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Original article

Endostatin neoadjuvant gene therapy extends survival in an orthotopic metastatic mouse model of renal cell carcinoma

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ABSTRACT

Despite recent advances in targeted therapy, renal cell carcinoma (RCC) remains one of the most lethal urologic malignancies. Approximately 30% of patients with localised RCC will develop metastases after curative surgery. Presurgical therapy has been explored for treatment of localised RCC. Endostatin (ES) is a fragment of collagen XVIII that possesses antiangiogenic activity. In this study, we examined the potential use of an antiangiogenic agent as a neoadjuvant therapy in an orthotopic metastatic mouse model of RCC. BALB/c mice bearing Renca cells were treated before nephrectomy with NIH/3T3-LendSN cells. At the end of the experiment, ES serum levels were measured. Primary and metastatic tumour area and microvascular area were determined. In the survival studies, mice were monitored daily until they died. ES serum levels in treated mice were higher in the control group ($P < 0.05$). The median primary tumour area and the mean microvascular area were significantly lower in the ES-treated group compared to control group ($P < 0.05$). The proliferation of Renca cells in the ES-treated group was significantly reduced compared with the control group ($P < 0.01$). ES therapy led to a significant reduction in the number of pulmonary metastatic nodules compared with the control group ($P < 0.01$). Kaplan–Meier survival curves showed that the probability of survival was significantly higher in mice receiving ES therapy ($P = 0.0243$, Log-Rank test). Our results indicated that neoadjuvant ES gene therapy has the potential to decrease tumour burden, extend survival, and may have clinical benefit in the management of RCC.

1. Introduction

Kidney tumours comprise 2% of all adult malignancies and account for 208,000 new diagnoses and 102,000 deaths worldwide each year [1]. Renal cell carcinoma (RCC) encompasses the vast majority of all kidney tumours and is classified histologically as either clear cell (60–80%), papillary (10–15%), chromophobe (5-10%), or collecting duct carcinoma (< 1%). In the case of localised disease, RCC is curable with surgery. However, the prognosis is poor in patients with distant metastases [2]. Nephrectomy provides curative treatment for localised disease, but unfortunately, 30% of patients subsequently experience recurrence and metastasis and have a survival rate of less than 10% [3].

The treatment of metastatic renal cell carcinoma (mRCC) has advanced remarkably because of the greater understanding of the disease pathogenesis. Recently in the clinic, some multi-targeted drugs have achieved a high percentage of partial response and/or disease stabilisation in patients with mRCC significantly affecting patients' long-term survival [4,5].

Neoadjuvant therapy is the administration of a therapy prior to the main course of treatment [6]. The main goals of neoadjuvant therapy include reducing the tumour size to enable the tumour to be removed surgically, reducing the risk of metastasis and improving survival [7]. In the field of kidney cancer, presurgical or neoadjuvant studies have been limited because of poor responses in the primary tumour and significant toxicity associated with treatment [6]. A few recent publications demonstrate promising results with neoadjuvant antiangiogenic therapy including partial remission in the primary tumour [6–8].

Endostatin (ES) is a natural occurring, antiangiogenic peptide that has been shown to inhibit endothelial cell proliferation, migration, invasion, and tube formation. In addition, ES treatment has dramatically reduced tumour growth and metastasis in several mouse models with no serious side effects [9,10]. Previously, we showed that subcutaneous injection of ES-transduced cells resulted in a significant antitumour effect in a murine model of mRCC. A histological analysis of ES-treated tumours showed a decrease in microvascular density, necrosis, and foci of apoptotic

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cells, which was associated with leukocyte infiltration [10,11]. In this study, we analysed the efficacy of retroviral ES therapy as a neoadjuvant therapy for RCC using an orthotopic metastatic animal model of RCC.

2. Material and methods

2.1. Cell lines

The NIH/3T3-LendSN-clone 3 was utilised for ES expression [16]. The mock-transduced cell line (NIH/3T3-LXSN) served as the negative control. The cells were maintained in DMEM with high glucose content (4.5 g/L at 25 mM) and supplemented with 100 U/ml penicillin, 50 mg/ml streptomycin, and 10% foetal bovine serum (Life Technologies).

Renca, a murine cell RCC line of spontaneous origin derived from a BALB/c mouse, was kindly donated by Dr Isaiah Fidler, D.V.M., Ph.D. (University of Texas M. D. Anderson Cancer Center, USA). The cells were maintained in RPMI supplemented with 10% foetal bovine serum (Life Technologies) and buffered with fresh 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin, and 100 mg/ml streptomycin.

Both cell lines were maintained in a humidified chamber at 37 °C in an atmosphere containing 5% CO_2.

2.2. Animals

Male BALB/c mice (10 to 12 weeks old) were obtained from the Animal Facility of IPEN/CNEN-SP, São Paulo, Brazil. Mice were kept in a pathogen-free isolator unit and were fed autoclaved food and water. All animals were maintained on a daily 12-hr light/dark cycle.

2.3. Orthotopic metastatic RCC tumour model

Renca cells were harvested by trypsinisation, counted, and resuspended in sterile phosphate-buffered saline (PBS). An aliquot of the cell suspension was removed, and viability was tested by trypan blue dye exclusion. Cell viability was 90% or greater.

Mice were anesthetised by subcutaneous injection of a mixture of ketamine and xylazine (100 mg/kg and 10 mg/kg accounting to body weight, respectively). The left kidney was exposed through a left-flank incision and was partially exteriorised. Using a Hamilton's syringe with a 27-gauge needle, 2×10^5 Renca cells in 10 μl of PBS were injected under the renal capsule. The kidney was then allowed to fall back into the abdominal cavity. The body wall and the skin incision were closed separately with absorbable 5-0 vicryl sutures.

2.4. Neoadjuvant therapy

After three days, the animals were randomly divided into control and ES-treated groups (10 mice/group). The control group mice received 3.6×10^6 NIH/3T3-LXSN cells, and the ES-treated group received 3.6×10^6 NIH/3T3-LendSN cells (ES production level = 1.36 μg/mL). Cells were inoculated, subcutaneously, in the right hind flank of the mouse. [12]. After ten days of treatment, nephrectomy was performed. To perform the nephrectomy, the left kidney was accessed, the renal hilus was ligated using a 4-0 silk suture, and extirpation was performed. Seven days after nephrectomy, the animals were euthanized and exsanguinated.

2.5. Postsurgical therapy

The left kidney was removed by unilateral nephrectomy 7 days after Renca cells inoculation. The animals were randomly divided into two groups of ten mice each: one of them was the control, and the other received a subcutaneous inoculation of 3×10^6 NIH/3T3-LendSN cells. Ten days after nephrectomy, the animals were euthanized and exsanguinated.

All experiments were performed in accordance with the local institutional guidelines for animal care approved by the Animal Experimentation Ethics Committee.

For both pre- and postsurgical assay in the survival studies, ten mice/group were monitored daily until they died.

2.6. Histological analysis

Kidneys and lungs were fixed with methacarn (60% methanol, 30% chloroform, and 10% glacial acetic acid).

Histological analysis was performed on 4 μm sections stained with hematoxylin and eosin (HE).

For immunohistological analysis, sections (4 μm thick) were cut and dehydrated, and antigen retrieval was achieved by heating in citrate buffer (0.01 M, pH 6.0) for 15 minutes. Non-specific binding of antibody was blocked with Tris buffered saline, 5% BSA, and 0.1% Triton X-100 (Sigma-Aldrich) for 30 minutes. The slides were incubated overnight in a wet chamber at 4 °C with anti-mouse CD34 (MEC 14.7, Santa Cruz, CA, USA, 1:50) and anti-PCNA antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, 1:100). Antibody binding was detected by incubating the slides with a biotinylated rabbit anti-mouse immunoglobulin (Ig) G F(ab)2 antibody fragment for 40 minutes (Dako, Glostrup, Denmark) followed by incubation with a peroxidase-conjugated streptavidin-biotin complex for 40 minutes (Dako). The slides were developed using a diaminobenzidine-based detection system (Dako) and subsequently imaged.

The images were obtained with a DXM1200F digital camera (Nikon Instruments Inc., Melville, NY, USA) and analysed using the EclipseNet software for Nikon cameras.

2.7. Morphometry

Metastatic tumour nodules in the lungs were counted microscopically (five sections of ten mice per group).

The quantification of the primary tumour area was done in HE staining sections at 4× magnification using the using the ImageJ software.

The vascular and nodule areas as well proliferating cell nuclear antigen (PCNA) staining cells were quantified as follows: grids were projected on random fields of lung sections at a 40× magnification, and the number of vessels, tumour nodules or PCNA staining cells within the grids were counted. Twenty fields were counted per tumour. Data were expressed as the relative area of blood vessels within tumour parenchyma.

2.8. ELISA analysis

Serum ES level was measured obtained from all treatment groups using a Mouse Endostatin ELISA Kit (USCN Life Science & Technology Company, Double Lake, MO) according to the manufacturer's instructions.

2.9. Statistical analysis

Statistical analysis was performed using the Student test (parametric analysis), and the results are expressed as mean ± SE. For non-parametric analysis, the Mann-Whitney test was used, and the data are expressed as medians [25%–75%]. The differences in the incidence were evaluated by the X^2 test. A probability (P) value of less than 0.05 was considered statistically significant. Survival curves were obtained using the Kaplan–Meier method, and differences between groups were evaluated using the Log-Rank

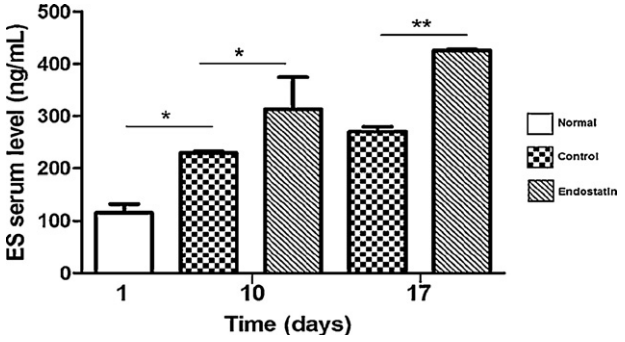

Fig. 1. ES serum levels of the control and ES-treated groups were measured just before Renca cells inoculation (1st day of assay) on the day of nephrectomy (10th day of assay) and at the end of the experiment (17th day of assay). The presence of tumour cells led to an increase in endogenous ES levels, as measured on the day of nephrectomy (day 10 – control vs. normal $P < 0.05$; day 17 – control vs. normal $P < 0.05$). Neoadjuvant subcutaneous inoculation of NIH/3T3-LendSN cells (ES-treated group) resulted in a significant increase in circulating ES levels in the ES-treated group compared with the control group on both day 10 and day 17 (day 10 – control vs. normal $P < 0.05$; day 17 – control vs. normal $P < 0.01$) (ANOVA).

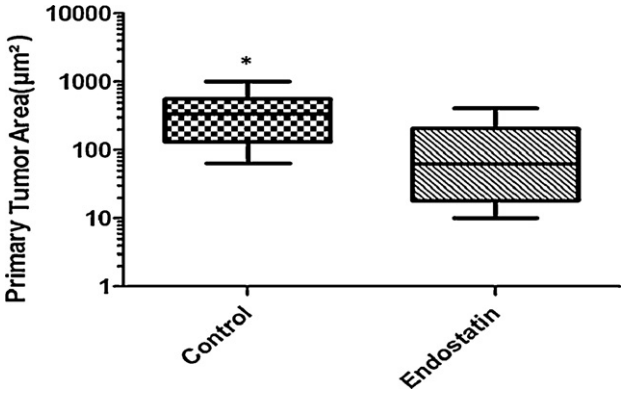

Fig. 2. Effect of neoadjuvant ES treatment on primary tumor area. The tumor area in the ES-treated group was significantly smaller than in the control group ($^{*}P < 0.05$) (Mann-Whitney test).

test. Excel 2007, GraphPad Prism 4.0 for Windows® (GraphPad® Software, San Diego, CA, USA) and Sigmastat 1.0 were used to perform these analyses.

3. Results

ES serum levels of the control and ES-treated groups were measured just before Renca cells inoculation (1st day of assay) on the day of nephrectomy (10th day of assay) and at the end of the experiment (17th day of assay). The presence of tumour cells led to an increase in endogenous ES levels, as measured on the day of nephrectomy (day 10 – control vs. normal $P < 0.05$; day 17 – control vs. normal $P < 0.05$). However, presurgical subcutaneous inoculation of NIH/3T3-LendSN cells (ES-treated group) resulted in a significant increase in circulating ES levels in the ES-treated group compared with the control group on both day 10 and day 17 (day 10 – control vs. normal $P < 0.05$; day 17 – control vs. normal $P < 0.01$) (Fig. 1).

3.1. Histopathological findings in the primary tumour after neoadjuvant ES therapy

ES treatment resulted in a significant reduction in the primary tumour area. As shown on Fig. 2, the median tumour area in the ES-treated group was $121.06 \pm [10.2–410.41]$ μm^2, and the median tumour area in the control group was $380.62 \pm [89–1001.41]$ μm^2 (control vs. treatment group, $P < 0.05$). As indicated on Fig. 3, the reduction in the primary tumour area in mice treated with NIH/3T3-LendSN cells was accompanied by a decrease in the microvascular area. MVA in the ES-treated group was significantly lower than that of the control group, $13.7.0 \pm 0.66$ μm^2 and 20.3 ± 1.76 μm^2,

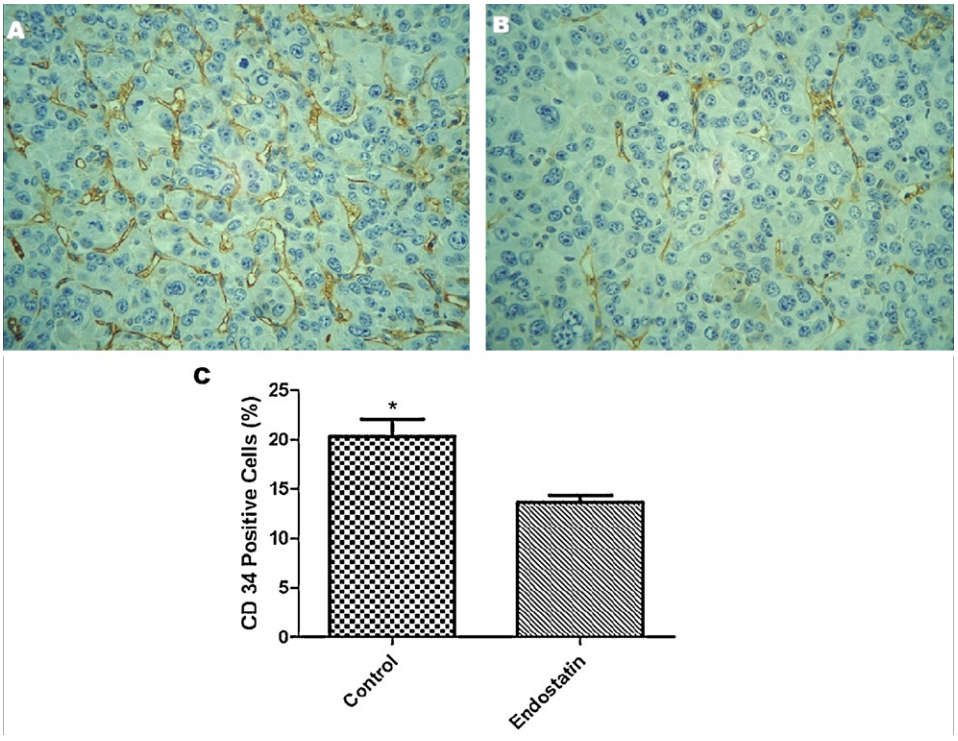

Fig. 3. CD34 immunofluorescence staining from primary tumors of control and ES-treated groups.
Representative photomicrographs of immunofluorescent stained sections of primary tumors from control group (A) and ES-treated mice (B). Tumor angiogenesis was quantified and represented as the percentage of positive CD34 staining using ImageJ software (C). ($^{*}P < 0.05$, Student's *t*-test) (magnification: 40×).

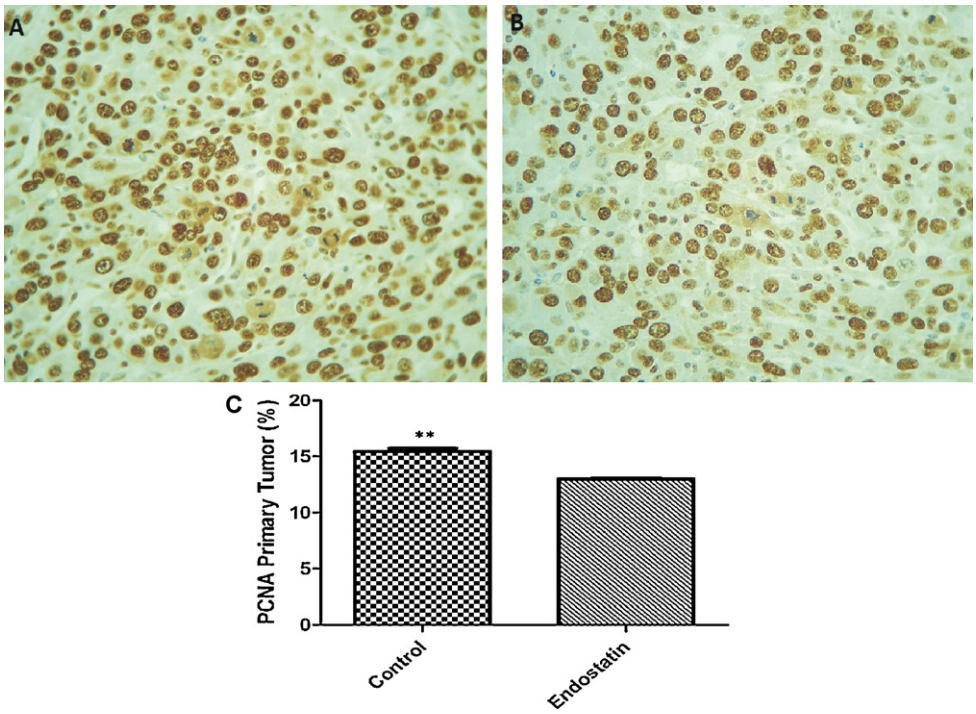

Fig. 4. PCNA immunofluorescence staining from primary tumors of control and ES-treated groups.
A. Control group: large number of PCNA positive cells. B. ES-treated group: decrease in PCNA staining in the primary tumour compared with the control group. C. Percentage of PCNA positive cells relative to primary tumour area in both the control and treated group ($^{**}P < 0.01$, Student's *t*-test) (magnification: 40×).

respectively ($P < 0.05$) (Fig. 3A–C). An analysis of the blood vessels in the tumour revealed an increase neovascularisation in the tumours of control group whereas neovascularisation was reduced and blood vessel abnormalities were detected in the ES-treated group (Fig. 3A,B).

An analysis of proliferating cells *in situ*, using PCNA antibody (Fig. 4A,B), indicated a significant decrease in PCNA positive cells within the tumours from the ES-treated animals ($P < 0.01$). A quantitative analysis of these sections showed that $7.45 \pm 0.96\%$ of the cells expressed PCNA in the ES-treated groups compared with $13.70 \pm 0.47\%$ in the control group (Fig. 4).

3.2. Reduced lung metastasis in ES-treated mice

In the ES-treated group, the incidence of lung metastasis was 13 out of 14 mice (92.8%) whereas 14 out of 14 mice (100%) in the control group had lung metastasis. There was no statistically significant difference between the groups ($P = 0.554$). However, neoadjuvant ES therapy led to a significant reduction in nodule tumour area compared with the control group ($P < 0.01$) (Fig. 5).

3.3. Survival of mice bearing RCC pulmonary metastasis

In the survival assay, mice (ten per group) were monitored daily until they died. The median survival time was 28 days for the control group and 38 days for the ES-treated group. The Kaplan–Meier survival curve demonstrated that the probability of survival was significantly higher for mice treated with ES therapy ($P = 0.0243$, Log-Rank test) (Fig. 6).

4. Discussion

The recurrence rates in patients with localised RCC ranges from 35% to 65% [13,14]. On the basis of the efficacy seen in patients

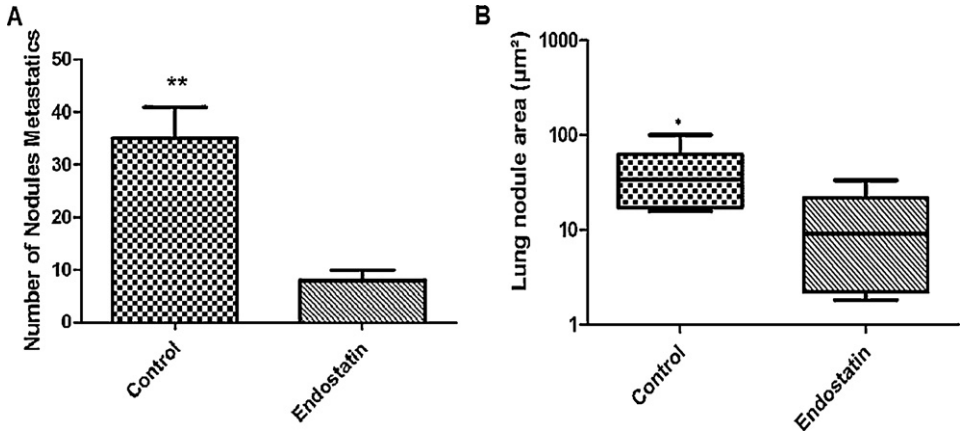

Fig. 5. The effect of neoadjuvant ES therapy on mRCC.
A. Number of metastatic nodules in the ES-treated group were significantly smaller than the control group ($^{*}P < 0.01$). (Student's *t*-test). B. Lung nodule area in the ES-treated group was significantly smaller than in the control group ($^{*}P < 0.05$) (Mann-Whitney).

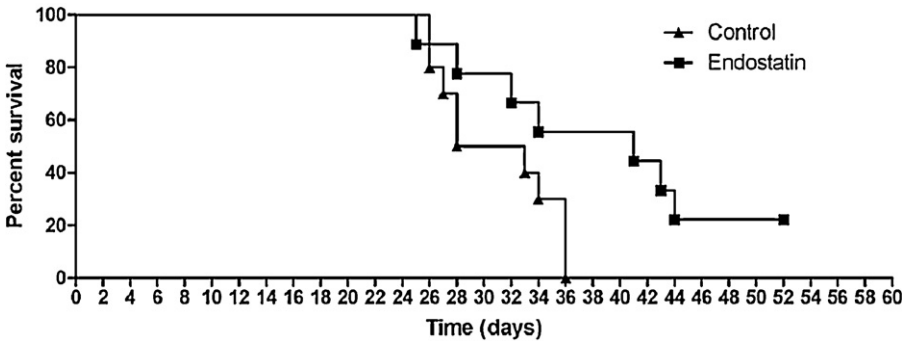

Fig. 6. Survival curves of tumour-bearing mice, were monitored daily until they died. Kaplan–Meier survival curves confirmed a strong correlation between neoadjuvant ES therapy and longer survival ($P = 0.0243$, Log-Rank test).

with metastatic disease, it has been proposed that neoadjuvant therapy with targeted agents may delay disease progression and improve overall survival [6–14,15].

In this work, we used an experimental animal model that mimics the clinical disease to evaluate the potential effectiveness of neoadjuvant ES therapy in the treatment of RCC.

In this model, it was observed that the presence of primary RCC resulted in an increase in plasma ES levels. Feldman et al. have shown that serum levels of ES and vascular endothelial growth factor (VEGF) are elevated in patients with RCC [16]. NIH/3T3-LendSN cells implanted in tumour-bearing animals induce a sustained increase in circulating levels of ES reaching a maximum level of 426 ng/ml at the end of the assay. Impairment of primary tumour growth was observed 7 days after treatment with NIH/3T3-LendSN cells, which provided exogenous ES. We also observed a decrease in tumour size with continued treatment. Our group, and others, have shown that exogenous ES can successfully control the growth of RCC *in vivo* [10–18].

ES treatment in mice with orthotopically transplanted Renca cells resulted in a 32% reduction of the vasculature. Other studies have also demonstrated the antiangiogenic and antitumour properties of ES in a wide variety of human and murine primary and metastatic tumours, as reviewed by Folkman et al. [19]. Reduced PCNA staining indicates that ES therapy has a significant antiproliferative effect within the tumour microenvironment. The resulting changes in the tumour microenvironment may impair invasion and metastasis of the tumour cells halting metastatic progression. These changes could explain the reduction in the number and area of lung metastatic nodules and more specifically the overall survival of ES-treated mice. The remarkable survival benefit seen with ES therapy could also be related to the lack of toxicity seen with ES treatment [19].

To date, few experimental and preclinical studies have evaluated antiangiogenic drugs in a neoadjuvant setting to treat RCC. Therefore, our results could contribute to the development of a new RCC treatment strategy.

In conclusion, the neoadjuvant ES gene therapy led to the reduction of primary tumor mass and the decrease of tumor cells escape into the blood circulation. These factors not only decreased tumour burden but also extended the animal survival. Altogether, neoadjuvant ES gene therapy may have clinical benefit in the management of RCC.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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