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Gene Therapy That Safely Targets and Kills Tumor Cells Throughout the Body

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ABSTRACT

The authors studied the therapeutic value of Sindbis vectors for advanced metastatic cancer by using a variety of clinically accurate mouse models and demonstrated through imaging, histological, and molecular data that Sindbis vectors systemically and specifically infect/detect and kill metastasized tumors *in vivo*, leading to significant suppression of tumor growth and enhanced survival. Use of two different bioluminescent genetic markers for the IVIS Imaging System (Xenogen Corp., Alameda, CA) permitted demonstration of an excellent correlation between vector delivery and metastatic locations *in vivo*. Sindbis tumor specificity is not attributable to a species difference between human tumor and mouse normal cells. Sindbis virus is known to infect mammalian cells using the Mr 67,000 laminin receptor, which is elevated in tumor versus normal cells, and downregulated expression of laminin receptor with small interfering RNA significantly reduces the infectivity of Sindbis vectors. Tumor overexpression of the laminin receptor may explain the specificity and efficacy that Sindbis vectors demonstrate for tumor cells *in vivo*. Laser capture microdissection of mouse tumor implants showed equivalent laminin receptor expression levels in the different tumor metastases in the peritoneal cavity. Incorporation of antitumor cytokine genes such as interleukin-12 and interleukin-15 genes enhances the efficacy of the vector. These results suggest that Sindbis viral vectors may be promising agents for both specific detection and growth suppression of metastatic ovarian cancer.

INTRODUCTION

Sindbis virus vectors

THE AUTHORS' LABORATORY has been developing a Sindbis viral vector system for the treatment of cancer. Sindbis virus, a member of the alphavirus genus in the *Togaviridae* family, was first isolated in August 1952 from a pool of mosquitoes (*Culex pipiens* and *C. univittatus*) trapped in the Egyptian Sindbis district.[1–3] Sindbis virus is widespread throughout the world and is associated with transient diseases. There have been clinical reports as a result of Sindbis infection, with symptoms including mild fever and rash.[4] Sindbis virus is a single-stranded, enveloped, positive-strand RNA virus, that replicates only in the cell cytoplasm and do not integrates in the host cell DNA genome.[5] Within hours of infection Sindbis virus amplifies highly his genome, having each cell an average of 10^5 viral RNA copies. In nature, Sindbis virus is transmitted via mosquito bites to mammals; it is a blood-borne virus able to cross the blood–brain barrier[6] and reach cells through the body via the bloodstream.

These characteristics of Sindbis virus life cycle confers safety to the viral vectors, avoiding

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the risk of chromosomal integration or germ line modification of patients and also makes them highly efficient for gene transfer throughout the body.

The Sindbis viral vectors we used in these studies are replication incompetent, so they cannot infect, replicate, and cause viremia unless recombination occurs. Recombinant competent virus (RCV) usually are below detection limits[7] and can be minimized and monitored.

Another advantageous property of Sindbis vectors is that they have been shown to induce apoptosis in mammalian cells.[8–11] This cytotoxicity could eradicate cancer if specific and complete tumor targeting is achieved.

Sindbis vectors infect human tumor cell lines

The aim of the first experiments was to determine if Sindbis vectors could infect and induce apoptosis in human tumor cells lines, with comparable efficiency as for baby hamster kidney cells (BHK). The vectors infected *in vitro* with high efficiency colon (LS174T and HT29), ovarian (ES-2 and SKOV-3), pancreatic (CFPAC), prostate (PC-3), and liver (HuH-7) cancer cell lines, and had a lower efficiency of infection for kidney (A498), bladder (HT1197), and epidermoid carcinoma of the vulva (A431) cell lines.

In vivo experiments were performed injecting subcutaneously (sc) the aforementioned colon and pancreatic cell lines in severe combined immunodeficient SCID mice, and after 4 weeks when tumors reached substantial size, mice started daily intraperitoneally (ip) treatment with Sindbis vectors. Statistically significant reduction of tumor size compared with nontreated animals was achieved after 2 weeks of treatment and complete regression of colon or pancreatic tumors was observed in some mice after 42 and 46 treatments, respectively.[12]

Sindbis vectors are capable of disseminating through the bloodstream and systemic targeting of tumor implants at different body locations, as demonstrated by the IVIS Imaging System, a noninvasive system able to detect the bioluminescence of luciferase activity *in vivo*. This technique allows monitoring the delivery and infection of the vector as well as the tumor cells in live mice. Immunodeficient SCID mice bearing BHK subcutaneous or intrapancreatic tumor implants were treated ip with Sindbis vector. Specific infection and tumor regression was achieved in both models. Furthermore, in a lung tumor model induced by injection of BHK cells intravenously (iv) in SCID mice, one single iv administration of Sindbis vectors was able to specifically target the tumor implants.[13] These encouraging results led to further vector characterizations in new developed cancer models that more accurately mimic patient pathology.

OVARIAN CANCER MODELS

Epithelial ovarian cancer remains a disease with a grim prognosis, the majority of patients are diagnosed with stage II or III disease, because of the uncommon and nonspecific (if present) early symptoms. Current treatment combines systemic chemotherapy and cytoreductive surgery. Initially, 70% of ovarian cancer patients respond to platinum-based chemotherapy, but the majority experience recurrence. The overall 5-year survival is approximately 20% for advanced-stage disease.[14]

MATERIALS AND METHODS

Cells and vector preparation

BHK and ES-2 cells were obtained from the American Type Culture Collection (ATCC). BHK cells were maintained in αMEM (JRH Biosciences, Inc., Lanexa, KS) with 5% FBS. ES-2 cells were derived from a patient with clear cell carcinoma, a type of ovarian cancer that has a poor prognosis and is resistant to several chemotherapeutic agents including cisplatin. ES-2 cells were cultured in McCoy's 5A medium (Mediatech, Inc., Herndon, VA) with 5% FBS. All basal media were supplemented with 100 μg/mL of penicillin-streptomycin and 0.5 μg/mL of amphotericin B (both from Mediatech).

Sindbis/luc vector was produced as described previously.[8] Briefly, the plasmids carrying the Sindbis replicon SinRep/Luc or Sindbis helper sequences DHBB were linearized with *Not*I or *Xho*I, respectively, before *in vitro* transcription using the mMACHINE RNA transcription kit (SP6 version; Ambion, Inc., Austin, TX). Both

helper and replicon RNA transcripts (20 μL each) were then coelectroporated into BHK cells and incubated in 10 mL of αMEM containing 5% FBS at 37°C for 12 h. The medium was replaced with 10 mL of Opti-MEM I medium (GIBCO-BRL, Grand Island, NY) and after 24 h, culture medium was collected and stored at -80°C. To determine Sindbis/luc vector titer, supernatants from coelectroporated BHK-21 cells containing viral particles were collected and serial diluted to infect 2×10^5 cells/dilution, for 1 hour at room temperature. After washing with PBS, cells were incubated with 2 mL of medium at 37°C for 24 hours. Cell lysates were then assayed for *firefly* luciferase activity using the Steady-Glo® Luciferase Assay (Promega, Madison, WI). Vector titers were estimated as the last dilution having detectable reporter activity, which refers to the number of infectious particles or transducing units per milliliter of supernatant (TU/mL).

Animal models

All animal experiments were done in accordance with National Institutes of Health and institutional guidelines. Sindbis vectors targeting experiments were done in C.B-17-SCID mice (female, 6 to 8 weeks old; Taconic, Germantown, NY) injecting ip 2×10^6 ES-2 cells in 0.5 mL of McCoy's 5A medium on day 0. Five days later both ES-2 inoculated mice and tumor-free control mice received a single treatment of Sindbis/Fluc vector, and the bioluminescence signals were monitored using the IVIS system 100 series (Xenogen, Alameda, CA) the next day (day 6) as described.[3]

For laser capture microdissection experiment, a C.B-17-SCID mouse was ip injected with 10^6 ES-2 ovarian tumor cells, at necropsy stage tumor metastases on the diaphragm, omentum, and the liver surface were removed and flash-frozen in OCT.

Laser capture microdissection

Ten micrometer–thick frozen sections were prepared onto uncharged glass slides and immediately fixed and dehydrated in fresh acetone for 5 minutes. These tissues were then stained with DEPC prepared hematoxylin and then stained in eosin, dehydrated through graded alcohols (70% to 100%) and two washes of xylene. Slides were placed into a vacuum desiccator overnight and microdissected the next day.

Real-time quantitative RT-PCR

Total RNA was extracted from laser captured microdissected tissues using commercial RNA extraction kit (Arcturus, Mountain View, CA). RNA was reverse transcribed into cDNA for 1 h at 42°C in a 20-μL reaction containing 15 u of ThermoScript™ RNAse H-Reverse Transcriptase (Invitrogen Co., San Diego, CA), dNTPs (1 mM), oligodT (2.5 μM), RNAse inhibitor (40 u), DTT (5 mM), and 1× buffer. Real-time quantitative PCR was performed on a iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA) in a 20-μL reaction mix containing 4 μL RT product, reaction buffer (1×), dNTPs (200 μM/each), GAPDH or hLR primers (0.5 μM/each), 1U of Taq Polymerase (Fisher Scientific, Pittsburg, PA), fluorescein (100 nM), and 1 μL of SYBR Green I (10,000× diluted to 1:75,000 v/v). Thermocycling was carried out over 40 cycles of 95°C 30s, 60°C 30s, and 72°C 1 m. The sequences of the primers used were as follows: human laminin receptor forward primer (on exon 2), 5-CTCAAGAGGACCTGGGAGAAGC-3; human laminin receptor reverse primer (on exon 3), 5-TGGCAGCAGCAAACTTCAGC-3; human GAPDH forward primer, 5-CACCAGGGCTGCTTTTAACTCTGGTA-3; human GAPDH reverse primer, 5-CCTTGACGGTGCCATGGAATTTGC-3.

Human GAPDH was chosen as the housekeeping gene for comparative analysis. The fold change in laminin receptor relative to the GAPDH endogenous control was determined by: fold change $2–\Delta(\Delta\ C_T)$, where $\Delta\ C_T = C_{T(laminin\ receptor)}–C_{T(GAPDH)}$ and $\Delta(\Delta C_T) = \Delta\ C_{T(tumor)}–\Delta C_{T(omentum)}$CT is the threshold cycle determined at 84°C for fluorescence data collection.

RESULTS AND DISCUSSION

The authors developed a highly reproducible and clinically accurate SCID xenograft model, employing a human ES-2 cell line that has been derived from a clear cell ovarian carcinoma that is resistant to cisplatin among other chemotherapeutic agents.

Five days after ip inoculation of 2×10^6 ES-2 cells into female SCID mice, no tumor implants in the peritoneal cavity were visible except for a few small (<2-mm) unattached tumor clusters. However, histologic analysis of the tissues revealed microscopic tumor metastases on the omentum, mesentery, and diaphragm at this early stage of disease. Without any treatment, the mice developed grossly visible ascites and tumor growth on mesentery and omentum within 2 weeks.[13]

Once the accuracy of this aggressive human ovarian cancer model was established, the specificity of Sindbis vectors in tumor infection and its ability for disease suppression were studied.

Colocalization of vector infection and metastases *in vivo* was studied using IVIS imaging, measuring two consecutively independent bioluminescent signals in the same mouse that were generated by two different luciferases; Renilla and firefly. ES-2/Fluc cells express the firefly *luciferase* gene, and Sindbis vector (Sindbis/Rluc), carries the soft coral *Renilla renifomis* (*Rluc*) *luciferase* gene. Using two different substrates, it is possible to detect in the same animal in two consecutive images: first the *Renilla* bioluminescence corresponding to the vector activity, and then the firefly activity present in the tumor cells. Quantitative analysis of the bioluminescence signals generated in the same animal showed highly significant correlation. Thus, a single ip delivery of Sindbis vectors leads to efficient infection of the metastasized tumor cells throughout the peritoneal cavity.

The colocalization results did not eliminate the possibility of additional nonspecific vector targeting of vital organs in the peritoneal cavity. More detailed analysis of infection of organs were performed by IVIS system but in this case using as vector reporter the firefly luciferase, which has stronger bioluminescence. These vector-targeting experiments were performed in SCID female mouse bearing 5 days ES-2 tumor implants and tumor-free control mouse. Both received a single dose of Sindbis vector that carries firefly *luciferase* gene (Fig. 1). The next day the animals were IVIS imaged to detect vector activity. No detectable signals were observed in the peritoneal cavity of tumor-free control mouse. In contrast, vector was detected only in the ES-2 tumor-bearing mouse (Fig. 1A). Organ analysis showed vector only in tumor implants on the pancreas-omentum, bowel, and diaphragm (Fig. 1C). No nonspecific vector infection of vital organs (e.g., liver, hear, brain, kidneys) was detected in any mouse on repeated experiments, confirming the specificity of Sindbis vectors.

Monitoring ovarian tumor growth *in vivo* was made possible by inducing tumors in mice with ES-2/Fluc cell line that constitutively express the firefly *luciferase* gene. The day after ip injection of 10^6 ES-2/Fluc cells in SCID female mice, the authors were able to detect microscopic tumor metastasis using the IVIS system. Because it monitors disease progression without sacrificing the mice, the effect of Sindbis vector in tumor regression can be established periodically in the course of treatments. To determine therapeutic effects, after 5 days of ES-2/Fluc cells ip injection daily treatments were started with Sindbis/LacZ, which carries the bacterial β-galactosidase gene; Sindbis/IL-12; and Sindbis/IL-15, with mouse IL-12 and IL-15 genes, respectively. IL-12 and IL-15 are known to activate natural killer cells, eliciting antitumor activities.[15,16] However, in these experiments cytokine alone was not effective. Total whole body photon counts were determined by IVIS imaging on days 1, 5, 13, and 20 to analyze disease progression of ES-2/Fluc metastases. The Sindbis/LacZ vector significantly suppressed disease progression compared with untreated control mice. The IL-12 and IL-15 cytokine genes further enhanced the antitumor activity of Sindbis vectors compared with mice treated with Sindbis/LacZ.[17] Tumor suppression results correlate with significant improvement in survival of mice treated with Sindbis vectors, especially for the IL-12 and IL-15 groups.

To address the possibility that the tumor specificity of Sindbis vectors could be caused by preferential tropism for human cells and that immune responses could affect the targeting or therapeutic efficacy of Sindbis vectors, the authors also studied them in a syngenic ovarian cancer model developed by Roby et al. Mouse ovarian MOSEC cells were ip inoculated into fully syngenic immunocompetent C57BL/6

mice. As for the ES-2 cancer model, Sindbis vectors specifically targeted MOSEC cells and effectively suppressed disease progression.[17]

In further experiments the relation of Sindbis vector–specific tumor targeting with the laminin receptor (LAMR), the Sindbis virus natural receptor, was studied.[18]

LAMININ RECEPTOR

The 67-kDa high-affinity laminin receptor (LAMR) has been described as the surface receptor on mammalian cells for Sindbis infection;[18] it is substantially upregulated in numerous human cancers.[19–27] Increasing invasiveness and malignancy of different cancers has been associated with higher expression of LAMR.[28,29] On cancer cells, the majority of the LAMRs are not occupied by laminin, in contrast to normal cells.[30,31]

To determine whether high levels of unoccupied LAMRs in tumors contribute to Sindbis vector infectivity *in vivo,* immunohistochemical staining was performed on tumor sections with a LAMR specific antibody. ES-2/Fluc and MOSEC cell lines express higher levels of laminin receptor than normal tissues.[17]

Correspondence between LAMR and Sindbis vectors infection was studied in experiments using the cell line ES-2/Fluc/LRP, which stably expresses a small interfering RNA specifically against laminin receptor messenger. Real-time PCR of this new cell line has a reduction of 40% in LAMR expression compared with its parental ES-2/Fluc. Infection of ES-2/Fluc and ES-2/Fluc/LRP cells with Sindbis/LacZ vectors at MOIs of 100, 10, and 1 revealed markedly less infectivity by Sindbis vectors in ES-2/Fluc/LRP cells compared with ES-2/Fluc. These data indicate that the Sindbis infectivity of ES-2 cells correlates with the expression levels of laminin receptor.[17]

In the experiments done in the ovarian cancer tumor models, complete remission could not be achieved, although significant tumor regression was observed and the treatment prolonged the survival of the animals. Organ analysis of treated mice showed that tumor implants in the omentum were less susceptible to necrosis and reduction by Sindbis vector that tumor implants in other locations.

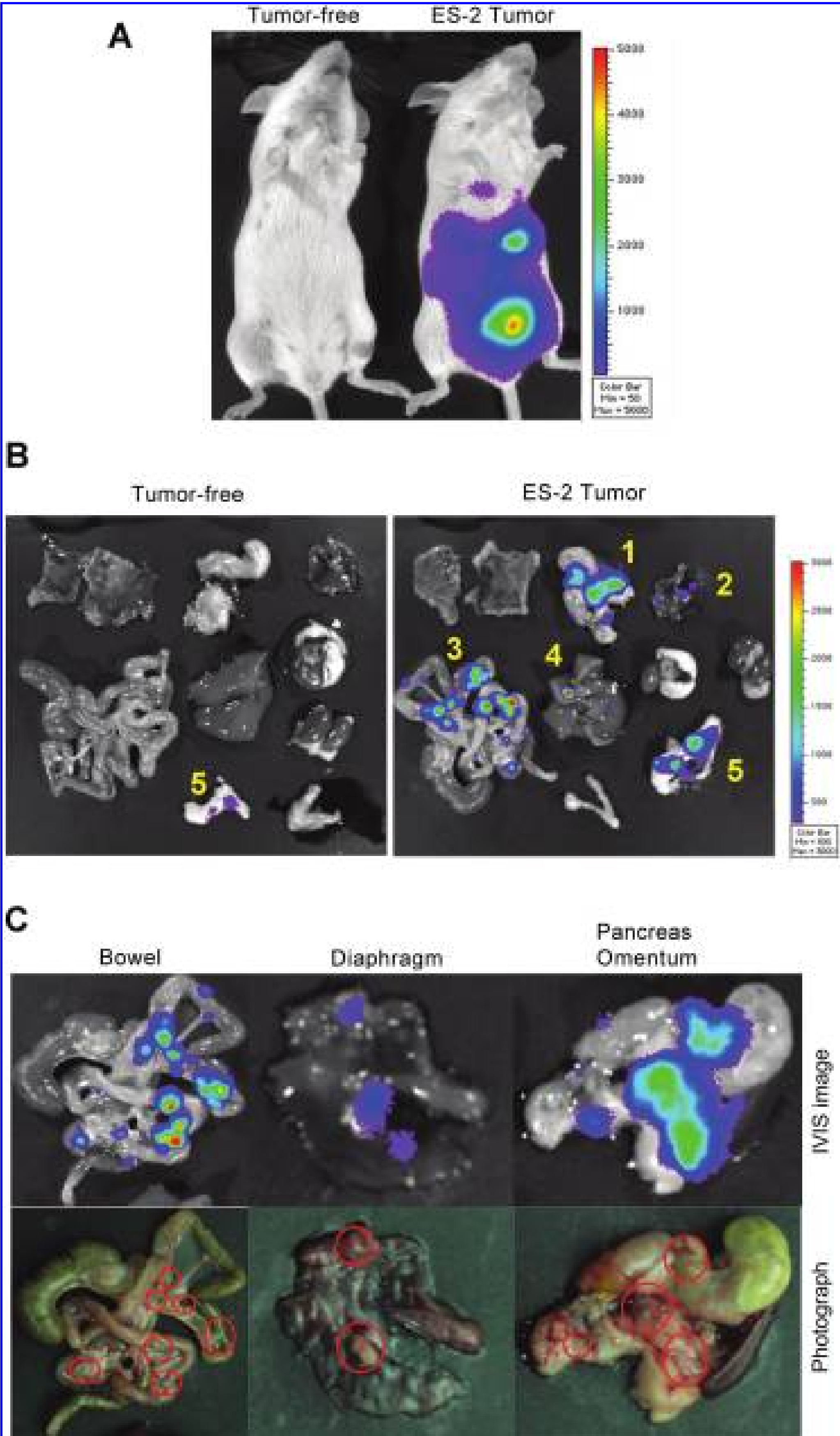
An explanation for this observation might be a difference in vector infectivity of these implants. Although all tumor implants are induced with the same cell line, during the oncogenesis process, cells in different tumor implant areas my differently express laminin receptor and therefore affect Sindbis vectors infectivity. The authors addressed this possibility using the laser capture microdissection technique. Briefly, a C.B-17-SCID female mouse was ip injected with one million ES-2 ovarian tumor cells. At the necropsy stage, tumor metastases on the omentum, diaphragm, and liver surface were removed and frozen. Ten mm thick frozen sections were hematoxylin/eosin stained to allow tumor visualization, and then tumor cells were cut and collected under light microscopy in a cap for RNA extraction (Fig. 2A). This technique allows collecting RNA only from tumor implants and performing accurate real-time quantitative PCR of LAMR. Results indicate comparable expression levels for laminin receptor gene in the three implants (Fig. 2B).

The authors conclude that other factors probably explain the lesser response of omentum implants to Sindbis vectors.

AR-339 NEW SINDBIS VECTORS

Although Sindbis vector targeting *in vivo* leads to marked reduction of tumor growth and increased survival; complete survival of all

FIG. 1. Sindbis vectors specifically infects ES-2 tumor implants. **A.** SCID mice received inoculations of 2×10^6 ES-2 cells on day zero and were ip treated with a single injection of Sindbis/Fluc vector on day 5. On the next day (day 6) the bioluminescence signals, resulting from vector infection of ES-2 cancer cells, were monitored using the IVIS system (*right panel*). Tumor-free control mice that also were treated with the vector did not show vector infection (*left panel*). **B.** The organs of these mice were harvested and imaged. Specific vector infection was exclusively observed in ES-2 metastases in pancreas-omentum (1), diaphragm (2), bowel (3) and liver (4). Treated and control mice had background infection in the fat tissue (5). **C.** The tumor metastases corresponding to IVIS bioluminescence are shown in *red circles*.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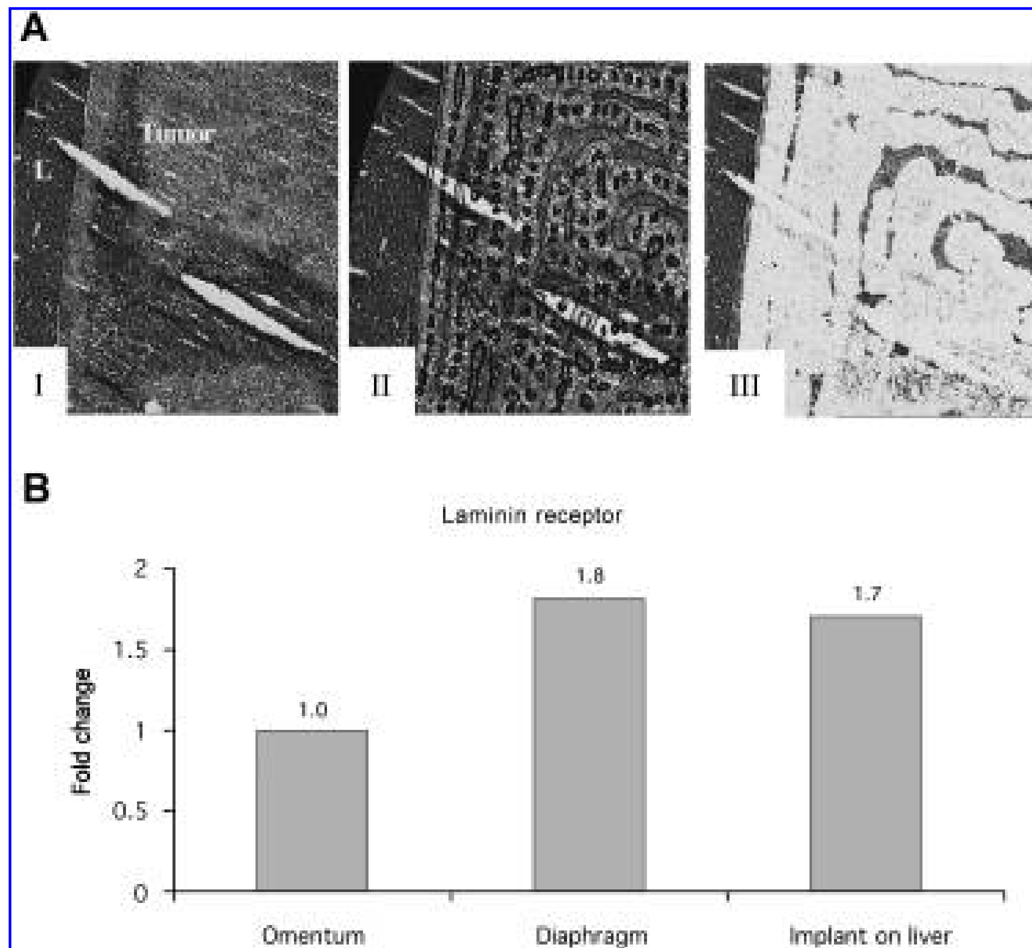

FIG. 2. LAMR Quantitative RT-PCR of laser microdissected ES-2 tumors. **A.** A SCID mouse was ip injected with 10^6 ES-2 ovarian tumor cells. At necropsy stage, tumor metastases on the diaphragm, omentum, and liver surface were removed and frozen. Ten micrometer–thick frozen sections were stained with hematoxylin/eosin, and tumor tissue was laser capped and microdissected. *(I)* Tumor implant on liver, precapture of tumor; *(II)* capture of tumor; *(III)* postcapture, tumor was specifically removed and nontumoral liver tissue was left in the slide. **B.** RNA was extracted from captured ES-2 tumor tissues. Quantitative RT-PCR for laminin receptor gene showed similar expression levels of LAMR for omentum, diaphragm, and implant of tumor.

mice in these ovarian tumor models has not yet been achieved. Different strains of Sindbis virus have been described to have diverse cell infectivity.[32] The Sindbis vectors used in all these experiments were developed from a wild-type laboratory adapted strain called Toto1101. To explore whether the mutations were acquired by Toto1101 Sindbis virus during the adaptation to growth in BHK cells *in vitro*, a new Sindbis vector was constructed from a Sindbis wild-type, Ar-339 Sindbis vector.[33]

As for the previous vectors, the authors performed targeting, suppression of disease progression, and survival experiments in the aforementioned ES-2 ovarian model. A single ip delivery of new Ar-339 Sindbis vector also resulted in very efficient infection of the metastasized tumor cells throughout the peritoneal cavity. Suppression of disease progression and survival results were comparable to results obtained from the previous vector.

Studying both vector sequences revealed a number of mutations, some of which were of special interest, such as those located in the E2 envelope protein at the Sindbis receptor-binding domain. Three Ar-339 reverse mutants were constructed that combined E2 envelope protein residues from both strains, and its implications in tumor infectivity were studied.

Targeting experiments in ES-2 model revealed that residue at position 70 of E2 is critical for *in vivo* tumor targeting. Further studies of E2 mutants should lead to a better understanding of the mechanisms used by Sindbis vectors to target and infect tumors *in vivo*.

The present studies demonstrate the advantages of Sindbis viral vectors for gene therapy of cancer. These vectors can specifically infect and detect micro and macro metastasis without unspecific infection of vital organs in mice upon systemic delivery. Sindbis vectors have the ability to disseminate through the bloodstream, allowing targeting of tumors growing subcutaneously, intrapancreatically, intraperitoneally, or in the lungs, after systemic administration. Sindbis viral vectors can deliver and highly amplify a chosen gene in the host cell, providing a powerful tool for tumor metastases detection as well as specific drug delivery. In this ovarian cancer model, the treatment of animals with Sindbis carrying cytokine genes markedly enhanced the antitumor effects of the vector alone. The development of new protocols combining therapeutic agents and genes with Sindbis vectors hopefully will lead to the total eradication of disease. Parallel approaches involving the study of the vectors sequences will broaden the knowledge of Sindbis specificity in tumor targeting and hopefully improve vectors infectivity.

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