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● *Original Contribution*

DUAL-TARGETED MICROBUBBLES SPECIFIC TO INTEGRIN αVβ3 AND VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR 2 FOR ULTRASONOGRAPHY EVALUATION OF TUMOR ANGIOGENESIS

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Abstract—Aggressive tumors are characterized by angiogenesis that promotes the migration and dissemination of tumor cells. Our aim was to develop a dual-targeted microbubble system for non-invasive evaluation of tumor angiogenesis in ultrasound. Avidinylated microbubbles were conjugated with biotinylated arginylglycylaspartic acid and vascular endothelial growth factor receptor 2 (VEGFR2) antibodies. Subcutaneous MHCC-97H liver carcinoma models were established. Non-targeted, αvβ3-targeted, VEGFR2-targeted and dual-targeted microbubbles was intravenously injected in series while acquiring ultrasound images of the tumor. The microbubbles were destroyed by a high-mechanical-index pulse 4 min after the injection. Peak intensity (PI) before and after the destructive pulse was recorded to compare contrast enhancement by different microbubbles. The targeting rates of the integrin-targeted, VEGFR2-targeted and dual-targeted groups were 95.02%, 96.04% and 94.23%, respectively, with no significant differences. Tumors in all groups were significantly enhanced. The time–intensity curve indicated no significant differences in arrival time, PI, area under the curve, amplitude and mean transit time. The difference in ultrasound signal intensity before and after the destructive pulse (⊿PI) for all targeted microbubble groups was significantly greater than that for the non-targeted microbubble group (all p values < 0.05), and the difference for the dual-targeted microbubble group was significantly greater than those of both mono-targeted groups ($p < 0.05$). (E-mail: puguang61@126.com)

Key Words: **Targeted ultrasound microbubbles, Biotin–avidin, Vascular endothelial growth factor receptor 2, Integrin αvβ3, Tumor angiogenesis.**

INTRODUCTION

Targeted ultrasound contrast agents enhance the detection sensitivity and specificity of cancer using ultrasonography (Borkowetz et al. 2017). Microbubble-based ultrasound contrast agents are chemically stable microparticles 1 to 10 μm in diameter (Barua et al. 2014). Microbubbles conjugated with peptides or antibodies facilitate targeted ultrasound molecular imaging, allowing evaluation of biological processes associated with cancer metastasis.

Ultrasonography, mammography, computed tomography (CT), magnetic resonance imaging (MRI) and combined positon-emission tomography (PET)/CT are common non-invasive pre-operative methods used in the diagnosis of axillary lymph nodes. Vascular endothelial growth factor receptor 2 (VEGFR2) and integrin αvβ3 are two important biomarkers closely correlated with tumor angiogenesis (Bachawal et al. 2015; Wei et al. 2014; Yan et al. 2015; Zhou et al. 2016; Zuo et al. 2017). During cancer progression, tumor cells secret vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs) and other factors to promote tumor neovascularization. In addition, tumor cells express VEGFRs, integrin receptors, and so forth to activate intracellular signaling pathways associated with vascular growth (Presta et al. 2017; Wei et al. 2017). High levels of both integrin receptor and VEGFR2 have been found in the epithelium of tumor neovasculature.

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VEGF/VEGFR2 signaling is critical for vessel development during tumorigenesis. VEGFR2 is present in both liquid and solid tumors, such as leukemia, non-small cell lung cancer (NSCLC), gastric cancer and breast cancer (Goel and Mercurio 2013). In breast cancer, VEGFR2 has been reported to promote the proliferation, survival and metastasis of breast cancer cells *in vitro* and *in vivo*. The tumor-promoting effects of VEGF/VEGFR2 have been reported in other cancer types, including neuroblastoma, prostate cancer and hepatocellular carcinoma (Lee et al. 2015). Together, these studies suggest that the VEGF signaling axis is involved in multiple aspects of cancer development and may be a good prognostic and therapeutic target for cancer patients. Many monoclonal antibodies targeting VEGF and VEGFR have been developed, for example, bevacizumab (Avastin, Genentech, San Francisco, CA, USA), aflibercept (Zaltrap, Regeneron Pharmaceuticals, Tarrytown, NY, USA) and ramucirumab (Cyramza, ImClone Systems Inc., Bridgewater, NJ, USA) (Lupini et al. 2015; Wang and Jia 2016).

As far as is known, there are 24 human integrin subtypes and 8 integrin dimers: αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, α5β1, α8β1 and αIIbβ3, which can recognize the arginylglycylaspartic acid (RGD) motif within extracellular matrix (ECM) proteins and play important roles in cancer and their metastasis (Schittenhelm et al. 2013). Among the 8 integrin dimers, integrin αvβ3 was the first to be identified and was proven to be involved in regulation of cell contractility, cell/ECM stiffness and cell movements. In tumors, αvβ3 expression also provoked increased chemokine receptor expression, leading to enhanced migration/invasion during tumor cell metastasis (Upheber et al. 2015).

Because VEGFR2 and integrin are highly expressed in tumor tissues, they serve as valuable targets for ultrasound molecular imaging and tumor treatment (Lopes-Aguiar et al. 2017; Zang et al. 2017). Our previous research indicated that molecular ultrasound imaging with VEGFR2-targeted microbubbles is non-invasive, feasible and reproducible for the assessment of tumor angiogenesis. Studies on the microbubbles targeted to VEGFR2 or integrin αvβ3 have been developed. However, less work has been done to develop ultrasound microbubbles targeted to both biomarkers. In the study described here, our aim was to develop dual-targeted microbubbles for imaging of tumor vessels.

METHODS

Ethical approval

This study was approved by the ethics committee of Zhongshan Hospital of Fudan University, Shanghai, China.

Materials

The hepatocellular carcinoma (HCC) cell line MHCC-97H was obtained from the Shanghai Institute of Liver Diseases, Zhongshan Hospital, Fudan University (Shanghai, China). A total of 10 male BALB/c-nu/nu nude mice (6 wk old) were provided by the Experimental Animal Centre of Fudan University (Shanghai, China). All mice were housed in a 25°C specific pathogen-free (SPF) feeding room under a 12-h light/12-h dark cycle and were allowed free eating and drinking. Dulbecco's modified Eagle's medium for cell culture, fetal bovine serum and cyan–streptomycin solution were all from Gibco (Waltham, MA, USA); USphere Labeler perfluorocarbon microbubbles were from Trust Bio-sonics (Zhubei, Hsinchu, Taiwan); fluorescein isothiocyanate (FITC)-labeled mouse anti-rat IgG2, biotinylated RGD cyclic peptide and VEGFR2 antibody were from Biolegend (San Diego, CA, USA); and peridinin–chlorophyll–protein complex (PerCP)-conjugated cyclic peptide antibody was from Boster (Wuhan, Hubei, China).

Microbubble preparation and particle diameter measurement

The USphere Labeler microbubbles were transferred into the high-speed oscillator at room temperature and oscillated for 40 s for avidin labeling. After labeling, a microbubble solution of approximately 2.5×10^{10} bubbles/mL was produced according to the manufacturer's recommendations. Microbubbles were then harvested and mixed with the biotinylated VEGFR2 monoclonal antibody and biotinylated cyclic peptide either separately or simultaneously at room temperature. The mixing ratio of 0.8 nmol antibody/mL USphere Labeler microbubbles was adopted. After gentle mixing for 15 min at room temperature, the modified microbubbles were diluted with phosphate-buffered saline solution, washed at 4 °C and centrifuged at 1000 rpm/min for 3 min. Thereafter, the supernatant was discarded. Microbubbles were rinsed three times. The resultant microbubbles were diluted 1000 times with phosphate-buffered saline solution before particle diameter measurement.

Immunofluorescence staining and flow cytometry

Microbubbles of each group were characterized by fluorescence imaging and flow cytometry. RGD cyclic peptides were labeled with PerCP, and VEGFR2 antibodies were labeled with FITC-labeled mouse anti-rat IgG2a, respectively. Florescence-labeled non-targeted microbubbles, microbubbles targeted at integrin-αvβ3 (αvβ3-targeted) and VEGFR2 (VEGFR2-targeted), as well as microbubbles targeted to both integrin αvβ3 and VEGFR2 (dual-targeted) were prepared with the aforementioned methods in a dark environment. A small volume of microbubbles was diluted 1000 times for examination of morphology