VEGFR-1 targeted DNAzyme via transcatheter arterial delivery influences tumor vasculature assessed through dynamic contrast-enhanced magnetic resonance imaging

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Abstract. DNAzymes are synthetic single-stranded DNA oligonucleotides that bind and cleave target mRNA in a sequence-specific manner. Although the therapeutic potential has been demonstrated in both preclinical and clinical settings, the efficient delivery and *in vivo* assessment of the DNAzyme efficacy remain the vital unsolved issue. In the present study, we examined the feasibility of using transcatheter arterial chemoembolization (TACE) strategy to deliver a DNAzyme targeting VEGFR-1 and monitoring its effect on tumor angiogenesis *in vivo* via dynamic contrast enhanced magnetic resonance imaging (DCE-MRI). In a rabbit liver cancer model (VX2), we showed that the DNAzyme was efficiently delivered into the tumor by TACE. DCE-MRI revealed that the VEGFR-1-targeted DNAzyme affected the tumor vasculature through inhibiting VEGFR-1 expression *in vivo*, which was reflected by a reduction of K^{trans} and K_{ep}, the parameters of tumor microvascular permeability. Our findings offer an efficient strategy of delivery and assessment of the VEGFR-1 DNAzyme, and further demonstrate the feasibility of DNAzyme for cancer therapy.

Introduction

Hepatocellular carcinoma (HCC) was one of the most common malignant tumors in 2012, with incidence and mortality rate of 20.7 and 19.4/100,000, respectively (1). Surgical removal of the tumor and chemotherapy is the first line treatment for HCC. However, chemoresistance is one of major issues in the clinical treatment of liver cancer. The application of intervention in the clinical transcatheter arterial chemoembolization (TACE) has been widely used for non-surgical method for advanced liver cancer. Recently, TACE in combination with different therapeutics has been intensively investigated at both preclinical and clinical settings for HCC (2).

Angiogenesis plays a critical role in tumor growth, progression and metastasis. Hypoxia induces the hypoxia inducible factor (HIF) family proteins via degradation of the von Hippel-Lindau (VHL) tumor-suppressor (3), and production of angiogenic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived, epidermal and placental growth factors, and angiopoietins-1 (4). The two major tyrosine kinase receptors are VEGFR-1 and VEGFR-2. Unlike fibroblast growth factor receptors, both VEGF receptors are selectively expressed on endothelial cells (ECs) (5). Depending on the tumor type, VEGF and its two receptors may function via either the autocrine or paracrine mechanisms in humans (6-8). In comparison to the surrounding normal tissue vasculature, in tumor-associated ECs in a variety of tumors, both VEGFR-1 and VEGFR-2 are upregulated (5). Recently, it has been shown that HCC has robust nuclear and cytoplasmic staining for active, phosphorylated VEGF receptor-1. Autocrine VEGF signaling directly promotes HCC cell proliferation and affects the sorafenib treatment outcome *in vitro* and *in vivo* (9).

As the elevated expression of VEGF and its receptors have been closely correlated with tumor vascularity, progression and metastasis, targeting of VEGF/VEGFRs becomes a worthwhile approach to cancer therapy. Several strategies have been explored to inhibit VEGFR activity, which include the use of antisense, ribozymes and DNAzymes against VEGFR-1 or VEGFR-2 to suppress the formation of VEGF-induced new blood vessels (10-12).

DNAzymes are synthetic, single-stranded DNA catalysts that bind to their complementary sequence within a target mRNA by Watson-Crick base pairing and subsequently cleave the mRNA at predetermined phosphodiester linkages (13-15). Numerous studies have demonstrated that specific DNAzymes, targeting tumor-associated genes, exerts anticancer effects,

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which supports the efficiency of specific DNAzymes in down-regulation of appropriate genes and highlights DNAzymes as potential therapeutic molecules. However, the major hurdle for application of gene-targeted therapeutics in clinical settings remains how to ensure an efficient *in vivo* delivery.

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), diffusion-weighted imaging (DWI), perfusion-weighted imaging (PWI) and other imaging methods are used to evaluate the efficacy of treatment (16-18). The dynamic contrast images obtained are used to quantitate parameters which characterize tumor microcirculation. For example, the volume constant for the transfer of CA from the plasma to the extravascular extracellular space (e.g., K^{trans}/minute) represents an important parameter, and is primarily related to blood perfusion and microvessel permeability. Over the past 10 years, DCE-MRI and extracted kinetic parameters have been applied to phase I and II clinical trials of anti-angiogenic drugs and vascular disrupting agents, with K^{trans} generally recognized as a marker of tumor blood flow and permeability. In addition, K^{trans} has been recommended as a primary endpoint for anticancer treatment trials by the US National Cancer Institute (19).

In the present study, we investigated whether it is feasible to utilize TACE in combination with the VEGFR-1-targeted DNAzyme for anti-angiogenesis approach to HCC treatment in tumor-bearing rabbits through interventional operation. DCE-MRI imaging is used to evaluate the efficacy of treatment. The present study showed the effect of VEGFR-1 targeted DNAzymes on tumor vasculature assessed by DCE-MRI.

Materials and methods

DNAzyme and oligonucleotides. DNAzyme and control oligonucleotides were commercially synthesized (Sangon Biotech, Shanghai, China) with an inverted thymidine at the 3' position and purified by high performance liquid chromatography (HPLC). The sequences of DNAzyme and its target were previously described (12) and are shown in Fig. 1A.

Cell culture and transfection. Corneal endothelial cells from rabbit cornea were isolated by trypsin (Gibco) digestion and cultured to assay the effect of targeted VEGFR-1 DNAzymes in the rabbit corneal endothelial cells. The present study was approved by the Animal Care and Use Committee of Central South University. Corneal endothelial cells were cultured with Opti-MEM (Gibco) and seeded into 6-well plates $5x10^6$/well one day before transfection. When cells reached 70-80% confluency, cells were washed with serum-free medium and tranfected VEGFR-1 DNAzymes (DZ) or Ctrl DNAzymes with FuGENE (Promega, Madison, WI, USA). Six hours after transfection, cells were used to assay transfection efficiency. At 24 h post-transfection, cells were allowed to recover in complete medium overnight and were harvested for real-time PCR. A 5' fluorescein isothiocyanate (FITC)-labeled DNAzyme oligonucleotide was used for assessing transfection efficiency *in vivo* of the VX2 HCC.

Fluorescence-activated cell sorter (FACS) analysis. The transfected corneal endothelial cells were incubated at 37˚C for 6 h. The cells were washed with pH 7.4 phosphate-buffered saline (PBS), harvested by treatment with trypsin-EDTA, resuspended in ice-cold PBS and immediately examined using a FACSort flow cytometer (FACS; Merck Millipore, Temecula, CA, USA). After 20,000 total events were acquired/sample, transfection efficiency was defined by assessing cell size and granularity in the forward scatter and side scatter channels. FITC fluorescence in the cell population was expressed as the geometric mean of positive events after subtraction of background fluorescence.

Animal model and DZ treatment. The present study was approved by the Animal Care and Use Committee of Central South University. Sixteen New Zealand white rabbits weighing 2.37-2.70 kg (2.54±0.11 kg) were used (The Third Xiangya Experimental Animal Center, Changsha, China), according to the Third Xiangya Hospital Animal Care and Ethics Committee rules [LLSC (LA) 2013-005]. Briefly, the hind limb donor rabbit was anesthetized with intravenous injection of 30 mg/kg pentobarbital sodium. The tumor tissues were removed avoiding the necrotic core and cut into 1 mm^3. These blocs were stored in sterile saline for implantation in 4˚C. After anesthetized, one bloc was planted into the left midial lobe of the rabbit. The treated rabbit was intramuscular injected 800,000 U penicillin (North China Pharmaceutical Group) for 3 days. All procedures were performed in a sterile condition. The VX2 HCC was incubated 10-14 days. Twelve tumor-carrying rabbits were randomly divided into 4 groups (n=3): group A, 10 μl Fugene (F) + 250 μl Lipiodol (L) + 50 μl targeted VEGFR-1 DNAzymes (DZ); group B, 10 μl Fugene + 250 μl Lipiodol + 50 μl control oligonucleotides (Ctrl); group C, 10 μl Fugene + 250 μl Lipiodol; group D, blank.

DCE-MRI scanning schedule and parameters. All MRI examinations were performed with a 3.0-T MR system (Signa HDxt 3.0T; GE Heathcare, WI, USA). The VX2 HCCs were grown for 10-14 days. At the 10th day after modelling, the first MRI scanning was performed and at the 13th day (before intervention as 0 h point) for second scanning. The third and fourth scannings were performed at 48 and 96 h after intervention, respectively. Scanning parameters were: T2 weighted imaging (T2WI) axial images used fast spin-echo (FSE) sequence, echo time (TE) = 85.1 msec, repetition time (TR) = 4,800 msec, field of view (FOV) =15 mm over a 160x256 matrix, number of excitations (NEX) = 4, layer thickness = 3 mm, layer number = 16, interlayer spacing = 0. T1W1 axial images used FSE sequence, TE = 7.9 msec TR = 520 msec, field of view (FOV) = 15 mm over a 128x128 matrix, number of excitations (NEX) = 2, layer thickness = 3 mm, layer number = 16, interlayer spacing = 0. DCE-MRI used volume acceleration-flexible (LAVA-Flex): TE = 1.2 msec, TR = 2.7 msec, flip angle 12°, FOV = 15 mm over a 128x128 matrix, actual layer thickness = 3.2 mm, layer number = 2,000, time resolution 4 sec with 50 time phase, total imaging time 8 min 50 sec. Scanning steps: 12 New Zealand white rabbits were anesthetized. 22G I.V. catheters were fixed on the left or right ear with a piece of hard paper. The rabbits lay supine placed onto a board, put in the eight-channel knee coil and surrounded by sponge aiming at reducing breathing movement. The advanced mode of scanning was feet first.

Before scan, we connected 22G I.V. catheters to the high-pressure injectors with disposable intravenous infusion needle (21GX3/4, 0.8x19 mm). On the first time phase, gadopentetate dimeglumine (Gd-BOPTA; Bracco, Milan, Italy), a blood-pool contrast agent, was injected via the tail-vein catheter, and the doses of 0.1 mmol/kg, 10 ml saline to flush.

Targeted drug delivery intervention. Twelve tumor-bearing rabbits were randomly divided into 4 groups. Drug admixture consisted of 10 μl Fugene and 50 μl VEGFR-1 DNAzymes or control. The drug admixture stayed at room temperature (RT) for 8 min, followed by adding 250 μl Lipiodol and 10 min at RT. A 2-3 cm long groin incision was made and blunt dissection down to the femoral bundle of the tumor-bearing rabbits. The femoral artery was separated gently from the accompanying nerve and vein, and punctured with arteriovenous catheter (22Gx1 " 0.9x25 mm) and catheterized with 4 F sheath and 4 F catheter. Catheter and micro-catheter was placed near the tumor blood vessels. After digital subtraction angiography (DSA) of the liver, the drug admixture was injected through the micro-catheter followed with 2 ml saline flushing, following pulling out the catheter and ligating femoral artery close to the incision. The treated rabbits were all intramuscular injected with 800,000 U penicillin for 3 days.

In vivo delivery efficiency analysis. Two VX2 tumor-bearing rabbits were used for assessing *in vivo* delivery efficiency. One was treated with the combination of 5 μl Fugene, 20 μl FITC-labeled DNAzyme and 200 μl Lipiodol, the other with the combination of 5 μl Fugene, 20 μl saline and 200 μl Lipiodol. The two rabbits were sacrificed 6 h after catheter delivery. The VX2 tumor tissue was removed for frozen sections. The 4 μm frozen sections was obtained with cryostat microtome (CM3050S) and examined with fluorescence microscope (both from Leica) directly.

DCE-MRI data analysis. These raw data of DCE-MRI were analyzed using NordicICE software (version 2.3.14: NordicNeuroLab, Bergen, Norway) without animal grouping information. The model for contrast agent permeability analysis was based on a two-compartment tissue kinetic model, which obtain quantitative parameters and the reconstruction of parameters map, such as K^{trans}, K_{ep} and Ve K^{trans} is a rate constant that represents the transfer of contrast agent from the arterial blood into the extravascular extracellular space (EES) (in ml/ml/min). K_{ep} is a rate constant that represents transfer of the contrast agent from the EES to the blood plasma (in/min). Ve is the EES volume of distribution (in milliliters of blood/milliliters of tissue), $Ve = K^{trans}/K_{ep}$. All region of interest (ROI) placement were manually performed (avoiding the necrotic core). The ROI areas were 18-25 mm^2. We selected the maximum level of tumor and performed two ROIs of each tumor, followed by reconstruction of the maps and the imaging.

Real-time PCR. The RNA was extracted from each tumor tissue with TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and reverse transcribed into cDNA with a reverse transcription kit (Thermo). Real-time PCR was performed by primers for VEGFR-1, 5'-TAGCATCACAAGGGCAGCT TTA-3' (sense) and 5'-CATCTACTATCTTGCACTAAGCC TCT-3' (antisense); GAPDH 5'-CATCATCCCTGCCTCCA CT-3' (sense), 5'-GCCTGCTTCACCACCTTCTT-3' (antisense). The amplification and dissolution curve of the real-time quantitative PCR (Bio-Rad, Hercules, CA, USA) was confirmed after the reaction.

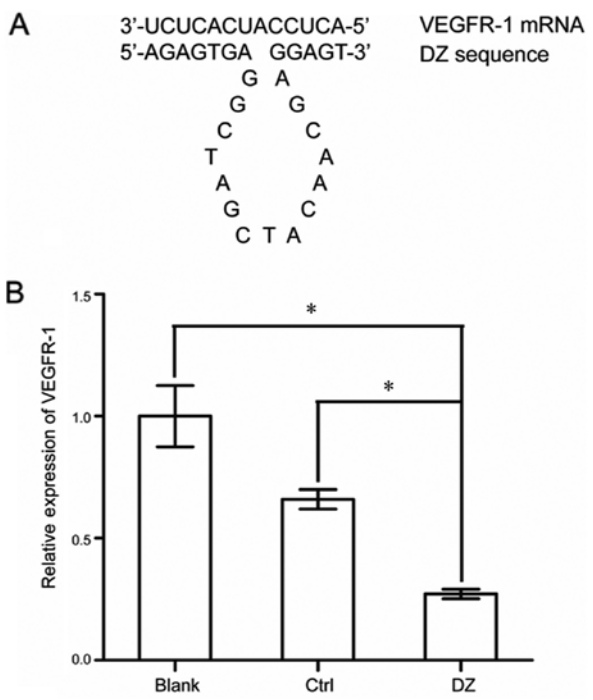

Figure 1. VEGFR-1-targetd DNAzyme and its effect on VEGFR-1 expression in rabbit cells. (A) Design of DNAzymes targeted to human VEGFR-1 mRNA. (B) Expression of VEGFR-1 in the rabbit corneal endothelial cells after transfection of the DNAzyme ($^*P<0.05$).

Statistical analysis. Data are presented as mean ± SD and were analyzed by t-test. Paired comparisons between multiple samples were tested by the Dunnett's test, t-test and Pearson's correlation analysis. All analysis was performed with 17.0 SPSS. A P-value of <0.05 was considered to indicate a statistically significant result.

Results

Downregulation of VEGFR-1 expression in rabbit corneal endothelial cells. VEGFRs, which belong to receptor tyrosine kinase family, play important roles in angiogenesis. We previously showed that VEGFR-1-targeted DNAzymes could suppress angiogenesis by inhibiting VEGFR-1 expression in human endothelial cells. In the present study, we investigated whether the DNAzyme could be delivered into liver and impact on tumor vasculature via a catheter that simulates TACE in rabbit hepatocyte carcinoma. To verify whether the DNAzyme could inhibit rabbit VEGFR-1 expression, the rabbit corneal endothelial cells were cultured and transfected with VEGFR-1-targeted DNAzyme using Fugene. The results showed that a marked decrease of VEGFR-1 expression was observed in the cells treated with VEGFR DNAzyme compared with control DNAzyme. While only a marginal decrease in mRNA level was observed in the

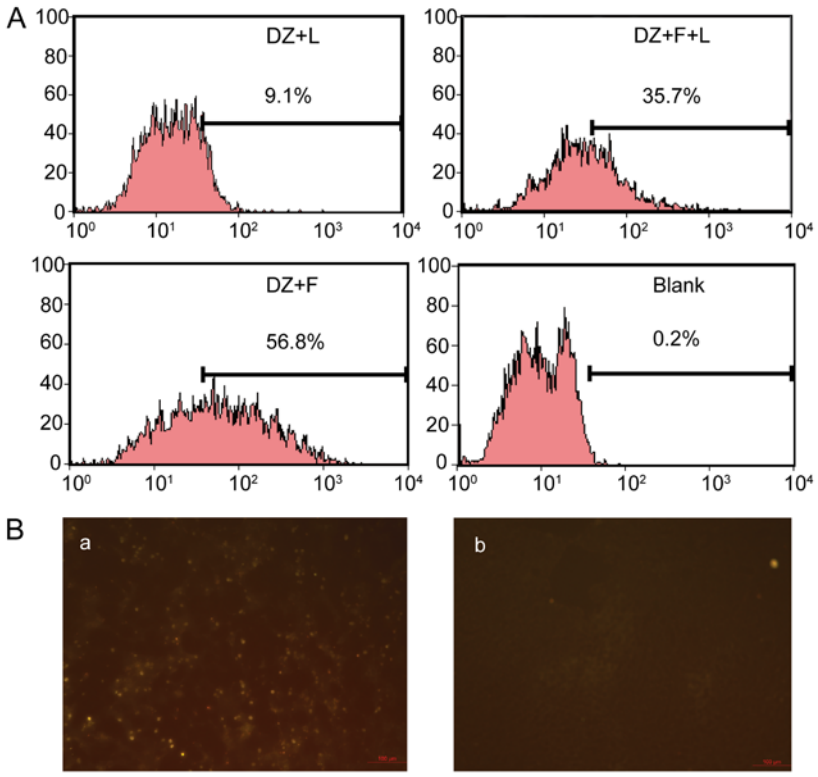

Figure 2. Assessment of the transfection efficiency of VEGFR-1 DNAzymes mixed with Lipiodol. (A) *In vitro* analysis of rabbit corneal endothelial cells by FACS. (B) *In vivo* analysis of liver tissues in tumor-bearing rabbits: a, FITC-labeled DNAzymes + Fugene + Lipiodol; b, non-labeled control + saline + Fugene + Lipiodol.

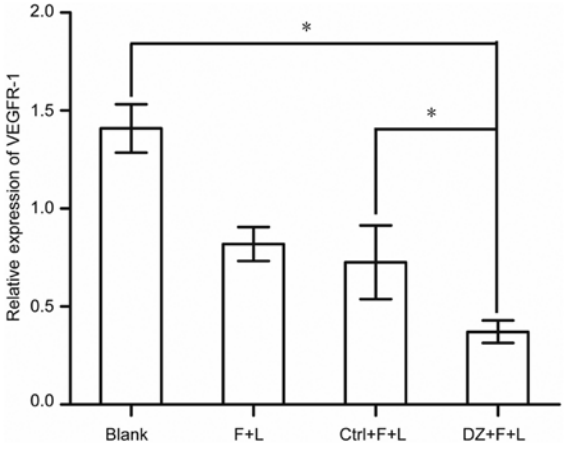

Figure 3. Expression of VEGFR-1 mRNA in the tumor tissues. The animals were sacrificed 8 days after treatment and qRT-PCR was performed. *P<0.05 compared with Ctrl group (n=3).

cells treated with control DNAzyme compared with blank, which may result from an antisense effect of the DNAzyme binding arms (Fig. 1B). Thus, these results demonstrated that VEGFR-1 DNAzyme is effective in downregulation of VEGFR-1 of rabbit origin.

Cellular uptake efficiency of Lipiodol-Fugene/DZ in rabbit corneal endothelial cells. Lipiodol can embolize the blood vessels and make the anticancer drugs selectively retainable at a high concentration in tumor tissues for a long time. To determine whether VEGFR-1 DNAzyme could be released from the Lipiodol-Fugene/DZ complex, DZ was labeled with FITC at the 5' end. Rabbit corneal endothelial cells were transfected with the complex, and the cellular uptake efficiency was analyzed by FACS. The results demonstrated the transfection efficiency of FITC-labeled DNAzymes + Fugene was 56.8%, FITC-labeled DNAzymes/Lipiodol/Fugene complex was 35.7% (Fig. 2A). Immunofluorescence also showed VEGDR1 DNAzyme released from Lipiodol (Fig. 2B). These results suggested that addition of Lipiodol in the transfection complex had some effect on transfection efficiency in cells, but still maintained the capacity of transfecting endothelial cells.

Downregulation of VEGFR-1 expression in tumor-bearing rabbits. To examine whether DNAzyme in Fugene/Lipiodol complex delivered via transcatheter into rabbit liver was biologically active, the tumor-bearing rabbits were treated with DNAzyme and control, and the liver cancer samples were collected 8 days post-treatment for RNA extraction for qRT-PCR. As shown in Fig. 3, VEGFR-1 mRNA level was downregulated in the DNAzyme-treated rabbit in comparison with the control. Both Lipiodol and control groups showed some effect of the treatment on VEGFR-1 expression, which may be due to the potential deleterious effect on both tumor and endothelial cells. The data suggested that the use of Lipiodol/Fugene to deliver DNAzyme via a transcatheter is feasible in an *in vivo* setting.

Effect of transcatheter-delivered VEGFR-1 DNAzyme on tumor vasculature permeability. Next, we investigated the impact of

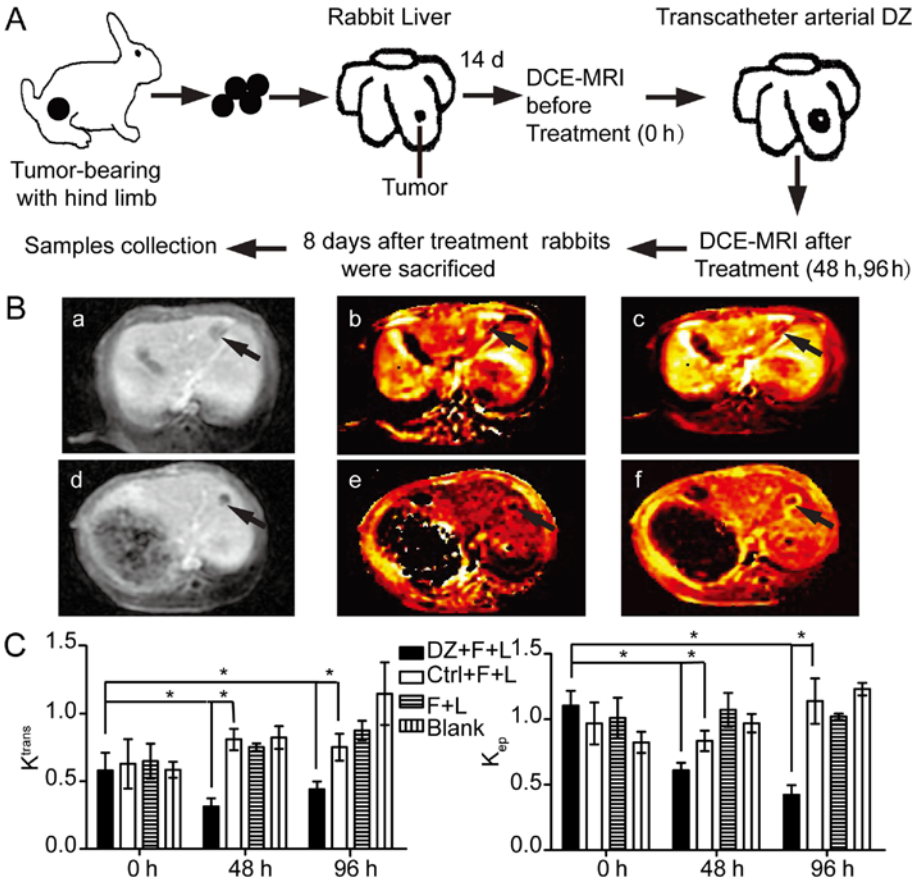

Figure 4. Effect of DZ on tumor angiogenesis assayed by DCE-MRI. (A) Schematic description of the experimental design. (B) DCE-MRI was performed 0, 48 and 96 h after DZ treatment. The representative dynamic enhanced MR images were presented for calculation of K^{trans} and K_{ep} from raw data of DCE-MRI by NordicICE software (v 2.3.14) at 48 h after treatment. a-c, Images from blank group; d-f, images from treatment group (DZ + F + L). (C) K^{trans} and K_{ep} values are expressed as means ± SE of three rabbits. *P<0.05 compared with the control group.

the VEGFR-1-targeted DNAzyme on tumor vasculature in tumor-bearing rabbits. DCE-MRI was used as a non-invasive *in vivo* method to analyze the change of the tumor vasculature. The parameter of K^{trans} derived from DCE-MRI reflects the condition of tumor microvascular permeability and has been widely used for evaluation of antitumor drugs. The schedule for treatment and acquiring raw data is shown in Fig. 4A and the K^{trans} and K_{ep} were calculated based on the images acquired from the animals (Fig. 4B). The results showed that the K^{trans} and K_{ep} values in DNAzyme-treated group were lower than that in control group after therapy at 48 h and 96 h time points ($P<0.05$) (Fig. 4C). These results indicated that VEGFR-1-targeted DNAzymes could downregulate the VEGFR-1 expression in rabbit liver cancer tissue, leading to change of tumor vasculature and vessel permeability.

Discussion

DNAzymes offer more advantages than other oligonucleotide agents. For example, DNAzymes, being composed of DNA, are not only easier and less expensive to synthesize, but also much more resistant to degradation *in vivo* than RNA molecules. A number of DNAzymes targeting c-Jun, LMP-1 and VEGFR-2, are being evaluated in human and animal trials, offering more therapeutic choices for patients (20-22). However, one of the principal barriers to the achievement of the clinical applications of DNAzymes is lack of efficient and safe delivery system. The present study assessed the feasibility of local delivery of the targeted VEGFR-1 DNAzyme via transcatheter in a rabbit liver cancer model. This approach has been clinically used in transcatheter arterial chemoembolization (TACE) for liver cancer treatment. Simulating clinical settings, we replaced chemotherapeutics with DNAzyme in Lipiodol-based embolism. The present study demonstrated that the DNAzyme can be delivered into the liver cancer in the designed schedule and caused disruption of the tumor vasculature, which proved the feasibility of the approach to HCC treatment via molecularly targeted agents in combination with TACE.

Over the past 10 years, DCE-MRI and extracted kinetic parameters have been used as an *in vivo* cancer imaging tool for the diagnosis, monitoring of treatment effect and evaluation of anticancer drugs. This method has been applied to phase I and II clinical trials of anti-angiogenic drugs and vascular disrupting agents (23,24). In particular, K^{trans} is currently recognized as a general marker of tumor blood flow, and has been recommended as a primary endpoint for an anticancer treatment trial by the US National Cancer Institute. K^{trans} is a factor that accounts for the complex functions of blood flow, endothelial surface area and endothelial permeability. However, it can also have multiple physiologic interpretations depending on the balance between capillary permeability and blood flow in a tissue. In high-permeability situations (where

flux across the endothelium is flow limited), K^{trans} is equal to the blood plasma flow/unit volume of tissue. Conversely, for low permeability conditions where tracer flux is permeability limited, the K^{trans} value is equal to the permeability surface area product/unit volume of tissue. Considering the pathological relevance of VEGFR-1 to angiogenesis, we previously showed that targeting VEGFR-1 by DNAzymes caused significant change of the tumor vasculatures (12). In the present study, the established rabbit HCC model provided a more operable and measurable system to examine the effect of TACE-mediated DNAzyme delivery on tumor vasculatures. The present study showed that VEGFR-1 DNAzymes caused a decline of the K^{trans} value for rabbit HCC as demonstrated by DCE-MRI, implying that VEGFR-targeted DNAzymes reduced the vessel leakiness and changed the vascular permeability.

In conclusion, DNAzyme therapeutics can be delivered into HCC via TACE in a much more clinically relevant manner. *In vivo*, the therapeutic effect of VEGFR-1 targeted DNAzyme can be assessed by DCE-MRI for its impact on tumor vasculature.

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