Anti-G250 nanobody-functionalized nanobubbles targeting renal cell carcinoma cells for ultrasound molecular imaging

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Abstract

Traditional imaging examinations have difficulty in identifying benign and malignant changes in renal masses. This difficulty may be solved by ultrasound molecular imaging based on targeted nanobubbles, which could specifically enhance the ultrasound imaging of renal cell carcinomas (RCC) so as to discriminate benign and malignant renal masses. In this study, we aimed to prepare anti-G250 nanobody-functionalized targeted nanobubbles (anti-G250 NTNs) by coupling anti-G250 nanobodies to lipid nanobubbles and to verify their target specificity and binding ability to RCC cells that express G250 antigen and their capacity to enhance ultrasound imaging of RCC xenografts. Anti-G250 nanobodies were coupled to the lipid nanobubbles using the biotin-streptavidin bridge method. The average particle diameter of the prepared anti-G250 NTNs was 446 nm. Immunofluorescence confirmed that anti-G250 nanobodies were uniformly distributed on the surfaces of nanobubbles. In vitro experiments showed that the anti-G250 NTNs specifically bound to G250-positive 786-O cells and HeLa cells with affinities of 88.13% ± 4.37% and 71.8% ± 5.7%, respectively, and that they did not bind to G250-negative ACHN cells. The anti-G250 NTNs could significantly enhance the ultrasound imaging of xenograft tumors arising from 786-O cells and HeLa cells compared with blank nanobubbles, while the enhancement was not significant for xenograft tumors arising from ACHN cells. Immunofluorescence of tumor tissue slices confirmed that the anti-G250 NTNs could enter the tissue space through tumor blood vessels and bind to tumor cells specifically. In conclusion, anti-G250 nanobody-functionalized targeted nanobubbles could specifically bind to G250-positive RCC cells and enhance the ultrasound imaging of G250-positive RCC xenografts. This study has high-potential clinical application value for the diagnosis and differential diagnosis of renal tumors.

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1. Introduction

Renal cell carcinoma (RCC) is the most common type of renal malignant tumor, accounting for approximately 90% of such tumors. Like other tumors, early diagnosis of RCC is essential [1]. Traditional imaging examinations, such as ultrasound and computed tomography (CT), can detect kidney lesions that are 0.5 cm in size or even smaller; however, there are still serious deficiencies in the identification of benign and malignant changes in kidney lesions, and approximately 20% of kidney tumors suspected to be malignant based on imaging examination were confirmed as benign lesions by pathology after surgery [2, 3]. Therefore, it is necessary to explore more effective imaging diagnostic techniques that can be used to improve the diagnosis and differential diagnosis of renal tumors.

Contrast-enhanced ultrasound (CEUS) is a noninvasive procedure that is relatively inexpensive, provides high soft tissue contrast, and does not expose the patient to radiation. The accuracy of ultrasound diagnosis can be improved by intravenous injection of ultrasound contrast agents (UCAs), and the use of such agents can significantly increase overall diagnostic accuracy by more than 30% [4–6]. With the extension of UCA research, especially research on targeted UCAs that incorporate specific antibodies or ligands that bind specifically to tissues expressing defined targets, ultrasound imaging has developed into an imaging technique at the molecular and cellular level [7–9]. Currently, microbubbles (MBs) with diameters of approximately 1–8 μm are widely used as an ultrasound contrast agent; however, MBs cannot penetrate the blood vessels to reach the tissue space and cannot therefore target the tumor cells or specifically image the tumor. Moreover, MBs have poor stability and a short half-life (approximately 3.5 min) [10, 11]. However, nanobubbles (NBs) can penetrate tumor blood vessels and reach the tissue space, and specific binding of NBs to tumor cells can be achieved when specific antibodies or ligands are carried on the surfaces of the NBs. NBs can be used to screen and distinguish tumors by directly enhancing tumor imaging and can achieve a certain therapeutic effect by carrying therapeutic drugs [12, 13]. It has been reported that prepared lipid NBs have an average particle diameter of approximately 500 nm, and it has been confirmed that NBs can pass through tumor blood vessels into the tissue space to enhance tissue imaging; furthermore, targeting of NBs has been achieved by attaching monoclonal antibodies against cell membrane antigens to the surfaces of the NBs [7, 14–16].

The G250 antigen is a transmembrane protein that is highly expressed in most RCCs and is not expressed in normal kidney tissue [17]. Thus, G250 is known as an RCC-associated antigen and has become a target for RCC treatment and/or diagnosis [18]. In a previous study, we successfully prepared nanobodies against the G250 antigen and confirmed that they bind specifically to G250-positive HeLa cells but not to G250-negative ACHN cells [19]. In this study, anti-G250 nanobodies were connected to the surfaces of NBs by using the biotin-streptavidin bridge method to prepare anti-G250 nanobody-functionalized targeted nanobubbles (anti-G250 NTNs), and the resulting NTNs were used to target RCC cells for ultrasound molecular imaging (scheme 1). We aimed to confirm that the anti-G250 NTNs could specifically enhance the ultrasound imaging of RCC xenografts so as to provide a basis for the discrimination of benign and malignant renal tumors and the early diagnosis of RCC.

2. Materials and methods

2.1. Cell culture and establishment of the xenograft model in nude mice

The human RCC cell lines 786-O (expressing G250 antigen or G250-positive) and ACHN (G250-negative) and the human cervical cancer cell line HeLa (G250-positive) (purchased from the Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China) were cultured in RPMI-1640, MEM and DMEM medium (Gibco Co., New York, USA), respectively, containing 10% fetal bovine serum (Gibco Co., New York, USA), 100 IU/ml penicillin and 100 μg ml⁻¹ streptomycin in a 5% CO₂ incubator at 37°C and were passed or plated for assays when the cells had reached 80%–90% confluence. BALB/c-nu nude mice, which were 4–5 weeks old and 15–20 g, were purchased from Beijing Huafukang Bioscience Co., Ltd, China. Log-phase 786-O, HeLa and ACHN cells were injected subcutaneously into nude mice at a concentration of 1 × 10⁶/200 μl. These animals were housed in a specific pathogen-free environment. The animal experiments conducted in this study were approved by the Experimental Animal Ethics Committee of the Army Medical University, China.

2.2. Preparation of the anti-G250 NTNs

Lipid NBs were prepared using the membrane hydration method. First, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-SN-glycero-3-phosphoethanolamine (DPPE), 1,2-dipalmitoyl-SN-glycero-3-phosphoglycerol (DPPG), 1,2-dipalmitoyl-sn-glycero-3-phosphate (DPPA) (Corden Pharma, Liestal, Switzerland) and biotinylated 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-PFG2000 (DSPE-P2000-Biotin) (NANOCX, Boston, MA, USA) were dissolved at a 3:3:3:1:1 mass ratio in a mixed solution containing phosphate-buffered saline (PBS) and glycerol. The mixture was placed in a 55°C water bath for 60 min; it was then moved to a closed vial, and
μl f1, 1′-octadecyl-3,3′,3′,3′-tetramethylphthalocyanine perchlorate (DiI, Beyotime Biotechnology, Shanghai, China), a fluorescent cell membrane dye, was added in the dark at the same time. The air in the vial was replaced with octafluoropropane (C₈F₈, Tianjin Nuclear Industry Institute of Physics and Chemistry, Tianjin, China). Subsequently, the vial was placed in an ST-B series mixer and horizontally mechanically shaken for 90 s. The obtained liquid was first centrifuged at 300 g for 3 min to separate the undissolved lipid (lower layer) and MBs/NBs. After the lower undissolved lipids were removed, the vial was centrifuged at 300 rpm for 3 min to separate the NBs (upper layer) and MBs. Large-diameter MBs were removed, and purified NBs were obtained. A blood cell count plate was used to count the number of NBs. The NBs were dropped on the counting board and were then observed and photographed under an inverted optical microscope (3 fields of view were randomly selected). ImageJ software was used for counting analysis. Finally, streptavidin (Suolaibao Biotechnology Co., Ltd, Beijing, China) was added at a ratio of 3 μg/10⁷ NBs, and the mixture was incubated at 4 °C for one hour. The NBs were then washed three times, and biotinylated anti-G250 nanobodies prepared in our laboratory were added at a ratio of 0.64 μg/10⁷ NBs. The mixture was incubated at 4 °C for one hour; the NBs were then washed three times to obtain targeted NBs carrying anti-G250 nanobodies (anti-G250 NTNs). The above process was conducted in the dark.

Scheme 1. Illustration of anti-G250 nanobody targeted nanobubbles (anti-G250 TNTs) that bind specifically to RCC cells expressing G250 antigen and enhance the ultrasound imaging of RCC xenografts.

2.3. Basic characteristics of the anti-G250 NTNs

The distribution of the anti-G250 NTNs was observed microscopically after 100-fold dilution in PBS, and the concentration of the anti-G250 NTNs was calculated using ImageJ software (imagej.nih.gov). The particle diameter and zeta potential of the anti-G250 NTNs were measured using a Malvern Zetasizer nano ZS90 detector (Malvern Instruments Inc., Worcestershire, UK). The anti-G250 NTNs were observed and measured daily over a period of one week to evaluate changes in concentration and particle diameter with time (during this time, they were stored at 4 °C). All measurements were performed three times. The morphology of the anti-G250 NTNs was observed by JEM-1400 transmission electron microscopy (JEOL, Tokyo, Japan). Two hundred microliters of the DiI-labeled anti-G250 NTNs sample was mixed with 200 μL of anti-M13-HRP mouse monoclonal antibody (GE Healthcare, Beijing, China) diluted 1:50 in PBS; the mixture was left on ice for 45 min and was then incubated with 200 μL of goat anti-mouse Alexa-488 (Thermo Fisher Scientific, Rockford, IL, USA) diluted in PBS (1:200) in the dark for 45 min. A 10 μL sample was diluted and examined by a Zeiss 780 confocal laser scanning microscope (CLSM; Carl Zeiss AG, Oberkochen, Germany) to determine whether the biotinylated nanobodies had attached to the surfaces of the NBs. An MTS assay was used to evaluate the potential cytotoxicity of the anti-G250 NTNs to 786-O cells.
786-O cells in logarithmic growth phase were cultured in 96-well plates at a density of 5000 cells/well under 5% CO₂ at 37 °C for 12 h. After the cells had fully attached to the plates, various concentrations of the anti-G250 NTNs were added to the cells, and the plates were incubated for an additional 8 h. Subsequently, 20 μl of MTS solution (Cell Titer 96™ Aqueous One Solution Reagent, Beijing Promega Co., Beijing, China) was added to each well. One hour later, the absorbance of each well at 490 nm was measured using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) and was used to calculate the cytotoxicity of the anti-G250 NTNs.

2.4. Cellular immunofluorescence assay

786-O, HeLa and ACHN cells in logarithmic growth phase were placed in 20 mm glass petri dishes at 5 × 10⁴ cells/dish and cultured overnight. The cells were then fixed in 4% paraformaldehyde and blocked with PBS containing 5% bovine serum albumin (BSA-PBS). Then, 100 μl (1:500) of biotinylated anti-G250 nanobody was added, and the cells were incubated overnight at 4 °C. After three rinses with PBS, 100 μl of Alexa-488-labeled fluorescent secondary antibody (1:500) was added, and the cells were incubated at 37 °C for two hours in the dark. After rinsing with PBS, the cells were counterstained with 4’,6-diamidino-2-phenylindole (DAPI, Beyotime Biotechnology, Shanghai, China) for 10 min and rinsed three times, and the expression of G250 in the cells was observed by laser confocal microscopy.

2.5. In vitro cell assay to detect targeted binding of the anti-G250 NTNs

786-O, ACHN and HeLa cells in logarithmic growth phase were dissociated using trypsin and collected into Eppendorf tubes at 5 × 10⁵ cells per tube. After resuspension of the cells in 500 μl of PBS, 500 μl of the blank nanobubbles (BNs) and the anti-G250 NTNs at 2 × 10⁷ ml⁻¹ were added sequentially and gently mixed with the cells. Both types of NBs were labeled with Dil. The mixtures were incubated at 37 °C for 40 min. The supernatant was removed after centrifugation, the cell pellets were resuspended in 250 μl PBS, and the binding of the NBs to the cells was measured by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA). In addition, after fixation in 4% paraformaldehyde, each type of cell was divided into two groups. The first group was incubated with 50 μl of the anti-G250 NTNs at a concentration of 1 × 10⁷/ml, and the second group was incubated with 50 μl of BNs at a concentration of 1 × 10⁷/ml. After incubation at 4 °C for two hours, the cells were rinsed three times with PBS for 3 min each time and stained with 3,3′-dioctadecylcarbocyanineperchlorate (DiO, Beyotime Biotechnology, Shanghai, China) and DAPI for observation of the cell membrane and nucleus, respectively. Finally, the binding of the two types of NBs to the three types of cells was observed by fluorescence microscopy.

2.6. In vivo enhanced ultrasound imaging by the anti-G250 NTNs

When the diameter of the subcutaneous tumors formed in the tumor-bearing nude mice reached 5–10 mm, five mice in each group were randomly selected for ultrasound imaging on a small animal ultrasound imaging system—Vevo 2100 (VisualSonics Inc., Toronto, ON, Canada), scanning with an MS250 high-frequency probe (frequency range 13–24 MHz, central frequency 20 MHz). The mice were anesthetized using 2% isoflurane before imaging, and the surface of the tumor was covered with a coupling agent. The probe was fixed on the area containing the largest cross-section of the transplanted tumor. After collection of the two-dimensional gray image, the imaging mode was adjusted to the contrast-enhanced imaging mode (central frequency 20 MHz) with the focus center located in the center of the transplanted tumor tissue. Then, 100 μl of the NBs or the anti-G250 NTNs at a concentration of 1 × 10⁸/ml was injected into the posterior orbital venous sinus of the nude mouse, and continuous dynamic images were collected for 10 min. After observation for 30 min, the Burst function key of the ultrasonic instrument was used to conduct NB blasting to remove the remaining NBs from the body. Then, another type of NBs different from the first injection type was injected into the posterior orbital venous sinus, and dynamic images were collected under the same parameters. Each mouse was injected twice. The peak intensity (dB value) of the collected images was analyzed using dedicated software (Vevo 2100 onboard software, VisualSonics Inc., Toronto, ON, Canada), the time-intensity curve was plotted, and the area under the curve (AUC) was calculated.

2.7. Localization of the anti-G250 NTNs in tumor tissues

Five mice bearing tumors consisting of the above three types of cells were randomly selected, and 200 μl of Dil-labeled anti-G250 NTNs at a concentration of 1 × 10⁸/ml was injected into the posterior sinus of the eye of each mouse. The mice were sacrificed 5 min later, and the tumors and thigh muscle tissues were dissected for cryosectioning. After fixation in 4% paraformaldehyde, sections were incubated with 100 μl of goat anti-mouse CD31 monoclonal antibody (Abcam, Cambridge, UK) (1:200) for eight hours at 4 °C. After three rinses with PBS, 100 μl of FITC-labeled rabbit anti-mouse secondary antibody (Beyotime Biotechnology, Shanghai, China) (1:200) was added to the sections, and the sections were incubated at room temperature for two hours in the dark. Tumor cell nuclei were counterstained with DAPI. After fixation, the sections were scanned with an orbital venous sinus of the nude mouse, and dynamic images were collected for 10 min. The tissue sections were observed under laser confocal microscopy.

2.8. Statistics

Measurement data were expressed as x ± s and were analyzed using SPSS 23.0 statistical software. Data analysis was performed by one-way analysis of variance and paired t-test. P < 0.05 was taken to indicate statistical significance.
3. Results

3.1. Preparation and examination of the anti-G250 NTNs

On microscopic observation, the anti-G250 NTNs appeared to be uniformly distributed in a circular pattern (figure 1(A)). After tungsten negative staining, the anti-G250 NTNs appeared as a dark black circular region by transmission electron microscopy (figure 1(B)). The anti-G250 NTNs concentration was calculated by ImageJ grayscale measurement to be \((18.29 \pm 0.75) \times 10^8 \text{ ml}^{-1}\). Measurement by the Malvern Zetasizer nano ZS90 analyzer revealed the particle diameter of the anti-G250 NTNs to be \((446.3 \pm 43.8) \text{ nm}\) (figure 1(C)); the polydispersity index was 0.011 \pm 0.006, indicating that the distribution range of the particle diameter was narrow, and the average zeta potential was \((-24.3 \pm 3.1) \text{ mV} \ (n = 3)\). The negative charge makes the NBs stable in water and not prone to aggregation. The anti-G250 NTNs

Figure 1. Basic characteristics of the anti-G250 NTNs. (A) Morphology of the anti-G250 NTNs as viewed by optical microscopy. (B) Morphology of the anti-G250 NTNs as viewed by transmission electron microscopy. (C) Particle diameter distribution of the anti-G250 NTNs. (D) Histogram showing the time dependence of changes in the concentration of the anti-G250 NTNs. (E) Histogram showing the time dependence of changes in the particle diameter of the anti-G250 NTNs. (F) Results of MTS assays of the anti-G250 NTNs. * indicates \(P < 0.05\).
Figure 2. Verification of the coupling of nanobodies to NBs by immunofluorescence. Under laser confocal microscopy, the NBs with Dil-labeled lipid membranes showed red fluorescence (A), while the fluorescent Alexa-488-labeled G250 nanobodies showed green fluorescence (B). The complete overlap of the two types of fluorescence indicates that the G250 nanobodies were ligated to the surfaces of the NBs (C).

Figure 3. Expression of G250 antigen of the three types of tumor cells. Immunofluorescence showed that the G250 antigen was expressed on the cell membranes of 786-O cells (A)-(C) and HeLa cells (G)-(I) and not expressed on ACHN cells (D)-(F). The scale bar represents 20 μm.
were stored at 4°C, and the number of particles and the particle diameter were continuously observed for one week. The concentration and particle diameter of the anti-G250 NTNs on day 3 differed from those on day 1 (P < 0.05); the concentration of the anti-G250 NTNs decreased with time (figure 1(E)), and the particle diameter increased with time (figure 1(F)). Subsequently, the cytotoxicity of the anti-G250 NTNs to 786-O cells was examined using the MTS assay (figure 1(D)). The results showed that the cell viability at NTN concentrations exceeding \(5 \times 10^9/\text{ml}\) was 74.63% ± 6.38%, significantly lower than the viability of 85.82% ± 3.69% observed at an NTN concentration of \(1 \times 10^9/\text{ml}\) (P < 0.05).

The NBs were coupled to anti-G250 nanobodies using the biotin–streptavidin bridge method. Streptavidin has four subunits. We used streptavidin in which one subunit is linked to biotinylated DSPE-PEG2000 and the remaining subunits are available to associate with multiple biotinylated G250 nanobodies. Under laser confocal microscopy, NBs with DiI-labeled lipid membranes showed red fluorescence (figure 2(A)), while the fluorescent Alexa-488-labeled G250 nanobodies showed green fluorescence (figure 2(B)). The two types of fluorescence overlapped completely (figure 2(C)), indicating that the anti-G250 nanobody was attached to the surfaces of the NBs.

3.2. Cellular immunofluorescence

First, we determined the expression of G250 protein in the RCC cell lines 786-O and ACHN and the cervical cancer cell line HeLa using a cellular immunofluorescence method. The results showed that the G250 antigen was highly expressed on the surfaces of 786-O cell membranes (figures 3(A)–(C)) but not on the surfaces of ACHN cell membranes (figures 3(D)–(F)); the G250 antigen was also highly expressed on the surfaces of HeLa cell membranes (figures 3(G)–(I)).

3.3. Specific binding of the anti-G250 NTNs to cells

Flow cytometry detection showed that the anti-G250 NTNs combined with 786-O (figure 4(A)) and HeLa cells
(figure 4(B)) in large amounts, significantly higher than the amount of BNs that bound to these cells (P < 0.05). There was almost no combination between the anti-G250 NTNs and ACHN cells (figure 4(C)). The anti-G250 NTNs bound to 88.13% ± 4.37% of 786-O cells and to 71.8% ± 5.7% of HeLa cells (figure 4(D)), significantly different from the affinity of these cells for BNs (P < 0.05).

Examination by fluorescence microscopy showed that the anti-G250 NTNs bound to 786-O cells and HeLa cells in large amounts, while the BNs showed no binding to these cells. ACHN cells did not bind either to the anti-G250 NTNs or to the BNs (figure 5). These results verify that the anti-G250 NTNs bound specifically to G250-expressing 786-O and HeLa cells.

3.4. In vivo enhanced ultrasound imaging of the anti-G250 NTNs

Xenografts of nude mice injected with 786-O cells, HeLa cells and ACHN cells were randomly selected for experiments, and the imaging characteristics of the anti-G250 NTNs and BNs in the three types of tumors were dynamically analyzed. Tumor tissues from animals without injection of NBs showed low echo. Injection of the anti-G250 NTNs and the BNs, respectively, resulted in good enhancement of ultrasound imaging ability, indicating that both types of NBs can penetrate tumor blood vessels and enhance tumor tissue imaging. Subsequently, continuous dynamic images were collected for 10 min (figure 6(A)); the time-intensity curves (figures 6(B)–(D)) were then plotted, and the AUCs were calculated (figure 6(E)). The results showed that there was a significant difference between the AUCs of the anti-G250 NTNs and the BNs in 786-O cells and HeLa cell xenograft tissues (P < 0.05). However, the AUCs of the two types of NBs in ACHN cell xenografts were not significantly different (P > 0.05). Comparison of the in vivo ultrasound intensities of the two types of NBs (table 1) showed that the ultrasound intensities (dB values) of 786-O cell and HeLa cell xenograft tissues after injection of the anti-G250 NTNs and BNs were significantly different (P < 0.05), while the dB values of the two types of NBs in xenograft tumors of ACHN cells were not different (P > 0.05). The above results indicate that compared with the BNs, the anti-G250 NTNs significantly enhance the ultrasound imaging effect of G250-positive xenografts and that the difference is statistically significant. However, for G250-negative xenografts, the two types of NBs show no significant difference.

3.5. Localization of the anti-G250 NTNs in tumor tissues

In figure 7, green indicates the distribution of blood vessels in the tumor tissue, and red indicates the presence and the distribution of NBs in the tumor tissue. NBs were present in the extravascular and intercellular spaces of various tumors, providing strong evidence that NBs can penetrate tumor blood vessels. In the transplanted tumors arising from 786-O cells and HeLa cells, a large number of the DiI-labeled anti-G250 NTNs were visible (figures 7(A)–(H)). In the transplanted tumors arising from ACHN cells, a small number of DiI-labeled NBs were observed outside the blood vessels (figures 7(I)–(L)), while NBs were barely detectable outside blood vessels in normal muscle tissue (figures 7(M)–(P)), indicating that the anti-G250 NTNs can pass through the tumor blood vessels and bind to the G250 antigen-expressing tumor cells and aggregate, while binding at a low level or not at all to cells that do not express the G250 antigen. In the transplanted tumors arising from 786-O cells and HeLa cells, the signal intensities of the anti-G250 NTNs were (25.01 ± 4.0)% and (20.19 ± 1.60)%, respectively, of the total tissue intensity; these values are approximately 10-fold those found in ACHN cells and muscle tissue, demonstrating a significant difference (figure 7(Q)) (P < 0.05).
4. Discussion

The incidence of RCC is high; however, the clinical symptoms of RCC are such that approximately 25%–30% of patients already have localized invasion or metastasis at the time of diagnosis. Approximately 30% of patients with early RCC experience recurrence or distant metastasis after radical surgery. The cure rate of RCC is low, and the mortality rate is
high [20]. Therefore, early diagnosis and timely treatment are essential for improving the prognosis and prolonging the survival rate of patients with RCC. Ultrasound is currently used clinically for primary screening of renal lesions followed by further evaluation with CT and/or magnetic resonance imaging (MRI). Contrast-enhanced CT (CECT) and MRI diffusion-weighted imaging (DWI) can improve diagnostic accuracy in cases of RCC. However, some inflammatory lesions and complex benign cysts in normal and diseased kidneys are still misdiagnosed. Furthermore, CT and MRI examinations carry risks associated with exposure to radiation and contrast agents, have relatively high cost and relatively low sensitivity, and require a longer time to acquire images than ultrasound [21, 22]. Ultrasound is a widely used realtime imaging tool with the advantages of portability, low cost and no radiation, but there are some shortcomings in differentiating benign and malignant tumors. Targeted UCAs can improve the resolution and sensitivity of ultrasound imaging, achieve early diagnosis of malignant tumors, and provide a new method for the early diagnosis of renal cancers.

The current commonly used UCAs are gas-containing lipid microbubbles. They are classified into micron-sized and nanosized microbubbles according to their particle diameters. Micron-sized microbubbles are currently in clinical use. One representative agent is SonoVue, produced by the Braco Co. Due to its large particle diameter, SonoVue cannot enter the tissue space through the blood vessel wall; it is mainly used to enhance vascular imaging and thereby indirectly enhance tumor imaging. Our research team has conducted extensive research on UCA, mainly lipid N Bs; the work includes verification of tumor vascular permeability of the N Bs, small interfering RNAs to inhibit cell proliferation, and N Bs coupled with monoclonal antibodies or aptamers to achieve targeted binding and targeted imaging enhancement of tumors and has achieved satisfactory results [13-15, 23-25]. The particle diameter of the N Bs we prepared was generally approximately 500 nm (446.3 ± 43.8 nm in this experiment), and the dimensions of the intercellular spaces of the tumor neovascular endothelial cells are 380–780 nm, thus ensuring that the N Bs can pass through the tumor blood vessels into the tissue space and directly interact with the tumor cells. This is critical for targeted imaging and targeted therapy and is a specific characteristic of N Bs that makes them superior to micron-sized microbubbles [26].

To achieve NB targeting, antibodies or aptamers must be attached to the surface of the N Bs. To further reduce the volume of targeted N Bs, nanobodies were used in this study. Nanobodies are derived from the variable domain of heavy chain (VHH) of heavy-chain antibody from camel; these antibodies are also known as single-domain antibodies and are the smallest antigen-binding fragments of natural origin that have complete functions. They are of low molecular weight (15 kDa) and exhibit good stability, low immunogenicity, high specificity, ease of modification, and strong penetrating capacity compared with monoclonal antibodies. Moreover, their monomeric properties and the presence of the carboxyl terminus on the other side of the complementary site make them ideal candidates for a variety of modifications (e.g. protein tags, bivalent or bispecific constructs, and enzyme or toxin conjugates); therefore, they are ideal for targeting cancer cells [27–30]. The low molecular weight of nanobodies helps maintain the stability of the targeted N Bs by effectively preventing their removal by the reticuloendothelial system (RES) and allowing complete penetration of the tumor vascular system [31]. Although nanobodies are small in size, their binding sites are similar to those of other antibodies of the same type. The anti-G250 nanobody we prepared has a molecular weight of approximately 17 kDa. It has been shown by flow cytometry and cellular immunofluorescence that the anti-G250 nanobody specifically binds to G250 antigen-expressing tumor cells (786-O cells and HeLa cells).

The G250 nanobody targets the G250 transmembrane antigen of RCC cells, which is the carbonic anhydrase (CA) isoform CAIX. CAIX is expressed in most RCCs. The proteoglycan-like domain (PG) (59 aa) of CAIX is unique compared with other CA subtypes, and its ligand does not cross-react with other CA subtypes, a feature that is extremely important for the specific targeting of targeted N Bs [32]. In this study, we successfully prepared targeted N Bs carrying anti-G250 nanobodies by connecting the anti-G250 nanobodies to the surfaces of N Bs with the biotin-streptavidin bridge method. The prepared N Bs had a mean diameter of 446.3 ± 43.8 nm, uniform size, relatively dispersed distribution, and good stability. The G250 nanobodies were uniformly distributed on the surfaces of the targeted N Bs, as shown by double fluorescence microscopy. Under the light microscope, the anti-G250 N Ts were observed to specifically bind to 786-O cells and HeLa cells, which express the G250 antigen, but not to ACHN cells, which do not express

| Table 1. Comparison of ultrasound enhancement intensity (dB) of anti-G250 N Ts and N Bs in transplanted tumors. |
|---|---|---|---|---|
| Tissue | Contrast agent | 30s | 180s | 300s | 600s |
| 786-O Tumor | BN | 10.67 ± 0.29 | 7.64 ± 0.56 | 4.71 ± 0.15 | 3.10 ± 0.04 |
| | NTN* | 13.66 ± 0.16 | 11.59 ± 0.72 | 10.49 ± 0.23 | 9.16 ± 0.75 |
| HeLa Tumor | BN | 9.59 ± 0.68 | 5.36 ± 0.27 | 3.82 ± 0.45 | 2.60 ± 0.31 |
| | NTN* | 13.30 ± 0.60 | 10.43 ± 0.72 | 9.47 ± 0.98 | 7.38 ± 0.53 |
| ACHN Tumor | BN | 10.65 ± 0.56 | 6.15 ± 0.68 | 4.51 ± 0.76 | 2.45 ± 0.61 |
| | NTN* | 10.33 ± 0.77 | 5.99 ± 0.73 | 4.15 ± 0.74 | 2.98 ± 0.41 |

* Significantly different from the N Bs (P < 0.05).
Furthermore, the affinity of the anti-G250 NTNs for G250-positive cells determined by flow cytometry was as high as 88.13%, while the BNs hardly bound to these cells. In fact, G250-negative cells hardly bound to any of the tested types of NBs. These results indicate that nanobodies can be used to successfully achieve the targeting of NBs and show that the G250 nanobodies we prepared exhibit high binding to RCC cells.
An in vivo ultrasound imaging study showed that various NBs exhibit good tissue imaging enhancement effects that may be attributed to the enhanced permeability and retention (EPR) effect of NBs [33]. However, in G250-positive 786-O and HeLa cell xenografts, the imaging enhancement effect of the anti-G250 NTNs was significantly superior to that of the BNs, whereas there was no significant difference in the imaging enhancement of G250-negative ACHN cell xenografts. A possible reason for this difference may be that the anti-G250 NTNs penetrate the tumor blood vessel wall by the EPR effect and reach the tissue space, and that the G250 nanobodies on the surfaces of the anti-G250 NTNs participate in a specific antigen-antibody reaction with the tumor cells, leading to higher aggregation and retention in the tumor and resulting in higher peak intensity and longer duration. Histological analysis confirmed that the anti-G250 NTNs could enter the tissue space through the blood vessel wall. Importantly, the antigen-antibody reaction enhanced the stability of the anti-G250 NTNs and their binding to tumor cells, resulting in higher aggregation and retention of the anti-G250 NTNs in the G250-expressing xenografts at levels approximately 10-fold those observed in non-G250-expressing xenografts. The above results are consistent with the results of Gao et al [8, 34–36]. It is extremely important that the anti-G250 NTNs can significantly enhance the ultrasound imaging of RCC xenografts. This finding has potentially important value in the clinical diagnosis and differential diagnosis of benign and malignant renal tumors.

5. Conclusion

In this study, we prepared anti-G250 NTNs and demonstrated that they had high affinity and specificity for G250-expressing RCC in vivo and in vitro and could enhance the ultrasound imaging of RCC xenografts in nude mice. This study provides a novel ultrasound molecular probe that may improve the diagnostic accuracy of the renal tumors. This probe can also function as a potential carrier to deliver targeted therapeutic drugs in the treatment of RCC. The carried drugs can be released through the ultrasound-targeted nanobubble destruction technique (UTMD) to increase the local drug accumulation in tumor tissues and reduce systemic side effects and toxicity, thereby improving the cure rate. Related research work is being carried out.

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References

[1] Ljunghberg B et al 2015 EAU guidelines on renal cell carcinoma: 2014 update Eur. Urol. 67 913
[2] Nishikawa M, Miyake H, Kitajima K, Takahashi S, Sugimura K and Fujisawa M 2015 Preoperative differentiation between benign and malignant renal masses smaller than 4 cm treated with partial nephrectomy Int. J. Clin. Oncol. 20 150
[3] Erman M et al 2016 Renal cell cancer: overview of the current therapeutic landscape Expert Rev. Anticanc. 16 955
[4] Frohlich E, Jenssen C, Schuler A and Dietrich C F 2015 Contrast-enhanced ultrasound for characterization of focal liver lesions, practical advice Z. Gastroenterol. 53 1099
[5] Gatos I, Tsantis S, Spiliopoulos S, Skouroliaikou A, Theotokas I, Zoumpoulis P, Hazle J D and Kagadis G C 2015 A new automated quantification algorithm for the detection and evaluation of focal liver lesions with contrast-enhanced ultrasound Med. Phys. 42 3948
[6] D’Onofrio M, Crosara S, De Robertis R, Canestrini S and Mucelli R P 2015 Contrast-enhanced ultrasound of focal liver lesions AJR Am. J. Roentgenol. 205 W56
[7] Zhu L, Guo Y, Wang L, Fan X, Xiong X, Fang K and Xu D 2017 Construction and targeted nanoscale microbubbles in prostate cancer Ultrasound Med. Biol. 43 3585
[8] Perez-Herrero E and Fernandez-Medarde A 2015 Advanced targeted therapies in cancer: drug nanocarriers, the future of chemo-therapy Eur. J. Pharm. Biopharm. 93 52
[9] Zhang H, Ingham E S, Gagnon M K J, Mahakian L M, Liu J, Fosret J L, Willmann J K and Ferrara K W 2017 In vivo characterization and in vivo ultrasound molecular imaging of nucleolin-targeted microbubbles Biomaterials 118 63
[10] Abou-Elkacem L, Bachawal S V and Willmann J K 2015 Ultrasound molecular imaging: moving toward clinical translation Eur. J. Radiol. 84 1685
[11] Cai W B, Yang H L, Zhang J, Yin J K, Yang Y L, Yuan L J, Zhang L and Duan Y Y 2015 The optimized fabrication of nanobubbles as ultrasound contrast agents for tumor imaging Sci. Rep. 5 13725
[12] Perera R H, Hernandez C, Zhou H, Kota P, Burke A and Exner A A 2015 Ultrasound imaging beyond the vasculature with new generation contrast agents Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 7 593
[13] Tong H, Wang L, Guo Y, Li L, Fan X, Ding J and Huang H 2013 Preparation of protamine cationic nanobubbles and experimental study of their physical properties and in vivo contrast enhancement Ultrasound Med. Biol. 39 2147
[14] Wang L, Li L, Guo Y, Tong H, Fan X, Ding J and Huang H 2013 Construction and in vitro/in vivo targeted of PSMA-targeted nanoscale microbubbles in prostate cancer Prostate 73 1147
[15] Fan X, Wang L, Guo Y, Xiong X, Zhu L and Fang K 2016 Inhibition of prostate cancer growth using doxorubicin assisted by ultrasound-targeted nanobubble destruction Int. J. Nanomed. 11 3585
[16] Uemura H, Nakagawa Y, Yoshida K, Saga S, Yoshikawa K, Hirao Y and Oosterwijk E 1999 MN/CA IX/G250 as a potential target for immunotherapy of renal cell carcinomas Br. J. Cancer 81 741
[17] Stillebroer A B, Mulders P F A, Boerman O C, Oyen W J G and Oosterwijk E 2010 Carbonic anhydrase IX in renal cell carcinoma: implications for prognosis, diagnosis, and therapy Eur. Urol. 58 75
[19] Hu M, Wang L, Jiang J, Guo Y, Fan X, Li Z, Yu Z and Zhu T 2017 Preparation and identification of camel nanobodies against human G250 ectodomain *J. Third Mil. Med. Univ.* 39 541

[20] Puente Vázquez J, Alonso Gordoa T, Moreno J, Poma L, Díaz Rubio E, Gomez A, Blazquez J and Gonzalez Larriba J L 2015 New challenges in kidney cancer management: integration of surgery and novel therapies *Curr. Treat. Option Oncol.* 16 337

[21] Goyal A, Sharma R, Bhalla A, Gamanagatti S and Seth A 2018 Comparison of MDCT, MRI and MRI with diffusion-weighted imaging in evaluation of focal renal lesions: the defender, challenger, and winner *Indian J. Radiol. Imaging* 28 27

[22] Wei J, Zhao J, Zhang X, Wang D, Zhang W, Wang Z and Zhou J 2018 Analysis of dual energy spectral CT and pathological grading of clear cell renal cell carcinoma (ccRCC) *Plos One* 13 e195699

[23] Zhu L, Wang L, Liu Y, Xu D, Fang K and Guo Y 2018 CAIX aptamer-functionalized targeted nanobubbles for ultrasound molecular imaging of various tumors *Int. J. Nanomed.* 13 6481

[24] Wang L, Zhang M, Tan K, Guo Y, Tong H, Fan X, Fang K and Li R 2014 Preparation of nanobubbles carrying androgen receptor siRNA and their inhibitory effects on androgen-independent prostate cancer when combined with ultrasonic irradiation *Plos One* 9 e96586

[25] Fan X, Wang L, Guo Y, Tong H, Li L, Ding J and Huang H 2013 Experimental investigation of the penetration of ultrasound nanobubbles in a gastric cancer xenograft *Nanotechnology* 24 325102

[26] Ma J, Xu C S, Gao F, Chen M, Li F and Du L F 2015 Diagnostic and therapeutic research on ultrasound microbubble/ nanobubble contrast agents *Mol. Med. Rep.* 12 4022

[27] Kijanka M, Dorresteijn B, Oliveira S and van Bergen En Henegouwen P M 2015 Nanobody-based cancer therapy of solid tumors *Nanomedicine* 10 161

[28] Gonzalez-Sapienza G, Rossotti M A and Tabares-da R S 2017 single-domain antibodies as versatile affinity reagents for analytical and diagnostic applications *Frontiers Immunol.* 8 977

[29] Kruwel T, Nevoltris D, Bode J, Dullin C, Baty D, Chames P and Alves F 2016 *In vivo* detection of small tumour lesions by multi-pinhole SPECT applying a (99 m) Tc-labelled nanobody targeted the epidermal growth factor receptor *Sci. Rep.* 6 21834

[30] Hernt S et al 2012 Nanobody-coupled microbubbles as novel molecular tracer *J. Control. Release* 158 346

[31] Liu L, Ye Q, Lu M, Chen S T, Tseng H W, Lo Y C and Ho C 2017 A new approach to deliver anti-cancer nanodrugs with reduced off-target toxicities and improved efficiency by temporarility blunting the reticuloendothelial system with intralipid *Sci. Rep.* 7 16106

[32] Logsdon D P et al 2016 Regulation of HIF1alpha under hypoxia by APE1/Ref-1 impacts CA9 expression: dual targeted in patient-derived 3D pancreatic cancer models *Mol. Cancer Ther.* 15 2722

[33] Nakamura H, Jun F and Maeda H 2015 Correction to: development of next-generation macromolecular drugs based on the EPR effect: challenges and pitfalls *Expert Opin. Drug Deliv.* 12 691

[34] Gao Y, Hernandez C, Yuan H X, Lilly J, Kota P, Zhou H, Wu H and Exner A A 2017 Ultrasound molecular imaging of ovarian cancer with CA-125 targeted nanobubble contrast agents *Nanomedicine* 13 2159

[35] Perera R H, Wu H, Peiris P, Hernandez C, Burke A, Zhang H and Exner A A 2017 Improving performance of nanoscale ultrasound contrast agents using N, N-diethyacyrlamide stabilization *Nanomedicine* 13 59

[36] Duan S, Guo L, Shi D, Shang M, Meng D and Li J 2017 Development of a novel folate-modified nanobubbles with improved targeted ability to tumor cells *Ultrason. Sonochem.* 37 235