Int J Clin Exp Med 2016;9(7):13059-13064
www.ijcem.com /ISSN:1940-5901/IJCEM0021941

Original Article

Evaluating the effectiveness of Endostar therapy in human lung cancer xenografts using contrast-enhanced magnetic resonance imaging

Xiaoqin Li[1,2*], Hao Wang[1*], Shuang Ding[3], Hong Wang[1], Yunling Wang[1], Lin Jia[4], Wenxiao Jia[1], Yonghua Xu[5]

[1]Imaging Center, The Second Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang, P. R. China; [2]Affiliated Tumor Hospital of Xinjiang Medical University, Urumqi, Xinjiang, P. R. China; [3]Imaging Center, The First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang, P. R. China; [4]Department of Graduate student, The Second Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang, P. R. China; [5]Shanghai Clinical Center of Chinese Academy of Sciences, Shanghai Xuhui Central Hospital, Shanghai, P. R. China. [*]Equal contributors.

Received December 15, 2015; Accepted June 8, 2016; Epub July 15, 2016; Published July 30, 2016

Abstract: To investigate the feasibility of antiangiogenic effect of Endostar therapy in lung cancer xenografts using contrast-enhanced magnetic resonance imaging (DCE-MRI). Two groups of nude mice bearing human lung carcinoma cells (NCI-H460 cell) were injected with Endostar (experiment group) or phosphate-buffered saline (control group). DCE-MRI was performed on the 7th day after injection, followed by collection of tumor tissues for immunohistochemistry. Tumor volumes in the experimental group were smaller than in the control group (0.249±0.138 vs. 0.448±0.096 cm^3, P=0.032). The mean transfer constant (K^{trans}), extravascular extracellular space fractional volume (V_e), and incremental area under the curve (iAUC) of the experimental group (0.052±0.015/min, 0.280±0.191, and 11.912±7.738, respectively) were found to be lower than those of the control group (0.149±0.026/min, 0.558±0.052 and 36.463±12.534) (all P<0.05), except the rate constant of backflux (K_{ep}) (control: 0.334±0.111/min; experimental: 0.478 ± 0.294/min; P>0.05). Microvessel density (MVD) was lower in the experimental group than in controls (11.36±4.57 vs. 20.44±3.46 vessels/high power field; P<0.05). In conclusion, Endostar could significantly inhibit the development of lung tumor xenografts in mice. DCE-MRI could be used as an effective method in evaluating tumor angiogenesis and the effect of antiangiogenic drugs.

Keywords: DCE-MRI, Endostar, antiangiogenic therapy, NCI-H460, lung cancer

Introduction

There is a close relationship between biological behavior of tumors and angiogenesis. Vascular endothelial growth factor (VEGF) has now become the target of new therapeutic strategies directed against angiogenesis. In this regard, recombinant human vascular endostatin (Endostar®) is used to inhibit the generation of new blood vessels to inhibit tumor growth and metastasis. VEGF plays multi-target anti-angiogenic effect, indirectly leading to tumor dormancy or withdrawal [1].

Endostar is a specific vascular endothelial cell growth inhibiting factor that has direct antiangiogenic effects by inhibiting endothelial cell migration and inducing apoptosis. It also inhibits the expression and activity of proteolytic enzymes of the tumor cell surface. In vitro and in vivo experiments showed that Endostar treatment results in reduction of tumor angiogenesis and decreased microvessel density (MVD) [2, 3]. By adjusting the pro-angiogenic and anti-angiogenic factors, VEGF-A and thrombin-sensitive protein-1 (thrombospondin-1, TSP-1) regulate the new tumor vascular pericytes for more efficient formation of complete basement membrane, thereby promoting tumor vessel maturation and normalization [4]. Huang et al. showed that Endostar treatment in mice with Lewis lung cancer xenografts increased tumor microvessel collagen and stabilized the tumor vasculature [5]. Currently, the evaluation of tumor MVD is mostly performed using fixed paraffin-embedded tissues, but the technique requires

biopsy or surgical specimens, and the methods to obtain those are invasive.

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) is considered to be highly useful to measure hemodynamic parameters and has some advantages like being non-invasive, real time measurements, quantitative evaluation of the effects of antiangiogenic drugs, and provide effective clinical imaging for evaluating and plan treatments [6].

In the present study, DCE-MRI was used to explore the effects of Endostar treatment of lung cancer xenografts in nude mice, compared with MVD as the gold standard index for the evaluation of antiangiogenic drugs.

Material and methods

Animals and cell

Male Balb/c nude mice, 6-week old, weighing 18-22 g, were purchased from the Beijing Research Center Comparative Medicine Branch. The mice were maintained under specific pathogen-free (SPF) conditions in an animal facility and given a standard pelleted regular rodent diet and water ad libitum.

Non-small cell lung cancer NCI-H460 cells were incubated in RPMI-1640 medium with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C and under a 5% CO2 atmosphere.

Establishment of mouse lung cancer model

NCI-H460 cells were injected subcutaneously at 5×10^6 cells/mice (n=10) in the lower limb. The mice were divided into two groups. Tumor cell inoculation was done 10 days before administration of treatment. In the experimental group, 0.2 ml of Endostar (Shandong Harbinger Magenta, Tianjin Pharmaceutical Co., Ltd. P. R. China) was injected every day intraperitoneally for 7 days. In the control group, 0. 2 ml of saline was injected for 7 days.

DCE-MRI scanning and imaging analysis

DCE-MRI was performed on whole animals using a Siemens Magneto Vision (Siemens, Erlangen, Germany) using dedicated MRI coils for small animals. Unenhanced scan was performed using a fast pin echo (TSE) sequence: thickness 2 mm; T2WI coronal and transverse; TR 3000 ms; TE 61 ms; and field of view (FOV) 70 × 70 mm. A T1WI coronal scan was used to determine the anatomical location of the tumor: TR 650 ms, TE 24 ms, and FOV 70 × 70 mm. Ggadolinium dimeglumine (Haibo Lecco Xinyi Pharmaceutical Co., Ltd., Beijing, China) was used as a dynamic contrast agent. The contrast agent (0.1 ml) was used for 3D fast low angle shot sequence (FLASH): TR 5.0 ms, TE 2.0 ms, FOV 55 × 70 mm, 8 layers, and thickness 5 mm. Tumor volume was calculated as: a2b × 0.52, where a is the short axis and b is the long axis.

DCE-MRI data processing and image analysis was done using Tofts model [1]. In this model, a blood dual compartment kinetic model was used to represent the extravascular/extracellular space (EES) and the plasma space after injecting the contrast agent, which quickly reach the two spaces and does not change with time. The model describes quantitative dynamic enhancement parameters including the transfer of contrast agent's constant volume (Ktrans), EES volume percentage (Ve), and speed constant (Kep), according to the equation: Kep = Ktrans/(Ve×Kep). In addition, the area under the curve (iAUC) was calculated under the peak area of concentration. After the scan is complete, the raw data was processed using the Tissue 4D software (available from Siemens) to delineate the tumor. Each cross-section was treated as a unit to select regions of interest (ROIs), displayed as pseudocolor pictures.

Pathological examination

Immunohistochemistry of paraffin-embedded tumor tissue was conducted using mouse CD34 monoclonal antibody [QBEND-10] (Abcam Co., Cambridge, United Kingdom) labeled with DAB (3,3'-2-diaminobenzidinetetrahydrochloride). MVD was measured according to the method reported by Weidner [7]. To determine the tumor vascular density, tumor and its surrounding connective tissue was examined at low magnification (×100) to determine the zone with the highest MVD, and then the vessels were counted at high magnification (×200). The capillary number was counted, which was the tumor tissue MVD.

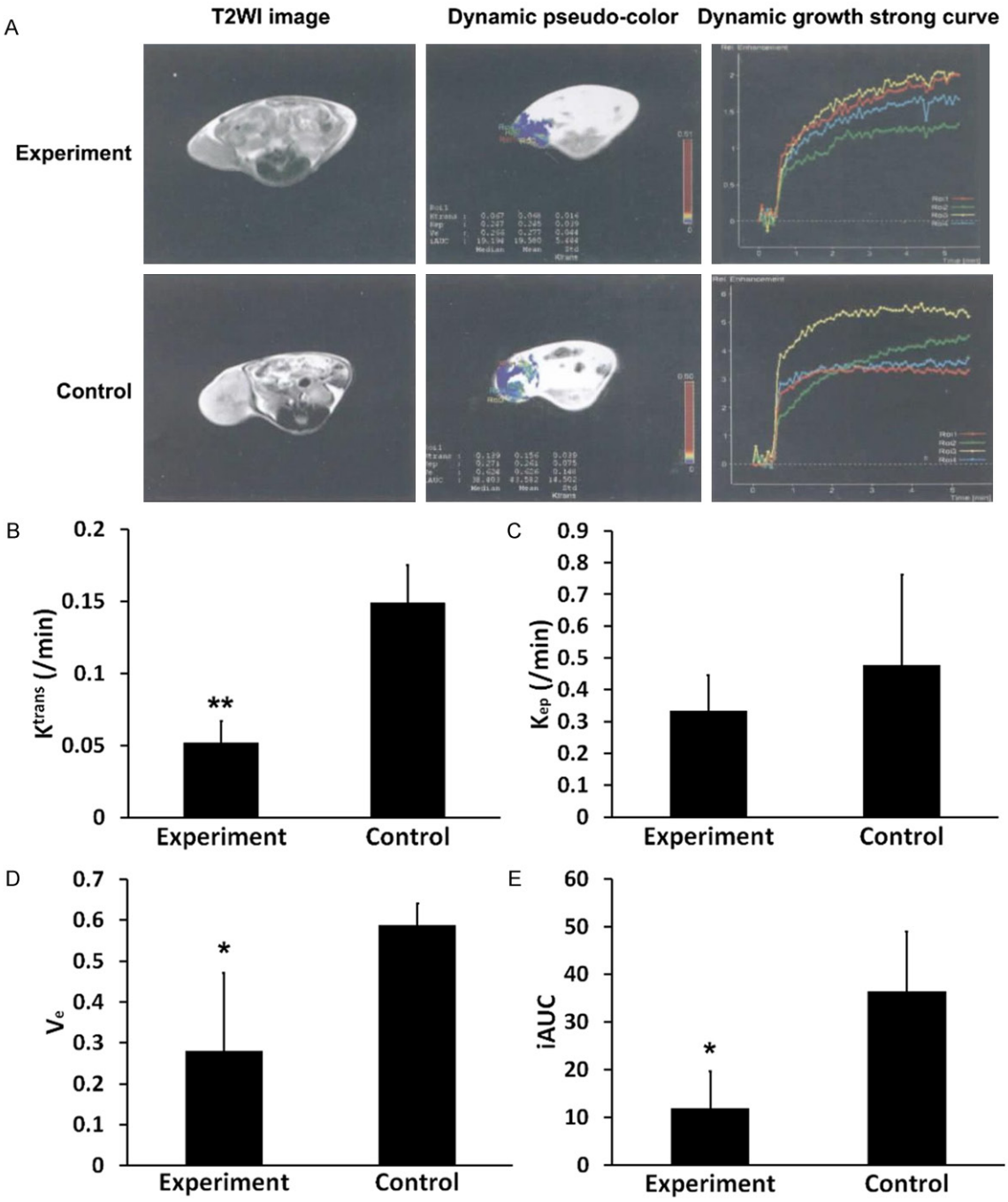

Figure 1. DCE-MRI quantitative analysis of the tumor vascular function in nude mice before and after treatment. A. T2WI image, quantitative dynamic pseudo-color (Ktrans = 0. 156/min). and dynamic growth strong curve of tumors. B-E. Tumor vascular function parameters (K^{trans}, K_{ep}, V_e, and iAUC values) of nude mice (mean ± standard deviation; n=5). *P<0.05, **P<0.01 vs. the control group.

Western blot

Western blotting was used to assess VEGF expression. Tissue sample was mechanically disrupted and mixed with SDS buffer. Samples were separated on SDS polyacrylamide gels. Proteins were transferred to PDVF membranes. Mouse VEGF antibody (manufacturer) was used

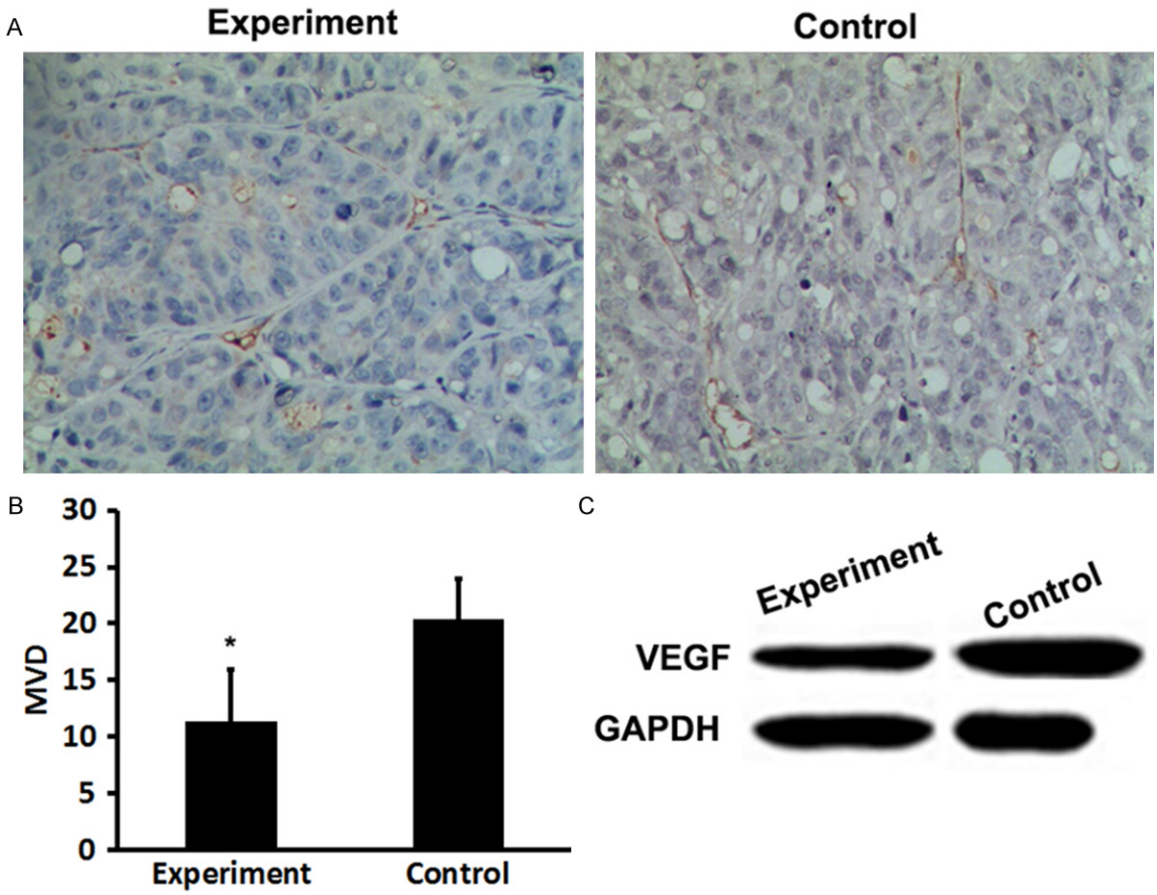

Figure 2. Microvessel density (MVD) and VEGF expression in tumors of nude mice. A. CD34 staining of tumors (SP ×200). B. Quantified MVD values of tumors (mean ± standard deviation, n=5). *P<0.05 vs. the control group. C. VEGF protein expression in tumors by western blot.

to detect the VEGF bands, which were revealed using an ECL kit (Pierce Chemicals, Dallas, TX. USA).

Statistical analysis

All data are presented as mean ± standard deviation. Comparison between groups was carried out using the Wilcoxon rank sum test. Statistical analysis was performed using SPSS 16. 0 (IBM, Armonk, NY, USA). Two-sided P-values < 0.05 were considered statistically significant.

Results

Tumor growth

Tumor volume in the Endostar mice was 0.249 ± 0.138 cm^3 compared with 0.448±0.096 cm^3 in the control mice (P<0.05).

DCE-MRI results

DCE-MRI results were used to analyze tumor vascular function parameter indexes in the two groups of mice (**Figure 1A**). The Ktrans, Ve, and iAUC values for the experimental group were significantly lower than in controls (Ktrans: 0.052±0.015 vs. 0.149±0.026/min; Ve: 0.280 ±0.191 vs. 0.588±0.052; iAUC: 11.91±7.74 vs. 34.46±12.53; all P<0.05), but there was no difference for Kep (0.334±0.111 vs. 0.588± 0.052/min, P>0.05) (**Figure 1B-E**).

MVD analysis and VEGF expression

MVD was 20.4 ± 3.5 vessels/high power field in controls compared with 11.4 ± 4.6 vessels/ high power field in controls (P<0.05) (**Figure 2A** and **2B**). In addition, VEGF expression in tumors from the Endostar group was lower than in controls (**Figure 2C**).

Discussion

Angiogenesis is a process through which new blood vessels are formed from pre-existing vessels. Angiogenesis plays a crucial role in tumor growth, invasion, and metastasis. Without angiogenesis, solid tumors could not exceed 1-2 mm3 [8]. The tumor vascular structure is different from that of normal blood vessels and invlude: (a) vessels, which are irregular, highly distorted, and dilated; (b) abnormal endothelial cells without complete basal membrane, presence of cracks, vascular structure brittleness, and high permeability. Because of its importance in tumor growth, angiogenesis has become a target for cancer therapy [9, 10]. Taking into account the non-tumor tissue vessel homogeneous conditions, there are differences in the structure and function in different regions. The application of anti-angiogenesis therapy has now been well explored in homogeneous tumors. Therefore, ROI selection is very important. Presently, ROI selection is made at the most obvious place for tumor parenchyma, but the ROI should be placed where the MVD is the most importabt, i.e. where MVD is measured.

In this study, data showed that the Endostar group had Ktrans, Ve, and values that were significantly lower than in the control group, suggesting that the control group had more immature tumor vessels, higher structural disorder, and more fissures. These factors may result in higher vascular permeability, characterized by high switching of the contrast agent. Endostar could reshape the tumor vasculature, and promote the normalization of the blood vessels, thus reducing vascular permeability. After treatment, the permeability decreased and the contrast agent from the tumor tissue was eluted at a slow rate and the residual gap within the organization leads to decreased vascular extracellular osmotic volume (Ve). AUC reveals the concentration of the contrast agent. It is a semi-quantitative parameter, and it shows the amount of blood flow into the tumor, tumor perfusion, and tumor tissue space. iAUC can comprehensively reflect the changes of Ktrans, Kep and Ve. After inhibition of tumor angiogenesis, microvessel permeability is reduced, thereby slowing the contrast agent elution rate, resulting in reduced clearance with a net decline in iAUC.

Tofts et al. [11] showed that Ktrans and Kep values are generally stable, but that Ve value is relatively unstable. In the present study, however, Kep value was not significantly different between the two groups. This could be due to the small sample size and experimental errors.

The results showed that DCE-MRI could successfully monitor the response to Endostar treatment in nude mice with lung cancer xenograft. Schnelletal [12] showed that NVP-BEZ235 inhibits BN472 tumor angiogenesis in mice. DCE-MRI scan in NVP-BEZ235 mouse tumors showed significant decline in Ktrans values than in the control group. Hillman et al. [13] also showed in the KCI-18 in human renal cell carcinoma model that DCE-MRI could be used to monitor the renal tumors vascular changes induced by sunitinib. Furthermore, DCE-MRI could be used in anti-angiogenic treatment to help deciding the drug dose and dosing regimens, thereby improving the efficacy of chemotherapy. Other studies also showed that the effective dose of drugs could be determined according to Ktrans [14-16]. Therefore, DCE-MRI could reflect the changes of the tumor after therapy and could be a minimally invasive technique to evaluate the efficacy of antiangiogenic drugs, especially for early evaluation of drug effectiveness.

In summary, the present study suggests that DCE-MRI parameters could be used for evaluating the effect of Endostar in the treatment of lung cancer in a mice model of subcutaneous tumor. DCE-MRI is minimally invasive and can be repeated for evaluating the tumor morphology.

Disclosure of conflict of interest

None.

Address correspondence to: Wenxiao Jia, Imaging Center, The Second Affiliated Hospital of Xinjiang Medical University, Urumqi 830063, Xinjiang, P. R. China. Tel: +86-13609979655; Fax: +86-021-64085875; E-mail: jwxxj@sina.com; Yonghua Xu, Shanghai Clinical Center of Chinese Academy of Sciences, Shanghai Xuhui Central Hospital, Shanghai 200031, P. R. China. Tel: +86-13609979655; Fax: +86-021-64085875; E-mail: smilel-g@163.com

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