Integrin α6 Targeted Near Infrared Fluorescent Imaging and Photoacoustic Imaging of Hepatocellular Carcinoma in Mice

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Received: 15 September 2021 | Revised: 15 February 2022 | Accepted: 17 March 2022 | Published: 12 April 2022

Abstract

Background and Aims: Hepatocellular carcinoma (HCC) is the fourth most common cause of cancer-related death and ranks sixth in terms of incident cases worldwide. The purpose of this study was to develop an effective and sensitive method to distinguish liver cancer tissues from normal tissues in HCC patients. Integrin α6 is a promising cell surface target for molecular imaging of HCC, where it is overexpressed and is a prognostic biomarker. We previously identified an integrin α6-targeted peptide CRYWDENAC (RWY) that has been used for positron emission tomography (PET) imaging of HCC in mouse models. Methods: We labeled the integrin α6-targeted RWY peptide with cyanine 7 (Cy7) to form an optical probe (Cy7-RWY) for near infrared fluorescent (NIRF) and photoacoustic (PA) imaging in HCC. Mice transplanted with subcutaneous HCC-LM3 or orthotopic HCC-H22 cells that overexpressed integrin α6 were intravenously injected with Cy7-RWY and its corresponding Cy7-control. NIRF and PA images of mice were collected from 0 to 48 h after injection. Results: Both NIRF and PA signals started to accumulate in the tumor 2 h after injection of Cy7-RWY and peaked at 24 h. Conclusions: Cy7-RWY is a promising optical probe for NIRF and PA imaging of HCC in mice, and has potential clinical application for HCC detection.

Introduction

Hepatocellular carcinoma (HCC) accounts for the majority of primary liver cancers. Worldwide, liver cancers are the fourth most common cause of cancer-related death and rank sixth in incident cases. According to GLOBOCAN 2018, the incidence of liver cancer is increasing, with an estimated 841,000 new cases and 782,000 deaths worldwide. Because of the absence of early symptoms and limitations of conventional imaging modalities for detection, the majority of HCC patients are diagnosed at an unresectable advanced stage with an extremely poor prognosis. Therefore, more effective methods for HCC detection are urgently required.

Targeted molecular imaging with high specificity and sensitivity is an attractive technique for HCC detection that has achieved promising results in animal models in recent studies. Near infrared fluorescent (NIRF) imaging (650–900 nm) is a noninvasive approach with high sensitivity and specificity imaging ability. It is widely used in basic science, medical research, and drug development. However, poor spatial resolution and insufficient depth penetration limit its application. Photoacoustic (PA) imaging is a novel noninvasive imaging modality combining the advantages of optical and acoustic imaging. It generates three-dimensional images that provide excellent spatial resolution in living organisms. Photoacoustic imaging (PAI) uses either endogenous signals like hemoglobin or an exogenous targeted contrast agent that is typically used for molecular imaging. The potential application of PA imaging in cancers has attracted intensive research interest.

In recent years, a series of side-viewing PA imaging systems have been developed and validated on small animal models, including in situ gastric cancer detection, melanoma in rat colorectum in vivo, and HCC xenograft tumors in vivo.

Integrin α6, an adhesion molecule located on the cell surface, and it is involved in cell attachment and multiple aspects of cancer invasion and metastasis. Many studies provide evidence that the integrin α6 is overexpressed in HCC tissues.
patients with particularly poor survival, and that it appears to have a significant role in tumor progression. We previously reported that integrin α6 overexpression occurs in approximately 94% of clinical early-stage HCCs and in mice is overexpressed in subcutaneous HCC-LM3 tumors compared with liver tissue. Several pathways have also been found to be involved in the function of integrin α6 in HCC development, such as the MAPK/ERK and PI3K/akt signaling pathways. The evidence suggests that integrin α6 is a potential target for molecular imaging in HCC detection. Among the molecular-targeted imaging probes, peptides have advantages of small size, low toxicity, highly efficient clearance from nontarget tissue, and deep penetration into tissue, which are of great interest. Some of the peptides that have been investigated and successfully used for tumor receptor imaging and cancer detection/targeted therapeutics are Arg-Gly-Asp (RGD), In-DTPA-octreotide, and anti-Her2/neu peptide (AHNP).

We previously identified a tumor-targeted peptide CRWYDENAC (RWY) by phage display technology and confirmed that it binds to integrin α6 with high specificity and affinity and can be used for nasopharyngeal carcinoma-specific nanotherapeutics. We previously identified a tumor-targeted peptide CRWYDENAC (RWY) by phage display technology and confirmed that it binds to integrin α6 with high specificity and affinity and can be used for nasopharyngeal carcinoma-specific nanotherapeutics. Recently, we translated this integrin α6-targeted peptide into a positron emission tomography (PET) probe, 18F-RWY, and explored its application for PET imaging of HCC in four different mouse HCC models. We also successfully constructed RWY peptide as an MR contrast agent, RWY-dL-(Gd-DOTA)4, for magnetic resonance (MR) imaging of HCC.

Methods

Peptide synthesis

The integrin α6-targeted fluorescent peptides (Cy7-RWY and Cy5-RWY) were synthesized by solid-phase chemistry and were purchased from the Chinese Peptide/Protein Core Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum and 1% pen/strep (100 U/mL penicillin and 100 U/mL streptomycin) and incubated at 37°C and 5% CO2.

Balg/c nude mice (18±2 g, 4 weeks of age) were obtained from the Animal Center of Vital River, Charles River China (Beijing, China). To obtain orthotopic transplantation tumor models, 1×106 of luciferase-tagged HCC-LM3 cells in 50% Matrigel (BD Biosciences, San Jose, CA) were injected into the left lateral lobe of the liver. For subcutaneous transplantation tumor models, 4×106 of HCC-LM3 or HCC-H22 cells were administered by subcutaneous injection in the right flank of mice. The growth of liver cancer was monitored by bioluminescence imaging.

Cytotoxicity assay

The in vitro cytotoxicity of Cy7-RWY peptide was determined by cell counting Kit-8 assay (CCK-8). Briefly, H22 cells were seeded in 96-well plates at a density of 3×104 cells in 100 µL of medium per well. After 24 h, cells were incubated with concentrations of Cy7-RWY peptide ranging from 0 to 500 nM for 48 h or 72 h. For assay, 10 µL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added to each well, and the cells were incubated for an additional 2 h. Absorption was measured at 450 nm by a TECAN Infinite M1000PRO microplate reader (TECAN, Victoria, Austria).

In vitro cellular uptake

For confocal fluorescence microscopy, HCC-LM3 cells were seeded on 24-well glass coverslips. After 24 h of attachment, cells were incubated with Cy7-RWY (6 nM) for 2 h, washed with phosphate buffered saline (PBS) three times, and fixed with 4% paraformaldehyde for 10 min. The cells were permeabilized with 0.2% Triton X-100/PBS for 20 min, blocked with 5% bovine serum albumen, and incubated with primary antibodies against integrin α6 (dilution 1:250; Cell Signaling Technology) overnight at 4°C. Slides were then washed and incubated with fluorescein-conjugated goat anti-rabbit secondary antibodies (dilution 1:1,000; Cell Signaling Technology) for 1 h at 37°C. Nuclei were stained with diamidino-phenylindole (dilution 1:1,000; Cell Signaling Technology) for 1 h at 37°C. Confocal microscopy was performed at various times after injection with an IVIS Imaging Spectrum System and IVIS 4.2 Living Imaging software (Perkin Elmer; Waltham, MA, USA).

PA imaging

PA imaging was performed with an Endra Nexus 128 PA tomography system (Endra, Michigan, USA) that provides wavelengths from 680 to 900 nm. The PA signals of Cy7-RWY at different concentrations were determined with a laser pulse of 750 nm. During image acquisition, a bowl-like tray with a dimple in the center was used for immobilizing the imaging positions in the center and filled with 1 mL water. Nude mice with subcutaneous HCC-LM3 tumors were placed in a water bath and anesthetized by intraperitoneal
injection of pentobarbital sodium (40 mg/kg). Cy7-RWY was intravenously injected into the tail vein (20 nM, 150 µL in PBS). PA imaging at 750 and 800 nm was performed before injection and at various times after injection. For ex vivo PA imaging, mice were sacrificed and the harvested organs were immediately placed in the PA imaging system.

**RWY biodistribution**

Tumor-bearing mice were sacrificed 48 h after injection of Cy7-RWY. The tissues were homogenized in PBS buffer and the homogenate was passed through 40 µm cell strainers to obtain clear tissue solutions. The distribution of RWY in the various organs was measured by Cy7 fluorescence and flow cytometry. The imaging studies were performed when the tumor volumes were about 100 mm³. The NIRF images of mice were obtained with the in vivo imaging system, a 740 nm excitation wavelength and a 780 nm filter. Immediately after imaging, the mice were sacrificed and heart, liver, spleen, kidney, lung, brain, and tumor were harvested for ex vivo imaging as described above.

**Bioluminescence imaging**

Bioluminescence imaging of luciferase-tagged HCC-LM3 cells or tumors was performed with a Xenogen IVIS Spectrum Imaging System and analyzed with Living Image software (PerkinElmer) by measurement of photon flux in the tumor region of interest in mice. Data were normalized to the background signal.

**Immunohistochemistry**

After sacrifice, the major organs were fixed in 4% formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. After blocking nonspecific antigens with 5% fetal bovine serum, sections were incubated overnight at 4°C with a primary antibody against integrin α6 (Abcam, USA). After washing with PBS Tween three times, sections were incubated with a secondary antibody for 1 h at room temperature and observed with a digital microscope (Leica QWin).

**Statistical analysis**

PA signal intensity was measured by region of interest (ROI) analysis with IVIS 4.2 Living Imaging software and the results were reported as means±SD. The statistical calculations were performed with GraphPad Prism v.5 (GraphPad Software, Inc.; San Diego, CA, USA) and p < 0.05 was considered to be statistically significant.

**Results**

**Binding of Cy7-RWY peptide to HCC cells**

The expression of integrin α6 in HCC cell lines was evaluated by western blotting. As shown in Figure 1A, integrin α6 was highly expressed in HCC cell lines. Fluorophore Cy7 was used to label the N-terminus of RWY peptide to form an imaging probe (Cy7-RWY, Supplementary Fig. 2). The binding affinity of Cy7-RWY was confirmed by confocal microscopy using HCC-LM3 cells. Strong red fluorescence signals of the Cy7-RWY were observed in HCC-LM3 cells, whereas the Cy7-control without targeting, showed no fluorescence response (Fig. 1B). Flow cytometry analysis was further performed to investigate the binding ability of Cy7-RWY. Cy7-RWY peptides displayed significantly higher cellular uptake than nontargeted peptides (Fig. 1C). Cytotoxicity of Cy7-RWY was evaluated by CCK-8. As shown in Supplementary

Fig. 1. Integrin α6-target probe Cy7-RWY binds to HCC cells. (A) The expression of integrin α6 in the HCC cell lines. (B) Colocalization of Cy7-RWY peptide and integrin α6 in HCC-LM3 cells imaged by confocal microscope. (C) Flow cytometry assay of the binding affinity of Cy7-RWY to HCC cells. HCC, hepatocellular carcinoma.
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Figure 3, no significant cytotoxicity was observed in H22 cells at increasing concentrations of Cy7-RWY, indicating the biocompatibility of the peptide. The results showed that Cy7-RWY had a high binding affinity to HCC cells in vitro.

**Biodistribution and tumor-targeting ability of Cy7-RWY**

NIRF imaging was performed to evaluate the tumor-targeting ability of Cy7-RWY in vivo using a subcutaneous HCC-LM3 tumor model. Figure 2A shows NIRF imaging of tumors before and after injection of Cy7-RWY. A strong fluorescence signal intensity was observed in the tumor site but little accumulation of fluorescence signals was observed in the Cy7-control group. In an ex vivo analysis, the mice were sacrificed 24 h post-injection for harvesting and imaging of tumor tissue and organs (heart, liver, spleen, kidneys and brain) for IVIS Lumina XR system quantitative biodistribution analysis. The biodistribution of Cy7-RWY and Cy7-control in the corresponding mice groups was evaluated by drawing ROI along the excised tumors and organs. As shown in Figure 2B, consistent with the in vivo observation, a strong signal was exhibited by tumors treated with Cy7-RWY peptide, indicating selective targeted of the tumor site. Quantitative analysis of the accumulation of dye intensity in organs is shown in Figure 2C. These results show that Cy7-RWY effectively and specifically accumulated in tumors.

In vivo fluorescence imaging of the HCC orthotopic transplantation tumor model carried out at various times showed that the fluorescence intensity reached the strongest level in orthotopic HCC-LM3 tumors at 24 h after systemic delivery (Supplementary Fig. 4). Figures 3 and Supplementary Figure 5 also show the localization of Cy7-RWY in orthotopic HCC-H22 tumors by in vivo fluorescence imaging. After the mice were sacrificed, ex vivo fluorescence images of tumor tissue and major organs from mice were obtained 48 h after injection. The high level of the fluorescence signal found in the tumors of the mice that received Cy7-RWY suggested effective accumulation of the peptide in the tumor at the time of observation. After the imaging procedures, we observed strong staining of integrin α6 of tumor sections by immunohistochemistry (Supplementary Fig. 6). Confocal examination of tumor sections found significant colocalization of Cy7-RWY (red fluorescence) and integrin α6 receptor in HCCs from mice (Supplementary Fig. 7), which confirmed highly efficient tumor targeting by Cy7-RWY. Major organs were harvested to analyze the biodistribution of Cy7-RWY. Flow cytometry revealed that, compared with the control group, liver cancer lesions had significantly higher fluorescence signals (Supplementary Fig. 8). We evaluated the in vivo toxicity of the imaging dose of Cy7-RWY. H&E staining did not find significant cell and tissue damage in major organs including the heart, spleen, lung, kidney and brain compared with the control group (Supplementary Fig. 9). We further assessed the biosafety of Cy7-RWY peptide in excess dose in normal mice and mice bearing subcutaneous HCC-LM3 tumors by routine blood examination (Supplementary Fig. 10) and H&E staining of the heart, liver, spleen, lung, and kidney (Supplementary Fig. 11). The results provided strong evidence of the high tumor-targeting efficiency of the Cy7-RWY peptide.

**Photoacoustic properties of Cy7-RWY peptide**

Cy7-RWY was used as an integrin α6 tracker for PAI in vivo
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In the UV-vis spectrum, with increasing excitation, Cy7-RWY yielded a characteristic peak at 744 nm at a concentration of 100 nM (Fig. 4B). Photoacoustic signals at different concentrations of Cy7-RWY increased as the Cy7-RWY concentration increased (Fig. 4C), and the fluorescence intensity of Cy7-RWY was positively correlated with Cy7 concentration (Fig. 4D). The results support the use of Cy7-RWY as a probe for biological applications.

Photoacoustic imaging with Cy7-RWY in mice with subcutaneous HCC

We performed in vivo PA imaging in mice bearing subcutaneous HCC-LM3 cells at various times. Mice were intravenously injected with Cy7-RWY, followed by PAI before injection, and at 1, 2, 4, 6, and 24 h after injection. The photoacoustic signal was calculated by drawing an ROI around the tumor. As shown in Figure 5A, before injection only large blood vessels in the tumor could be visualized using hemoglobin as an endogenous contrast agent (at 750 nm and 800 nm). One hour after intravenous injection of Cy7-RWY, we found consistently increasing photoacoustic signals, indicating that Cy7-RWY passively targeted tumor tissues in a short period of time. The PA signals reached maximum accumulation at 24 h for Cy7-RWY (Fig. 5A), indicating prolonged circulation time in the bloodstream. Imaging of tumor blood vessels was possible with an endogenous hemoglobin agent at 800 nm. Photoacoustic signals were observed in the important metabolic organs, such as the liver and kidney (Fig. 5B). Quantitative analyses of photoacoustic signal enhancement in the tumor regions at different times after injection (Fig. 5C) provided substantial evidence for the tumor-targeting ability of Cy7-RWY.

Discussion

Previously, we used phage display technology to identify a tumor-targeted peptide RWY, and confirmed its target as integrin α6. In this study, we developed an integrin α6-targeted NIRF and PA imaging agent, Cy7-RWY and focused on its use in HCC. We confirmed that integrin α6 was expressed at a relatively high level in all HCC cell lines, and that the RWY peptide specifically bound to HCC cells in vitro. The integrin α6-overexpressing HCC cells HCC-LM3 and HCC-H22 were used to establish subcutaneous and ortho-
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**Fig. 4.** Characteristics of the Cy7-RWY peptide. (A) Schematic of the photoacoustic imaging system. (B) UV-vis-NIR spectra of Cy7-RWY peptide. (C) PA signals with different concentrations of Cy7-RWY at 760 nm and the linear relationship between PA signal intensity and the different concentrations of Cy7-RWY. (D) The near infrared fluorescence signal of the Cy7-RWY. PA, photoacoustic.

**Fig. 5.** Photoacoustic imaging in mice bearing subcutaneous HCC-LM3 tumors. (A) In vivo PA imaging of the tumor areas before (0 min) and 1, 2, 4, 6, and 24 h after injection of Cy7-RWY. (B) Ex vivo quantification of PA signals of major organs and tumors from mice 24 h post-injection. (C) Quantitative analyses of PA signals of tumor tissues in vivo. Student’s t-test, n=3, ***p<0.001. PA, photoacoustic.
tropic HCC tumors in mice. In vivo PA/NIRF imaging showed Cy7-RWY effectively and specifically accumulated in HCC tumors. The data indicate that Cy7-RWY has potential as an agent for PA imaging of α6-overexpressing HCCs.

Cell targeting peptides are a class of targeting moieties used as delivery vehicles for HCC diagnosis and imaging. Recent advances in developing different targeted peptides provide exciting new possibilities for targeted therapy in HCC. Using mass spectrometry, Lee et al. identified L5 (Thr-Tyr-Phe-Leu-Thr-Arg-Gln) peptide targeting glypican-3 expressing HCC cells, which supports its use as suitable homing moiety for HCC diagnosis and targeted therapy. Du et al. showed that the peptide A54 screened from the phage display library was a novel targeting therapy vector in doxorubicin delivery for HCC target therapy. In addition, LO et al. demonstrated that the conjugation of SP94 and liposomal doxorubicin enhanced the therapeutic efficacy against HCC xenografts.

Molecular imaging is an emerging diagnostic approach for oncology applications. Recently, interest in peptides as probes for dual-modality tumor imaging there has increased. Chen et al. described the use of plectin-1 targeted nanoparticles for fluorescence/MR dual-modal imaging in pancreatic cancer. Dual-modality (MRI/SPECT) molecular imaging was shown to be a powerful diagnostic tool for the early diagnosis of mesothelin-expressing cancers. The results of this study showed that the novel optical probe, Cy7-RWY peptide, bound specifically to integrin α6 in HCC in vitro and in vivo. Cy7, an NIR fluorophore with longer wavelength absorption and emission provides deeper tissue penetration to achieve good signal and should be a strong contrast agent for NIRF and PA imaging. Therefore, optical imaging with the Cy7 labeling method has great potential for the highly sensitive and accurate visualisation of tumor microfoci that overexpress integrin α6. In this study, we found that integrin α6 was expressed at a relatively high level in HCC cell lines. Colocalization of Cy7-RWY and integrin α6 was observed in HCC cells, and fluorescence activated cell sorting supported the results. The in vivo integrin α6 binding specificity was confirmed biodistribution and imaging studies. In both subcutaneous and orthotopic HCC tumor xenografts, the results demonstrated the presence of NIRF signals localized exclusively in the HCC-LM3 or HCC-H22 tumor model in a time-dependent manner with relatively low uptake by nontargeted organs. However, spatial resolution is the major limitation of the technique. Therefore, mice bearing subcutaneous HCC-LM3 tumors were given Cy7-RWY through vein tail to allow systemic circulation and were subjected to photoacoustic radiation at different times. Before injection, there was only a weak PA signal detected in the tumor tissue. After injection, enhanced PA signals were visible in tumor tissue, and they increased gradually with time, indicating good tumor accumulation of Cy7-RWY after systemic administration. Compared with NIRF imaging, PA had significantly higher spatial resolution. The PA signal in the tumor was very strong at 24 h, suggesting high tumor-targeting efficiency. In our study, the fluorescence signal reached a steady level already after 6 h and was sustained until 24 h post-injection, thus providing a broad clinically useful time window for imaging. Imaging agent toxicity is a concern for which there is relatively little data. The Cy7-RWY probe showed no toxicity in major organs including the heart, lung, liver, spleen, and kidney, and had fast renal clearance. Given the data, Cy7-RWY provided extremely high sensitivity for the detection of liver tumor foci. Studies of intraoperative resection, drug carriers, therapy monitoring, and cancer diagnosis will benefit from Cy7-RWY peptide guided imaging.

There are several limitations in our study. Firstly, although the PA signal has good tissue penetration, systemic imaging is rarely done because of the limited scanning range of the machine. It is challenging to use for whole-body imaging as applied in diagnostic systems and intraoperative imaging for clinical applications. Secondly, it is difficult for the targeted Cy7-RWY peptide to detect early HCC lesions with a small diameter without optimization. Thirdly, we did not determine whether Cy7-RWY peptide is suitable for malignant metastatic HCC. We are now exploring the feasibility of our peptide in differentiating tumors from abnormal liver tissues such as fibrosis, and from regenerative nodules.

Conclusions

In summary, we successfully constructed an integrin α6-targeted probe, Cy7-RWY with promising biocompatibility and good optical stability. It is suitable for NIRF and PA imaging of HCC. Upon further development and validation, the Cy7-RWY probe may contribute to the accurate delineation of liver tumor foci and may have wide clinical application in early diagnosis and accurate surgical excision in the future.

Funding

This study was funded by the National Natural Science Foundation of China (Grant: 81972531), Fundamental Research Funds for the Central Universities (Grant: 19ykpy174), GuangDong Basic and Applied Basic Research Foundation (Grant: 2020A1515011374) and GuangDong Basic and Applied Basic Research Foundation (Grant: 202102020138).

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Performance of experiments, collection of data, analysis of results, and writing of the manuscript (YZL, YW, XCY, DHC), provision of technological and associated help (YJP, JD, WJL, MYT, LZ, JMC, MXL, RBL, WGZ), design and supervision of experiments, and modification of the manuscript (JYL and GKF).

Ethical statement

All animal experiment procedures were approved by the Experimental Animal Welfare and Ethical Committee of Sun Yat-Sen University Cancer Center and were performed following the guidelines for the care and use of laboratory animals of Sun Yat-sen University Cancer Center.

Data sharing statement

No additional data are available.

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