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Original article

Vascular endothelial growth factor as a biomarker for endostatin gene therapy

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ABSTRACT

Renal cell carcinoma (RCC) is characterized by high vascular endothelial growth factor (VEGF) production and, consequently, excessive angiogenesis. Several strategies have been developed to target angiogenesis as a method for treating metastatic RCC (mRCC). Endostatin (ES) is a C-terminal fragment of collagen XVIII that has antiangiogenic activity. The aim of this study was to investigate the predictive value of circulating VEGF-A in a murine model of mRCC after ES gene therapy. ES therapy did not affect the levels of collagen XVIII/ES or ES in the tissue. The circulating level of ES was increased in the control and ES-treated groups (normal vs. control, $P < 0.05$ and ES-treated vs. control, $P < 0.001$), and the intratumoral vessels were significantly decreased (ES-treated vs. control, $P < 0.05$). ES therapy decreased the VEGF mRNA levels. The tissue and circulating levels of VEGF in the control group were significantly higher than normal ($P < 0.01$ and $P < 0.05$, respectively). Treatment with ES significantly reduced the VEGF concentrations in both compartments ($P < 0.001$ for tissue and $P < 0.05$ for plasma). Our findings indicate that in addition to the directly targeted tumor vessels, ES exerts its antitumor effect by down-regulating *VEGF* gene expression in renal tumor cells. Additionally, our findings point to the predictive value of VEGF for ES therapy.

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1. Introduction

Angiogenesis is the growth of new vessels from a pre-existing blood supply and is essential for the growth and metastasis of solid tumors [1]. To promote angiogenesis, tumors upregulate the proangiogenic cytokine production. However, they also generate angiogenesis inhibitors, and decreased expression of these endogenous antiangiogenic factors is another important feature of tumor angiogenesis [2,3].

Renal cell carcinoma (RCC) is a group of malignances comprising different histological subtypes. Clear cell renal cell carcinoma (ccRCC) is the most frequent renal tumor subtype, accounting for 70% of cases. Inactivation of the von Hippel-Lindau (VHL) tumor suppressor via mutation, hypermethylation, and loss of heterozygosity frequently occurs in ccRCC [4]. VHL protein (pVHL) is a part of an E3 ubiquitin ligase complex that ubiquitylates the hypoxia inducible factor α (HIFα) subunit, leading to its rapid degradation. Loss of the VHL protein prevents the VHL complex from targeting HIF for degradation, resulting in accumulated HIF. An increased level of HIF results in the increased transcription of its downstream targets, including platelet-derived growth factor (PDGF) β, carbonic anhydrase 9 (CA9), transforming growth factor (TGF) α and vascular endothelial growth factor (VEGF or VEGF-A) [4–6]. VEGF is the most important angiogenic factor associated with inducing and maintaining the neovasculature in tumors, thereby exerting its mitogenic effect by binding to its receptor, VEGFR2 (human KDR; mouse Flk-1) [7].

Over the last decade, there has been a better understanding of the molecular pathways involved in RCC pathogenesis, which result in the development of VEGF (the therapeutic agents target) and the mammalian target of the rapamycin (mTOR) pathway. Receptor tyrosine kinase inhibitors ([TKIs], sorafenib, sunitinib, pazopanib and axitinib), VEGF antibodies (bevacizumab) and mTOR inhibitors (everolimus, temsirolimus) were approved to treat metastatic kidney cancer [8].

In oncology, a biomarker is a biological indicator of the disease, a tool for patient prognosis and treatment response [9]. A prognostic biomarker provides information about the progression of a disease without considering any treatment. Conversely, a predictive biomarker can provide information about the effect of a specific treatment [10].

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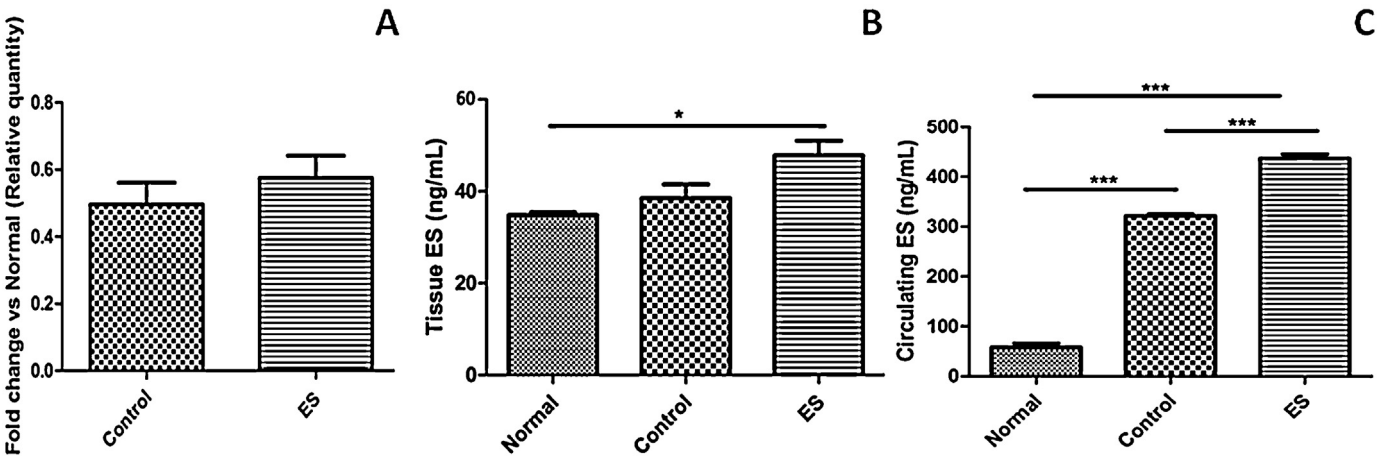

Fig. 1. Tissue and circulating endostatin profile in a metastatic renal cell carcinoma murine model. The endostatin mRNA expression in metastatic lungs was examined using RT-qPCR. The results are shown as the folds increase relative to normal levels following normalization against a housekeeping gene. A. Endostatin gene expression was not modulated by the endostatin treatment ($P = 0.3168$). B. The lung endostatin concentration was significantly higher than that of normal lungs (normal vs. endostatin-treated $P < 0.05$). C. Circulating endostatin in the control group showed a pronounced increase compared with the normal group (normal vs. control groups, $P < 0.001$); in the endostatin-treated mice the plasma levels of endostatin were significantly higher than that of the control mice (control vs. endostatin-treated mice, $P < 0.001$).

In RCC, VEGF has been considered a prognostic biomarker because plasma VEGF levels have been associated with shorter survival. However, its predictive value is unclear [11,12].

Endostatin (ES) is a cleavage product from the C-terminal portion of collagen XVIII, which has been shown to exhibit antiangiogenic activity and has been effective in the experimental treatment of diseases with neovascularization; it is also involved in the pathogenesis of some diseases, such as arthritis, diabetic nephropathy and tumor growth. *In vitro*, ES has shown many biological activities, including inhibiting endothelial cell migration and inducing and proliferating apoptosis [13–16]. The apoptotic effect of ES is associated with decreased levels of anti-apoptotic proteins, such as Bcl-2 and Bcl-xl [17]. It was demonstrated that soluble ES inhibits endothelial cell migration by binding to integrin $\alpha 5$ and αv [18]. Oligomeric ES can also bind to heparan sulfate on cell surface to regulate morphogenesis and cell migration. Kim et al. demonstrated that the inhibition of endothelial proliferation and migration is caused by the direct interaction of ES with KDR/Flk-1 receptor [19].

In vivo, the antitumor activity of ES has been demonstrated in different tumor types [20–22]. Over the last 10 years our group has demonstrated that ES gene therapy resulted in increased levels of plasma ES and in significant antitumor effects in a murine model of primary and metastatic RCC [23–25]. Treated tumors showed a decreased microvascular density, reduced nodule areas and a decreased proliferation of tumor cells. In addition, tumor-bearing treated mice showed a significantly prolonged survival rate [26].

The aim of this work was to evaluate the potential of VEGF as a predictive biomarker for ES therapy.

2. Results

Balb/c mice bearing mRCC were treated with ES for 10 days, and then the ES and VEGF levels of mRNA and protein were assessed.

2.1. Tissue and circulating endostatin profile in a metastatic renal cell carcinoma murine model

The RT-qPCR revealed that the ES mRNA expression in the metastatic lungs was not modulated by the ES treatment (control vs. ES-treated groups, $P = 0.3168$) (Fig. 1A). ES concentration in the

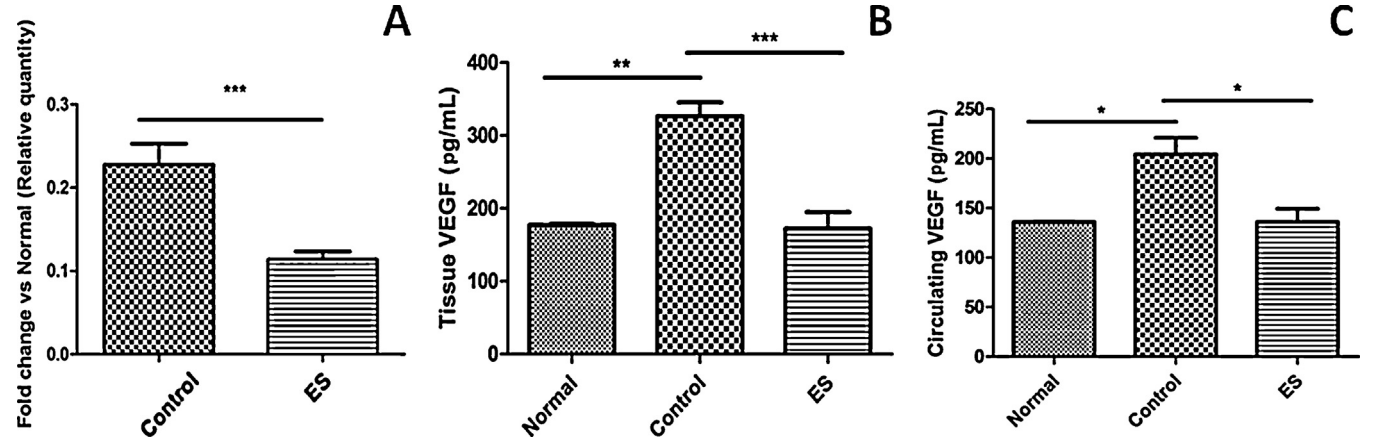

Fig. 2. Tissue and circulating vascular endothelial growth factor profile in a metastatic renal cell carcinoma murine model. The expression of the vascular endothelial growth factor gene in metastatic lungs was examined using RT-qPCR. The results are shown as the folds increase relative to normal following normalization against a housekeeping gene. A. The expression of mRNA vascular endothelial growth factor decreased after the endostatin treatment ($P < 0.001$, Student's *t*-test). B. The levels of vascular endothelial growth factor in the lungs of mice in the control group was significantly increased (normal vs. control groups, $P < 0.01$), and after the endostatin treatment, these levels were reduced (control vs. endostatin-treated groups, $P < 0.001$). C. Compared to normal mice, the circulating levels of vascular endothelial growth factor in the control group increased (normal vs. control groups, $P < 0.05$), and the endostatin treatment led to a significant reduction in the plasma vascular endothelial growth factor concentration (control vs. endostatin-treated groups, $P < 0.05$, analysis of variance [ANOVA]).

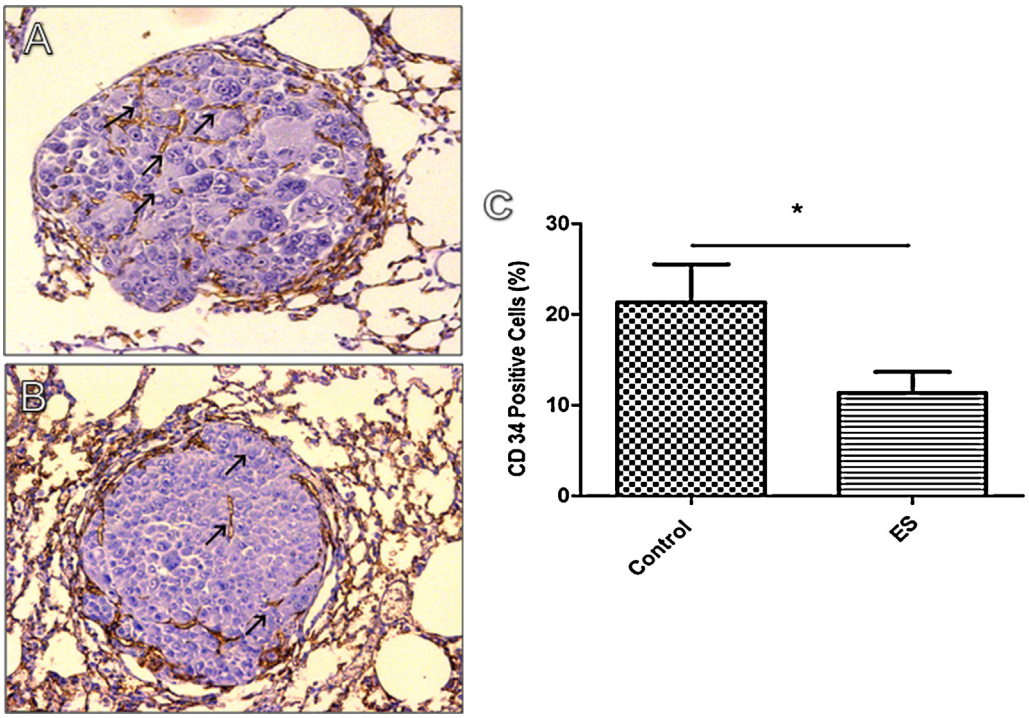

Fig. 3. CD34 immunohistochemistry and blood vessel quantification in nodules from the controls and endostatin-treated animals. CD34 immunostaining (20×) of endothelial cells and representative vessels in sections (indicated arrow) from the control (A) and endostatin-treated groups (B). (C) Blood vessel quantification in tumor tissues was performed using ImageJ software (http://rsb.info.nih.gov/ij/). The percentage of CD34-positive cells reduced significantly after the endostatin therapy. Bars, means ± SE (control vs. endostatin-treated groups, $P < 0.05$, Student's test-*t*).

lungs of control mice was similar to that presented in the lung of normal mice. However, tissue ES levels were significantly increased in the ES-treated group compared to the normal group (normal vs. ES-treated, $P < 0.05$) (Fig. 1B).

Circulating ES levels were also measured, and the control group showed a pronounced increase in the endogenous ES concentration. The presence of tumors cells lead to a 3-fold increase in the ES levels (normal vs. control groups, $P < 0.001$). As expected, the

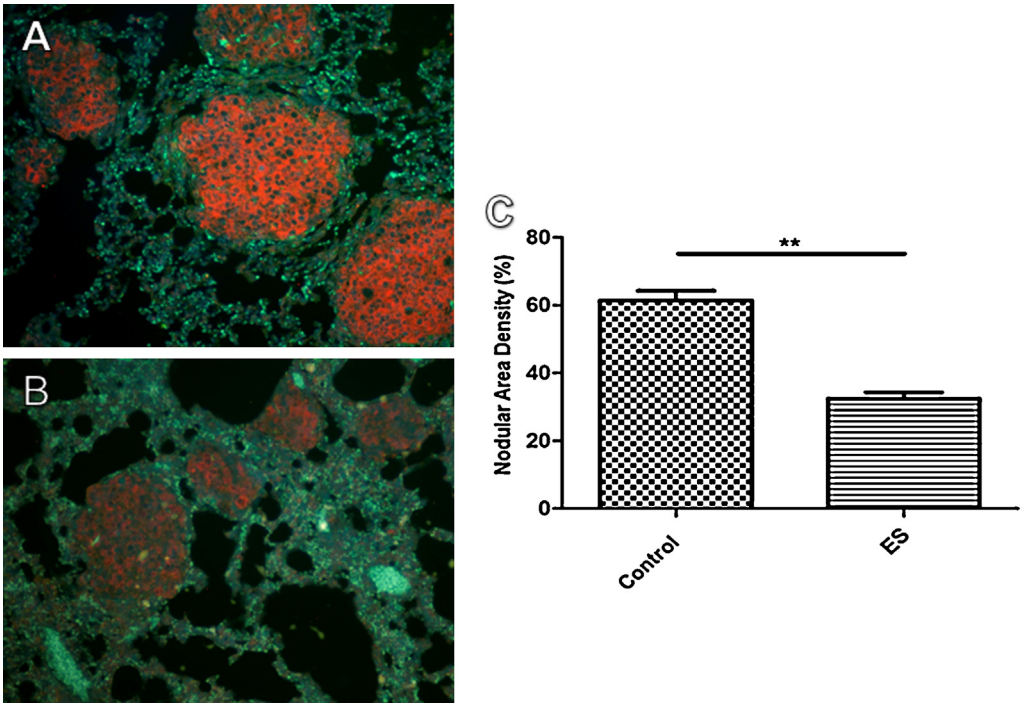

Fig. 4. Vascular endothelial growth factor immunofluorescence staining in the lung tissues from mice in each experimental group. Representative sections are shown. A. Intense cytoplasmic vascular endothelial growth factor labeling (red) was observed in the tumor cells of the control mice. B. Vascular endothelial growth factor staining decreased markedly after the endostatin treatment. C. A semi-quantitative analysis was performed using ImageJ software. Original magnification (20×). Bars, means ± SE (control vs. endostatin-treated groups, $P < 0.01$, Student's *t*-test).

subcutaneous inoculation of NIH/3T3-LendSN-clone 3 cells resulted in a significant increase in circulating ES levels in the ES-treated group (control vs. ES-treated, $P < 0.001$) (Fig. 1C).

2.2. Tissue and circulating vascular endothelial growth factor profile in an metastatic renal cell carcinoma murine model

The RT-qPCR revealed that VEGF gene expression was modulated by the presence of ES gene therapy. On average, VEGF mRNA expression decreased nearly 2.0-fold in response to the ES treatment (control vs. ES-treated mice, $P < 0.001$) (Fig. 2A).

In the lung tissue of the control group, the VEGF levels increased by approximately 84% compared to the normal group (normal vs. control groups, $P < 0.01$). As a result of the ES treatment, the VEGF levels decreased, nearly returning to levels similar to that of the normal group (ES-treated vs. control mice, $P < 0.001$) (Fig. 2B).

The circulating VEGF levels of the normal, control, and the ES-treated groups were impacted by the presence of metastatic tumors; these results were similar to those obtained in lung tissue (normal vs. control groups, $P < 0.01$; control vs. ES-treated mice, $P < 0.001$) (Fig. 2C).

2.3. Histological analysis

To determine the extent of angiogenesis, lung tissues were also stained with antibodies against the endothelial marker CD34. As indicated on Fig. 3, ES-treatment resulted in a reduced number of intratumoral vessels (Fig. 3A, 3B). A quantitative analysis showed a decrease of approximately 60% in the tumor microvessels (Fig. 3C).

The expression levels of VEGF in the metastatic lungs were also assessed using immunofluorescence analysis. Cytoplasmic fluorescence staining corresponding to the VEGF expression was observed in the lung tumor cells from both the control and treated groups. Metastatic cancer cells from the control group showed strong and positive VEGF staining (Fig. 4A). Semi-quantitative immunofluorescence analysis of these sections showed a reduction of approximately 66% in the VEGF expression, as previously shown using the ELISA (Fig. 4B).

3. Discussion

Tumor growth is dependent on angiogenesis, which is controlled by proangiogenic and antiangiogenic molecules. VEGF is a central regulator of tumor angiogenesis and is highly expressed in many types of tumors, including RCC [27–31]. Thus, both angiogenesis and VEGF have been confirmed as targets of anticancer therapeutics [32].

The use of molecular targeted drugs has changed the therapeutic practice and also brought many challenges to oncology. One challenge is finding biomarkers.

In the last 10 years, our group has been working with ES in treating an experimental model of mRCC, with promising results [23–25]. In this study, using the same animal model, we sought to determine the predictive value of circulating VEGF.

ES treatment showed interference neither in the collagen XVIII/ES gene expression nor in the lung ES protein expression. Moreover, corroborating our previous data, the ES plasma levels increased significantly in the control group and, as expected, in the ES-treated group. This profile indicates that the increased plasma levels of endogenous ES in the control group results from cleavage of collagen XIII by metalloproteinases (MMPs) and proteases and its release into the circulation [33]. The expression of MMPs, such as MMP-2 and MMP-9, correlated with tumor progression [34]. Additionally, elevated serum levels of ES endogenous and VEGF correlated with stage IV clear cell renal cancer [31]. The increased plasma levels of endogenous ES did not control the tumor growth. However, the implantation of engineered NIH/3T3 cells producing the ES fragment independently of proteolytic activation (recombinant ES) displayed antitumor action.

The increased tissue and circulating VEGF levels in the control group can justify high the intratumoral vascularization. Increased VEGF expression, either in the circulation or in tumor tissue, was found to correlate with worse prognoses [12]. Exogenous ES treatment caused a markedly reduced expression of VEGF-A mRNA in the metastatic lungs of the ES-treated groups. The down-regulation of VEGF expression by ES has been demonstrated in malignant murine keratinocytes and EF43.fgf-4 mammary carcinoma, indicating a direct effect of ES on tumor cells [35]. Circulating VEGF levels displayed a similar trend in tissue. This evidence shows that circulating VEGF could be directly derived from tumor tissues, thereby reflecting the biological activity of mRCC.

In summary, in mRCC, the antitumor effect of ES therapy seems to be mediated by both the reduction of VEGF gene expression by renal tumor cells and antiangiogenic activity. Additionally, our findings point to the predictive value of VEGF in ES therapy.

Disclosure of interest

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

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References

[1] Dewing D, Emmett M, Pritchard Jones R. The roles of angiogenesis in malignant melanoma: trends in basic science research over the last 100 years. ISRN Oncology 2012;546927.
[2] O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 1997;88:277–85.
[3] Sato Y. Molecular diagnosis of tumor angiogenesis and anti-angiogenic cancer therapy. Int J Clin Oncol 2003;8:200–6.
[4] Banks RE, Tirukonda P, Taylor C, Hornigold N, Astuti D, Cohen D, et al. Genetic and epigenetic analysis of von Hippel-Lindau (VHL) gene alterations and relationship with clinical variables in sporadic renal cancer. Cancer Res 2006;66:2000–11.
[5] Linehan WM, Bratslavsky MDG, Pinto PA, Schmidt LS, Neckers L, Bottaro D, et al. Molecular diagnosis and therapy of kidney cancer. Annu Rev Med 2010;61:329–43.
[6] Vaziri SAJ, Tavares ELJ, Golshayan AR, Rini BI, Aydin H, Zhou M, et al. Differing von Hippel-Lindau genotype impaired primary and metastatic tumors in patients with clear cell renal cell carcinoma. Front Oncol 2012;2:51.
[7] Kim YM, Hwang S, Kim YM, Pyun B, Kim TY, Lee ST, et al. Endostatin blocks VEGF-mediated signaling via direct interaction with KDR/Flk-1. J Biol Chem 2002;277:27872–9.
[8] Finley DS, Pantuck AJ, Belldegrun AS. Tumor biology and prognostic factors in renal cell carcinoma. Oncologist 2011;16(Suppl. 2):4–13.
[9] Murukesh N, Dive C, Jayson GC. Biomarkers of angiogenesis and their role in the development of VEGF inhibitors. Br J Cancer 2010;102(1):8–18.
[10] La Thangue NB, Kerr DJ. Predictive biomarkers: a paradigm shift towards personalized cancer medicine. Nat Rev Clin Oncol 2011;8(10):587–96.
[11] Djordjevic G, Mozetic V, Mozetic DV, Licul V, Ilijas KM, Mustac E, et al. Prognostic significance of vascular endothelial growth factor expression in clear cell renal cell carcinoma. Pathol Res Pract 2007;203:99–106.
[12] Otrock ZK, Hatoum HA, Musallam KM, Awada AH, Shamseddine AI. Is VEGF a predictive biomarker to anti-angiogenic therapy? Crit Rev Oncol/Hematol 2011;79:103–11.
[13] Hanai J, Gloy J, Karumanchi SA, Kale S, Tang J, Hu G, et al. Endostatin is a potential inhibitor of Wnt signaling. J Cell Biol 2012;158(3):529–39.
[14] Yin G, Liu W, An P, Li P, Ding I, Planelles V, et al. Endostatin gene transfer inhibits joint angiogenesis and pannus formation in inflammatory arthritis. Mol Ther 2002;5(5):547–54.
[15] Ichinose K, Maeshima Y, Yamamoto Y, Kitayama H, Takazawa Y, Hirokoshi K, et al. Antiangiogenic endostatin peptide ameliorates renal alterations in the early stage of a type 1 diabetic nephropathy model. Diabetes 2005;54(10):2891–903.

[16] Digtyar AV, Pozdnyakova NV, Feldman NB, Lutsenko SV, Severin SE. Endostatin: current concepts about its biological role and mechanisms of action. Biochemistry (Mosc) 2007;72(3):235–46.
[17] Dhanabal M, Ramchandran R, Waterman MJ, Lu H, Knebelmann B, Segal M, et al. Endostatin induces endothelial cell apoptosis. J Biol Chem 1999;274(17):11721–6.
[18] Rehn M, Veikkola T, Kukk-Valdre E, Nakamura H, Ilmonen M, Lombardo C, et al. Interaction of endostatin with integrins implicated in angiogenesis. Proc Natl Acad Sci U S A 2001;98(3):1024–9.
[19] Kim YM, Hwang S, Kim YM, Pyun BJ, Kim TY, Lee ST, et al. J Endostatin blocks vascular endothelial growth factor-mediated signaling via direct interaction with KDR/Flk-1. Biol Chem 2002;277(31):27872–9.
[20] Coutinho EL, Andrade LNS, Chammas R, Schor N, Bellini MH. Anti-tumor vector of endostatin mediated by retroviral gene transfer in mice bearing renal cell carcinoma. FASEB J 2007;21:3153–61.
[21] Peng F, Xu Z, Wang J, Chen Y, Li Q, Zuo Y, et al. Human endostatin normalizes tumor vasculature and enhances radiation response in xenografted human nasopharyngeal carcinoma models. PLOS 2012;7(4):e34646.
[22] Wang HL, Ning T, Li M, Lu ZJ, Xi Y, Peng Q, et al. Effect of endostatin on preventing postoperative progression of distant metastasis in a murine lung cancer model. Tumori 2011;97:787–93.
[23] Braga MS, Chaves KCB, Chammas R, Schor N, Bellini MH. Endostatin neoadjuvant gene therapy extends survival in an orthotopic metastatic mouse model of renal cell carcinoma. Biomed Pharmacother 2011;6(4):237–41.
[24] Chaves KCB, Peron JPS, Chammas R, Turaça LT, Pesquero JB, Braga MS, et al. Endostatin gene therapy stimulates upregulation of ICAM-1 and VCAM-1 in a metastatic renal cell carcinoma model. Cancer Gene Ther 2012;19:558–65.
[25] Chaves KCB, Turaça LT, Pesquero JB, Mennecier G, Dagli ML, Chammas R, et al. Fribronectin expression is decreased in metastatic renal cell carcinoma following endostatin gene therapy. Biomed Pharmacother 2012;66(6):464–8.
[26] Rocha FG, Calvo FB, Chaves KC, Peron JP, Marques RF, de Borba TR. Endostatinand interleukin-2-expressing retroviral bicistronic vector for gene therapy of metastatic renal cell carcinoma. J Gene Med 2011;3:148–57.
[27] Schips L, Dalpiaz O, Lipsky K, Langner C, Rehak P, Puerstner P, et al. Serum levels of vascular endothelial growth factor (VEGF) and endostatin in renal cell carcinoma patients compared to a control group. Eur Urol 2007;51:168–74.
[28] McDonald M, et al. Rapid vascular regrowth in tumors after reversal of VEGF inhibition. J Clin Invest 2006;116(10):2610–21.
[29] Zhao J, Yan F, Ju H, Tang J, Qin J. Correlation between serum vascular endothelial growth factor and endostatin levels in patients with breast cancer. Cancer Lett 2004;204:87–95.
[30] Zafirellis K, Agrogiannis G, Zachaki A, Gravani K, Karameris A, Kombouras C. Prognostic significance of VEGF expression evaluated by quantitative immunohistochemical analysis in colorectal cancer M.D. J Surg Res 2008;147(1):99–107.
[31] Feldman AL, Tamarkin L, Paciotti GF, Simpson BW, Linehan WM, Yang JC, et al. Serum endostatin levels are elevated and correlate with serum vascular endothelial growth factor levels in patients with stage IV clear cell renal cancer. Clin Cancer Res 2000;6:4628–34.
[32] Murukesh N, Dive C, Jayson GC. Biomarkers of angiogenesis and their role in the development of VEGF inhibitors. Br J Cancer 2010;102:8–18.
[33] Bellini MH, Malpighi TF, Calvo FB, Miranda AR, Spencer PJ, Cichy MC, et al. Immobilized kidney 28-kDa endostatin-related (KES28kDa) fragment promotes endothelial cell survival. Am J Nephrol 2010;31:255–61.
[34] Qiao ZK, Li YL, Lu HT, Wang KL, Xu WH. Expression of tissue levels of matrix metalloproteinases and tissue inhibitors of metalloproteinases in renal cell carcinoma. World J Surg Oncol 2013;11:1.
[35] Hajitou A, Grignet C, Devy L, Berndt S, Blacher S, Deroanne CF, et al. The antitumoral effect of endostatin and angiostatin is associated with a down-regulation of vascular endothelial growth factor expression in tumor cells. FASEB J 2002;16(13):1802–4.