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ORIGINAL ARTICLE

Therapeutic silencing of HPV 16 E7 by systemic administration of siRNA-neutral DOPC nanoliposome in a murine cervical cancer model with obesity

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Summary

Purpose: *To evaluate the effectiveness of a neutral DOPC nanoliposome system for the delivery of siRNA to tumor cells in an obese murine cervical cancer model.*

Methods: *In vitro silencing of E6-E7 mRNA and E7 protein using siRNAE6 or siRNAE7 was analyzed in TC-1 cells by RT-PCR and Western blot. Silencing and antitumor capacities of siRNAE7-DOPC-nanoparticles (NP) were tested in vivo in both normal and obese mice using qPCR. These NPs were administered twice a week for 15 days and tumor volume and weight were recorded.*

Results: *Levels of in vitro E6-E7 silencing were 90% for mRNA and 60% for protein when siRNAE7 was used. On the other hand when siRNAE6 was used, the levels of silencing were 50% for E6-E7 mRNA and only 20% for protein. In vivo E7 mRNA silencing by siRNAE7-DOPC-NP was similar (60%) in both non-obese and obese mouse models. The therapeutic study showed a 65% decrease in tumor volume and a 57% reduction in tumor weight as compared to the control groups.*

Conclusion: *There was no negative impact of obesity on the antitumor activity of siRNA-DOPC-NP in obese mice.*

Key words: *cervical cancer, DOPC, HPV, nanoliposomes, obesity, siRNA*

Introduction

Cervical cancer (CC) is the second leading cause of death in women around the world, responsible for 300,000 deaths annually. Eighty percent of these cases occur in developing countries, with approximately 600,000 new cases each year. In 2010, around 10,000 new cases of cervical cancer were documented in Mexico, and 50% of these ended in death (WHO, 2010). The persistent infection of high risk human papilloma virus (HPV) variants 16 and 18 (hrHPV 16 or 18) is considered the main factor in the development of CC [1]. HPV encodes the oncogenes E6 and E7, whose expression is essential for virus replication [2,3]. The integration of HPV into the DNA of the infected cell, and subsequent expression of the HPV oncoproteins E6 and E7, transforms normal cervical cells into malignant cells [4]. HPV16 E6 and E7 proteins have a high affinity for tumor suppressor proteins p53 and retinoblastoma (Rb), in comparison with other HPV variants. p53 promotes cell cycle arrest or initiates apoptosis in response to cellular stress. However, the antiproliferative role of p53 is abrogated by E6, which promotes its accelerated degradation via ubiquitination and subsequent proteosomal degradation [5]. On the other hand, E7 targets the Rb protein, a negative regu-

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lator of the cell cycle. Rb inhibition by E7 results in an upregulation of genes required for G1/S cell cycle transition. These phases are essential for viral genome replication and amplification [6]. For these reasons, E6 and E7 of HPV 16 or 18 have been proposed as target oncogenes to direct gene therapy strategies against CC.

Worldwide, obesity is the second leading cause of preventable mortality, just after smoking [7,8]. Elevated body mass index (BMI> 40) is associated with a high rate of death in both men and women, because obesity is a factor that increases the risk of mortal diseases such as cancer. Among obese men, esophageal, colorectal, liver, pancreas and kidney cancer are the most common, whereas obese women are mostly affected by breast, cervical, ovarian and endometrial cancer [9]. Ulmer et al. showed that obese patients with CC have a 12% higher risk of death in comparison with non-obese patients [10]. Overweight and obese women have an increased surgical morbidity [11], radiation dosimetry is difficult [12], and the paradigm of the required chemotherapy dose remains controversial [13]. Therefore, special considerations have to be taken for the correct treatment of obese cancer patients.

Introduction of double-stranded RNA (dsRNA) is recognized by the enzyme Dicer, and cleaved into small 19 base-pair long fragments (siRNAs). The siRNAs bind to a protein complex called RNA-induced silencing complex (RISC). The passenger strand of siRNA is then cleaved and discarded, while the guide strand directs RISC to the complementary target mRNA [14]. *In vitro* studies have shown that introduction of hrHPV E6 and E7-targeting siRNAs resulted in growth suppression of virus-positive cancer cells [15]. However, the use of siRNAs *in vivo* is limited due to various barriers such as rapid nuclease degradation in circulating blood, renal excretion, and low capacity of these molecules to be internalized into the cell. To overcome these drawbacks, our group developed a neutral nanoliposome system (1,2-dioleoyl-sn-glycero-3-phosphatidylcholine, DOPC) for *in vivo* delivery of siRNA, obtaining encouraging results in the silencing of genes in different murine models of cancer [16,17]. Clinical studies have demonstrated that CC patients develop obesity or become overweight, and these conditions are a negative combination that leads to problems in the effective treatment of malignancies [18]. Therefore, it is important to develop alternative therapies to help improve the quality of life of patients with cancer and obesity. In order to contribute to that issue, we evaluated the capacity of our neutral nanoliposome system in an obese murine cancer model. Thereby, our results demonstrated that the efficiency of the neutral nanoliposome system is the same in obese and non-obese models, allowing us to conclude that obesity is not an obstacle to the siRNA-DOPC-NP system in cancer therapy. This data opens up a range of possibilities for future studies to evaluate the efficiency of the nanoliposome system in patients with obesity and to offer alternatives in cancer treatment.

Methods

Cell line and culture conditions

TC-1 cells (murine C57BL/6 lung epithelial cells transformed with HPV16 E6, E7 and h-ras oncogenes) were kindly donated by Dr. Gomez-Gutierrez at University of Louisville. Cell line was mycoplasma-free and the expression of HPV16 E6 and E7 oncogenes and phenotype changes were continuously evaluated. Cells were maintained in RPMI-1640 medium (Invitrogen Co, USA) supplemented with 5 % of fetal bovine serum, 1% essential amino acids, and 0.02% G418 (all from Gibco Co, USA). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Design of siRNA and in vitro transfection

The siRNAs were designed using the RNAi Direct software and based on the published sequences in the NCBI database. All siRNAs were obtained commercially from Sigma Aldrich (St. Louis MO, USA) as follows: for E6 (E6, GenBank Accession NC-001526.2 Gene ID 1489078), siRNAE6 sequence (sense 5'-AAGUCAUAUACCUCACGUC-3'); for E7 (E7, GenBank Accession NC-001526.2 Gene ID: 1489079), siRNAE7 sequence (sense 5'UUUGUACGCACAACCGAAG-3'); and a commercial negative control siRNA (Cat. No. SIC001). For the transfection, $4x10^5$ TC-1 cells were seeded in 6-well culture plates containing 2 mL of RPMI medium with 5% FBS. Twenty-four hrs later, cells were transfected using RNAiFect Transfection Reagent (Qiagen, USA) according to the manufacturer's instructions. The cells were divided into 5 groups: UT (untreated cells), NC (7 μg siRNA non-silencing), TR (transfection reagent), E6 (7 μg siRNAE6), and E7 (7 μg siRNAE7). Treated cultures were harvested at 24, 48 and 72 hrs to analyze E6-E7 expression.

Semi-quantitative reverse transcriptase-PCR

Total cellular RNA was isolated with TRIZol Reagent (Invitrogen Co, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 5 μg of total cellular RNA using M-MLV kit (Invitrogen Co, USA). End point PCR was performed from 1.5 μl of cDNA products in a total volume of 25 μl, containing

10 μmol/L reverse primer and 10 μmol/L forward primer. The sequence primers used for PCR were: for E6-E7 bicistron, reverse 5'-GCCCATTAACAGGTCTTCCAAA-3', forward 5' -ACTGCAATGTTTCAGGACCCAC-3'; and for β-Actin, reverse 5'-GCTGCCTCAACACCTCAACCC-3', forward 5'-GTCCCTCACCCTCCCAAAAG-3'. PCR products were analyzed in 1.5% agarose gels and visualized on a High Performance Ultraviolet Transilluminator (UVP, USA). Optical density measurement of E6-E7 mRNA was determined by normalization to control of β-Actin using a Kodak Scientific Imaging Systems version 3.5.4.

Western blot analysis

After treatments, TC-1 cells were lysed in extraction buffer (150 mM NaCl, 25mM Tris, 1% Triton X-100, pH=7.6) containing a protease-inhibitor cocktail (Promega, USA). Lysates were centrifuged at 13,000 g at 4°C for 15 min; supernatants containing cellular proteins were collected and stored at −80°C until use. A total of 50 μg of protein were separated on 12% SDS-PAGE. Resolved proteins were transferred to PVDF membrane, which was blocked with 5% non-fat milk in tris-buffered saline with 0.1% tween 20. Immunodetection was performed using primary antibody for HPV16 E7 (1:200) (Santa Cruz Biotechnology, USA) or primary antibody for β-Actin (1:10,000) (Sigma Aldrich, USA). Membranes were then incubated with anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000). SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, USA) was used to detect signals, which were measured in a MicroChemie system (DNR Bio-Imagen Systems, Israel), β-Actin was used as the loading control.

siRNA-DOPC nanoliposomes preparation

siRNAs were encapsulated in 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) nanoliposomes. Briefly, siRNAs were mixed with lipid at a 1:10 ratio (w/w). Tween 20 was added to the mixture at a 1:19 ratio (Tween 20:siRNA/lipid) in an excess of tertiary butanol and lyophilized. Before animals were injected, the lyophilized lipid-siRNAs were reconstituted with 0.9% sterile saline to form liposomes [16,17].

In vivo experiments

Female C57BL/6 mice were used in all experiments. Animals were obtained from the institutional Animal Facility and experiments were approved by the Ethics Review Committee of CINVESTAV-I.P.N. (CICUAL-CINVESTAV Protocol No.0076-14). Mice were divided into 2 groups and were fed with either a normal diet (ND) or a high fat diet (HFD) for up 10 weeks. The HFD consisted of 58% energy from fat, whereas the normal diet contained 18% energy from fat. For the systemic delivery of siRNAs we used the DOPC neutral nanoliposomes. To determine the dosage plan of anti-HPVE7 liposomal siRNAs (siRNAE7-DOPC-NP), mice were injected subcutaneously into the right flank with $1x10^5$ TC-1 cells, and after 21 days a single dose of siRNAE7-DOPC-NP (0.45 or 1.5 mg/kg), nanoliposomes Negative Control siRNA (NCsiRNA-DOPC-NP; 1.5 mg/kg, n=5), or empty nanoliposomes (Empty-DOPC-NP, n=5) were administered into the tail vein. The animals were sacrificed 48 or 72 hrs later and necropsies were performed. To determine the effects of siRNAE7-DOPC-NP on tumor growth, 1x105 TC-1 cells were injected subcutaneously into mice, and 15 days later mice were randomized into 3 treatment groups (n=5 each) and received either Empty-DOPC-NP, NCsiRNA-DOPC-NP, or siRNAE7-DOPC-NP, biweekly for 2 weeks. Tumors were measured during the course of the experiment and at the end of the treatments all mice were sacrificed, necropsies were performed and final tumor sizes and weights were recorded.

Real-Time PCR

Real-time PCR was performed on MXP3000 system (Alligent Technologies, USA) using 100 ng of cDNA for each sample. All reactions were performed in 20 μl in triplicates. Brilliant III Ultra-Fast SYBER Green QPCR Master Mix (Alligent Technologies, USA) was used to detect products. 250 nM of the following primers were used: HPV16 E7, reverse 5'-GGTTTCTGAGAACAGATGGGGCA-3', forward 5'-CAGCTGGACAAGCAGAACCGGA-3', β-Actin: reverse 5'-GCTGCCTCAACACCTCAACCC-3', forward 5'-GTCCCTCACCCTCCCAAAAG-3 '. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. The relative expression of mRNA E7 was calculated by the ΔΔCt method and β-actin was included as housekeeping gene.

Statistics

Data is expressed as mean ± standard error of the mean (SEM). Statistical significance for the mean of each group was assessed by one-way analysis of variance (ANOVA) with a p value <0.05 being considered significant.

Results

Silencing of E6-E7 in vitro by siRNA

To examine the effects of the siRNAE6 and siRNAE7 on E6-E7 bicistron's mRNA expression, TC-1 cells were transfected with 7 μg siRNAs for 24, 48 and 72 hrs, then RT–PCR and Western blot (E7 protein) were performed. As shown in 1 (A and B), siRNAE6 produced an 80% knockdown of E6-E7 mRNA at 24 hrs, which went down to the basal level of expression by 72 hrs. Then, we used the same dose of siRNAE7 causing a 90% expression inhibition at 48 hrs, while at 72 hrs the expres-

sion inhibition was 60%. In the same way, we analyzed the expression levels of E7 protein. When siRNAE6 was used, we observed a 30% silencing at 72 hrs, while siRNAE7 treatment resulted in a 60% reduction after 48 hrs (Figure 1 C and D). Based on this data, we decided to use siRNAE7 for the rest of the study.

Use of siRNAE7-DOPC-NP in vivo in non-obese mice

Once silencing was standardized *in vitro*, we analyzed the in vivo effects of siRNAE7-DOPC-NP after 48 hrs of a single intravenous injection in tumor bearing non-obese mice (average weight of 23±1.0 g). Two different doses of siRNA were tested, dose A (0.45 mg siRNAE7-DOPC-NP/kg weight), and dose B (1.5 mg siRNAE7-DOPC-NP/kg weight). The maximum silencing effect in tumor tissue was obtained with dose B, reducing the E7 mRNA levels by 65% (Figure 2A). Furthermore, protein levels showed a 60% knockdown with the same treatment (Figure 2B).

Effect of siRNAE7-DOPC-NP in obese mice

As the best silencing in normal mice was reached with 1.5 mg of siRNAE7-DOPC-NP/kg weight, we decided to keep using the same dose to evaluate whether or not obesity has any effect on the nanoliposome system's efficiency. Mice fed with a HFD for 10 weeks showed a 50% increase in body weight when compared with those mice on ND (35±1 vs 23±0.6 g). Obese mice carrying TC-1 tumors were treated with a single dose of siRNAE7-DOPC-NP and 48 hrs later E7 mRNA expression levels in tumors showed a 60% reduction in comparison to controls (Figure 3A). However, protein levels showed only a 30% expression inhibition (Figure 3B), lower than that observed in the non-obese model (Figure 2B). Although we found a 30% difference in the expression inhibition between obese and non-obese animals, the silencing achieved in the obese animals was statistically different as compared to the controls (Figure 3B). Therefore, we decided to use this dose for the rest of the study. Next, we determined the effective si-

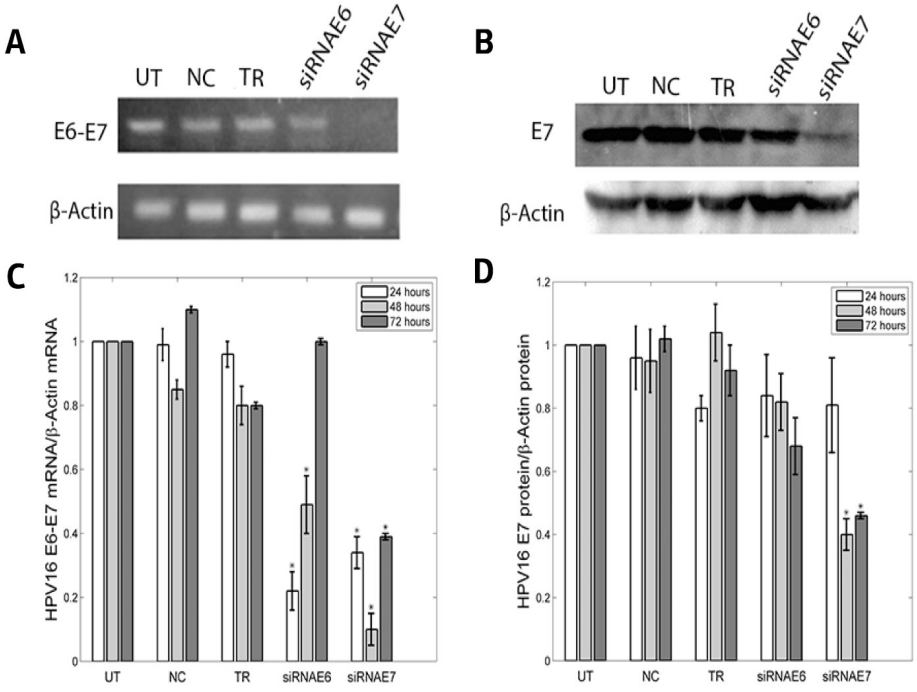

Figure 1. Effect of siRNAE6 and siRNAE7 in HPV16 E6-E7 expressing TC-1 cells. **A:** Semiquantitative RT-PCR depicting a decrease of E6-E7 (90%) with 7 μg of siRNAs for 48 hours. **B:** Levels of E6-E7 at 3 different times post-transfection. **C:** Western blot depicting silencing of E7 in TC-1 cells with 7 μg of siRNAs for 48 hrs. **D:** Levels of E7 at 3 different times post-transfection. β-Actin is used as loading control. Tests were performed in triplicate; *p< 0.01 significantly different to UT group. Bars and error bars represent the mean value and the corresponding SEM. UT: Untreated cells, NC: Negative siRNA Control, TR: Transfection Reagent, siRNAE6 and siRNAE7 groups of treatments.

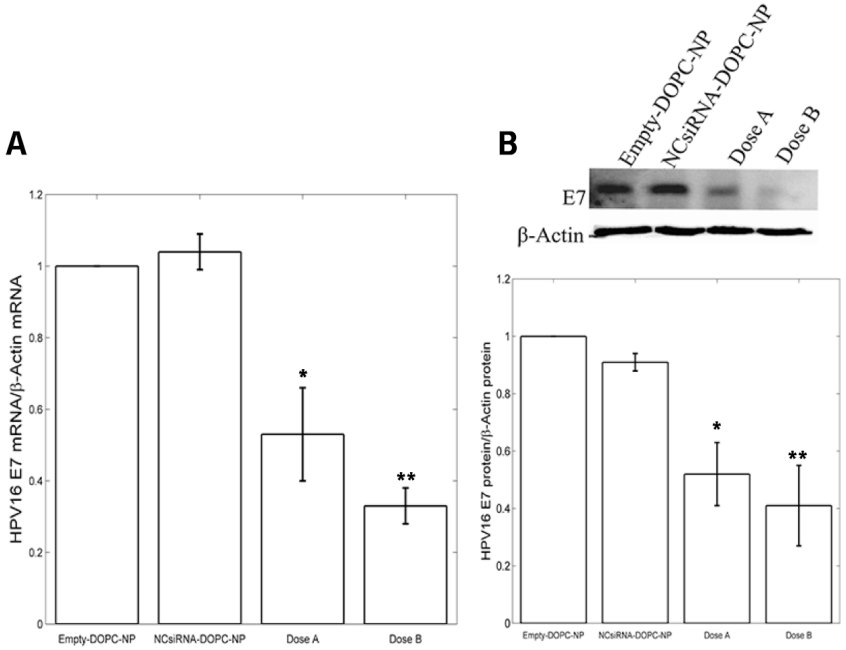

Figure 2. Silencing of E7 in non-obese mice. **A:** *In vivo* silencing of E7 48 hrs later of a single i.v. injection of Dose A (0.45mg/kg) or Dose B (1.5 mg/kg) siRNAE7-DOPC-NP, NCsiRNA-DOPC-NP, or Empty-DOPC-NP, as assessed by real time PCR. Each tumor (n=5 per group) was analyzed in triplicate. Bars and error bars represent the mean value and the corresponding SEM. β-Actin was used as housekeeping gene. **B:** Knockdown of E7 protein was observed in tumors after i.v. administration of siRNAE7-DOPC-NP. β-Actin was used as loading control. *p<0.05, ** p<0.01

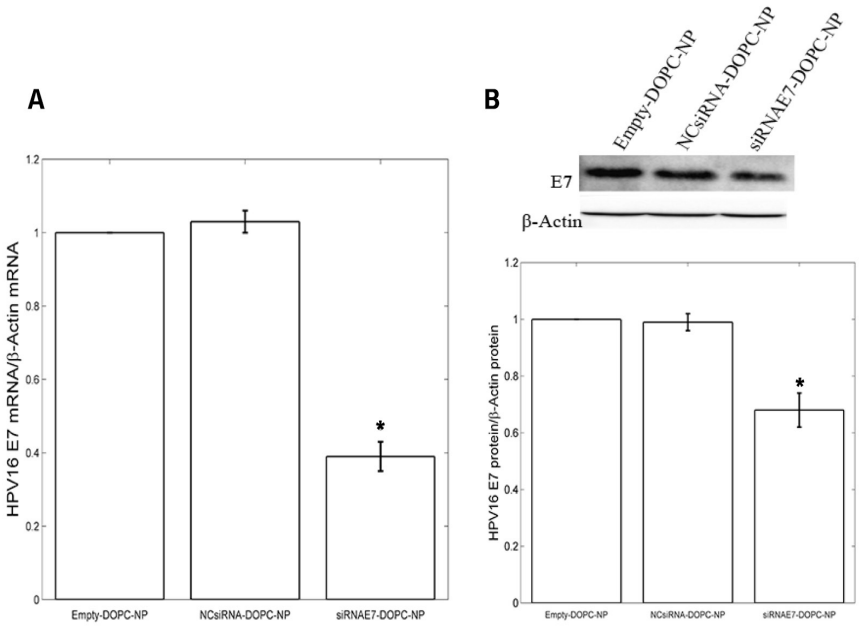

Figure 3. Knockdown of E7 in obese mice. **A:** Levels of mRNAE7 48 hrs after a single i.v. dose (1.5 mg/kg weight) of Empty-DOPC-NP, NCsiRNA-DOPC-NP, or siRNAE7-DOPC-NP were measured by real time PCR. Each tumor (n=5 per group) was analyzed in triplicate. Bars and error bars represent the mean value and the corresponding SEM. β-Actin was used as housekeeping gene. **B:** Western blot of E7 after receiving the indicated treatment. β-Actin was used as loading control. *p<0.01.

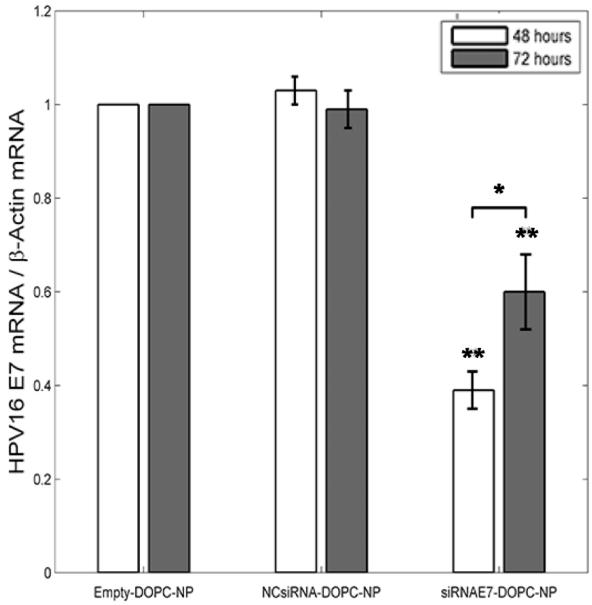

Figure 4. Silencing effects of a single siRNAE7-DOPC-NP dose. Data depicts optimal decrease of E7 expression at 48 hrs after single intravenous injection of siRNAE7-DOPC-NP compared to Empty-DOPC-NP treatment, with an expression recovery at 72 hrs. Each tumor (n=5 per group) was analyzed in triplicate. Bars and error bars represent the mean value and the corresponding SEM β-Actin was used as housekeeping gene. *p<0.05, **p<0.01.

lencing time period of a single dose of siRNAE7-DOPC-NP in the obese model. To achieve this, E7 mRNA expression levels were measured at 48 and 72 hrs after a single IV injection. Figure 4 shows that at 48 hrs a maximum silencing of 60% was obtained. By 72 hrs an expression recovery was observed, with only a 40% silencing. This indicates that two liposomes doses per week would be needed in order to keep low levels of E7 expression.

Inhibition of tumor growth by siRNAE7-DOPC-NP in obese mice

After establishing the optimal dose and time for our therapeutic experiment, we performed an anti-tumoral study in the obesity model. Tumor-bearing obese mice were treated with siRNAE7-DOPC-NP or controls (Empty-DOPC-NP or NCsiRNA-DOPC-NP) twice a week for a period of 15 days. As shown in Figure 5, the average tumor volume reached in the control groups was 1019 mm3, while tumors in the group treated with siRNAE7-DOPC-NP reduced to an average size of 435 mm^3, a 65% reduction. The average weight of tumors was registered at the end of the experiment, and for the control groups the values were 1150 mg (Empty-DOPC-NP) and 1098 mg (NCsiRNA-DOPC-NP) while in the siRNAE7-DOPC-NP group the value was 492.6 mg. This decrease in weight represents a 57% reduction in weight, in comparison with controls.

Discussion

CC is the second leading cause of death in women worldwide. The main cause of CC is the persistent infection with HPV; when the tumor is caused by infection of this virus, E6 and E7 oncoproteins, which are synthesized from a single mRNA (bicistron), are responsible for the malignant transformation of healthy cells [19]. Several *in vitro* studies have shown that silencing these two oncogenes by RNAi causes a decrease in cell proliferation and the expression of mRNA of E6-E7 bicistron [15,20].

Our *in vitro* results show that using siRNAE7 induces a 90% silencing of E6-E7 mRNA compared to untreated groups. However, little effect on expression of the E6-E7 bicistron was observed when siRNAE6 was used on TC-1 cells. This effect is consistent with the results of another research group in which they used a similar siRNAE6 silencing sequence and obtained 50% of E6-E7 bicistron inhibition in TC-1 cells [21]. These results lead us to conclude that siRNAE7 is a better op-

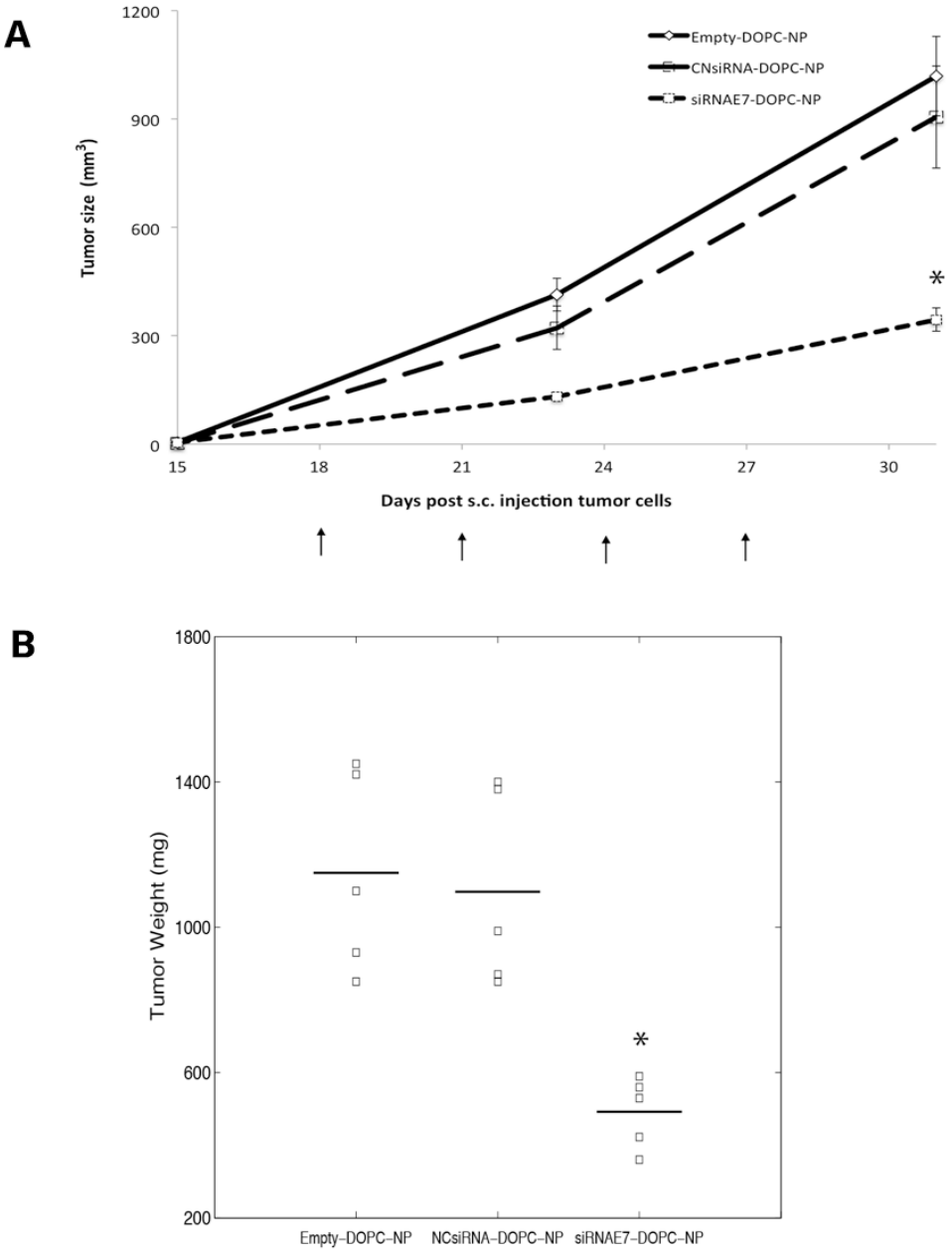

Figure 5. Antitumoral therapeutic effects of siRNAE7-DOPC-NP in obese mice. Obese tumor bearing mice were treated twice a week for two weeks with 1.5 mg siRNAE7-DOPC-NP/kg. **A:** Tumor development and treatment scheme (arrows indicate times of treatment). There is a significant decrease in tumor growth in mice treated with siRNAE7-DOPC-NP compared to mice treated with Empty-DOPC-NP (1019 mm^3 compared to 435 mm^3, respectively). **B:** Tumor weights were measured at the end of the experiment. There is significant inhibition of tumor weight between Empty-DOPC-NP (1150 mg) and siRNAE7-DOPC-NP (492.6 mg). *p<0.01.

tion, because it induces 40% more expression inhibition of the bicistron E6-E7 when compared to siRNAE6, hence our decision to use it for the *in vivo* studies.

The capacity of siRNAE7 to silence *E7* oncogene in a non-obese and immunocompetent murine model was evaluated. To achieve this, a neutral nanoliposome system based on the phospholipid 1,2-dioleoylsn-glycero-3-phosphatidylcholine (DOPC) was used to encapsulate siRNAE7 (siRNAE7-DOPC-NP). This formulation was tested at two different doses (0.45 and 1.5 mg/kg) of siRNAE7 in normal weight C57BL/6 mice bearing TC-1 tumors. Maximum E7 expression inhibitions

of 60 and 65% in the tumor tissue were reached at the 1.5 mg/kg dose after 48 hrs of treatment for protein or mRNA, respectively. This same nanoliposome system was used by Menashe Bar-Eli et al, who used a dose of 0.45 mg siRNA-PAR-1/kg weight and obtained 70% silencing of PAR-1 mRNA at 48 hrs in a nude murine model of melanoma [17]. The need for a higher siRNA-DOPC-NP dose in our study could be due to several factors like the difference in tumor model and the presence of a complete immune system.

At this point, we have demonstrated the effectiveness of our nanoliposome system in a normal weight immunocompetent murine model. However, our main objective was to analyze whether the characteristic elevated body fat content found in obesity had a negative effect on the silencing effectiveness of our liposomal system. To do this, we analyzed the effect of a single i.v. dose of 1.5 mg siRNAE7-DOPC-NP/kg in obese mice. A 60% E7 mRNA silencing was obtained at 48 hrs, which is similar to that observed in the non-obese mice. This shows that the nanoliposome system is able to deliver siRNA to the tumor cells in an obese animal as well as in the non-obese model. Therefore, the high amount of body fat present in the obese mice is not an obstacle for the nanoliposome system efficiency. These results were somehow unexpected, as we had hypothesized that nanoliposomes could be trapped in the body fat resulting in a lower silencing efficiency. Once we verified the silencing of E7 gene at 48 hrs, a time-response study was conducted. At 72 hrs the silencing level was down to 40%, indicating that the expression of the oncogene was recovering. Consequently, the optimal time to supply siRNAE7-DOPC-NP in an *in vivo* model is twice a week.

A therapeutic study using 1.5 mg siRNAE7-DOPC-NP/kg, twice a week was performed. The decrease of tumor volume of those animals receiving siRNAE7-DOPC-NP was observed as soon as the second dose of treatment was administered, this difference being 65% as compared to the control groups at the end of the experiment. This outcome is better than that reported in 2011 by Wu et al. who provided 3 siRNA doses (2 mg/kg body weight) for 15 days to C57BL/6 mice without obesity, reaching a 50% decrease in tumor volume as compared to the controls [21]. In addition to the better antitumoral effects, our liposome system is simpler and easier to produce, characteristics that make it a very good candidate for clinical application.

Our data indicates that the DOPC-nanoliposome system is efficient in delivering siRNA in a model with obesity. This provides a therapeutic option for patients who develop cancer and obesity.

Authors' contributions

Hector Chapoy-Villanueva: conception and design, development of methodology, acquisition of data, analysis and interpretation of data.

Ivonne Martinez-Carlin: development of methodology, acquisition of data.

Gabriel Lopez-Berestein: analysis and interpretation of data, review of the manuscript, material support.

Arturo Chavez-Reyes: conception and design, analysis and interpretation of data, study supervision.

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