purposes.

Thus, our two adenovirus vaccines proposals are CRT/E7m and CRT/E7dm. When cells were infected with these adenoviruses, we obtained a 75 kDa protein fusion on Western blot, of which 60k Da correspond to calreticulin, and 15 kDa to E7. Also, when cells were infected with Ad CRT/E7m and Ad CRT/E7dm, we observed that E7 expression was located in the ER, which was corroborated with the co-localization signal of calnexin, an ER-lumen marker. The latter is due to calreticulin fusion on E7 N-terminus that directs protein expression to the ER-lumen, which has been previously reported [9,10].

Once the fused proteins expression in the ER was proved, the antitumor effect was tested in an *in vivo* model. Mice immunized with Ad CRT/E7, Ad CRT/E7m and Ad CRT/E7dm showed a significant antitumor effect compared with the control group vaccinated with Ad LacZ at 22 days post-tumor cells injection.

Our results demonstrate that the antitumor effect generated by the new mutant variants of E7 fused to calreticulin was as efficient as the induced by the wild type E7 version. Importantly, our mutant E7 versions lack the oncogenic potential of E7, which is essential to create a vaccine for potential therapeutic use in humans. These results may have a promising impact on clinical research.

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Conflict of interests

The authors declare no conflict of interests.

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