Sodium Pump Na⁺⁺/K⁺⁺ ATPase Subunit α1-Targeted Positron Emission Tomography Imaging of Hepatocellular Carcinoma in Mouse Models

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Abstract

Purpose: Positron emission tomography (PET) imaging was not efficiently used in the early diagnosis of hepatocellular carcinoma (HCC) due to the lack of appropriate tracers. Sodium pump Na⁺⁺/K⁺⁺ ATPase subunit α1 (NKAα1) emerges to be a potential diagnostic biomarker of HCC. Here, we investigated the feasibility of ¹⁸F-ALF-NOTA-S3, a PET tracer based on an NKAα1 peptide, to detect small HCC.

Procedures: GEPIA database was searched to obtain the expression characteristics of NKAα1 in HCC and its relationship with the prognosis. PET/CT was performed in orthotopic, diethylnitrosamine (DEN)-induced and genetically engineered HCC mouse models to evaluate the use of ¹⁸F-ALF-NOTA-S3 to detect HCC lesions.

Results: NKAα1 is overexpressed in early HCC with a high positive rate and may correlate with poor survival. In orthotopic, DEN-induced and genetically engineered HCC mouse models, PET/CT imaging showed a high accumulation of ¹⁸F-ALF-NOTA-S3 in the tumor. The tumor-to-liver ratios are 2.56 ± 1.02, 4.41 ± 1.09, and 4.59 ± 0.65, respectively. Upregulated NKAα1 expression in tumors were verified by immunohistochemistry. Furthermore, ¹⁸F-ALF-NOTA-S3 has the ability to detect small HCC lesions with diameters of 2–5 mm.

Conclusions: NKAα1 may serve as a suitable diagnostic biomarker for HCC. ¹⁸F-ALF-NOTA-S3 shows great potential for PET imaging of HCC.

Key words: NKAα1, PET, Hepatocellular carcinoma, Peptide

Introduction

Liver cancer is a major health threat and results in huge disease burden worldwide. It ranks sixth for cancer incidence, fourth for cancer deaths, and is second leading cause of cancer years of life lost globally in 2015 [1]. Hepatocellular carcinoma (HCC) accounts for 75~85% cases of liver cancer [2]. Treatment for HCC patients diagnosed at an early stage (BCLC stage 0 to A) can lead to an average survival of 60 months. However, patients with HCC who have advanced or end-stage disease (BCLC stages C and D) at diagnosis have a poor prognosis, with expected median survival time of 6–8 months (stage C) and 3–4 months (stage D) [3]. Therefore, early diagnosis is one of the keys to improving survival for HCC patients.
Current imaging techniques for clinical diagnosis of liver cancer include ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI). The detection performance of these anatomically based imaging techniques closely correlates with tumor size. HCC ≥ 2 cm in diameter can be accurately localized (approximately 100%) by MRI and CT-enhanced scanning. However, for small HCC 1–2 cm in diameter, the sensitivity of MRI and CT-enhanced scanning gradually decreases, with MRI decreasing by 45–80% and CT decreasing by 40–75%[4]. Moreover, MRI and CT-enhanced imaging cannot detect extrahepatic lesions [5] and lack the ability to predict response to treatment. Positron emission tomography (PET) enables whole-body imaging based on function and metabolism. 2-Deoxy-2[18F]fluoro-d-glucose (18F-FDG) is the most widely used tracer for PET imaging. Yet, in more than half cases of HCC, significant uptake of FDG was not observed [6]. Most well-differentiated HCCs are negative for 18F-FDG PET. Thus, FDG PET scan is not recommended for early diagnosis of HCC. The detection rates of some other existing tracers (such as 11C-choline) are also unsatisfactory [7]. Nowadays, few imaging approaches are perfect for the early diagnosis of small HCC. As a result, more effective imaging methods are needed to improve the diagnosis of HCC.

Na+/K+-ATPase (NKA), an enzyme present in almost all higher eukaryotic cell membranes, is a member of the P-type ATPase family. Under physiological conditions, NKA plays a key role in cellular ion transport, metabolism, migration, and signal transduction [8, 9]. NKA contains 2 subunits, the α (including α1 ~ α4) catalytic subunit and the β (including β1 ~ β3) regulatory subunit [10, 11]. Among them, the α1 subunit, NKAα1, is abnormally overexpressed in a variety of cancers [12–15] and considered a potential target for anticancer drug development [16, 17]. NKAα1 was also found to be overexpressed in HCC, and knockdown of this gene led to cell cycle arrest and apoptosis in HCC cells [18]. These studies suggest that targeting NKAα1 may be of help to the treatment of HCC. Consequently, the development of NKAα1-targeted imaging methods may be used for both detection of HCC and assessment of related therapies in the future. Our previous studies used phage display to screen a breast cancer-target peptide of NKAα1, namely S3. The specific binding of S3 peptide to NKAα1 was validated. PET imaging was successfully performed in breast cancer mouse models after this peptide radio-labeled and the tracer’s in vitro and in vivo stability verified [19]. We believe this targeted S3 peptide could serve as a new approach for the imaging of HCC.

In this study, we first investigated the overexpression of NKAα1 in HCC based on public databases and established literature. Then, we performed PET/CT imaging using the tracer 18F-ALF-NOTA-S3 (dubbed 18F-S3 for short) in orthotopic implantation HCC mice and validated the feasibility of this targeted peptide in PET imaging of HCC. We subsequently performed PET/CT imaging on chemically induced HCC mice, as this model has much in common with human liver cancer in terms of morphology and biochemical features [20]. Lastly, we use genetically engineered HCC mice to further confirmed the feasibility of the tracer detecting small HCC lesions.

Materials and Methods

Cells and Animals

The HCC cell line used in this study is HCC-LM3 cells purchased from the American Type Culture Collection (ATCC). This cell line was cultured with DMEM containing 10% fetal bovine serum. BALB/c mice and C57BL6 mice in this study were purchased from Vital River, Charles River Laboratories China (Beijing, China). The animal experiments were accredited through the Institutional Animal Care and Use Committee (IACUC) in Sun Yat-sen University Cancer Center.

The immune incompetent BALB/c nude mice were used to construct orthotopic implantation HCC models. To facilitate the monitoring of tumorigenesis, HCC-LM3 cells were stably transfected with luciferase to form HCC-LM3-luciferase cells. An amount of 5 × 10^5 HCC-LM3 cells in logarithmic growth phase was passed into a 25-mm culture flask, and 6 ml of DMEM containing 10% fetal bovine serum was added. Five microliters of H7656 pLenti-CBh-3FLAG-luc2-tCMV-mNeonGreen-F2A-Puro (Obio Technology, Shanghai) was added and incubated together for 48 h (with the culture renewed once at 24 h). Puromycin of a final concentration of 2.5 μg/ml was used to screen for resistant cells. After serial passages of the screened cells, bioluminescence was performed to determine whether luciferase was stably expressed. Anesthetized mice were surgically exposing their livers in a sterile environment. Then, the HCC-LM3-luciferase cells were injected orthotopically to the left lobe of the liver under direct observation. One week after surgery, the tumorigenesis of mice was confirmed by bioluminescence imaging.

For the construction of the chemical-induced HCC mouse model, a single dose of diethylnitrosamine (DEN) (200 mg/kg) was administered into male C57BL6 mice to induce HCC. Mice were scanned via MRI to verify the presence of HCC lesions in the livers 36 weeks after injection. H11LNL-Myc knock-in HCC mouse model was developed by Shanghai Model Organisms Center, Inc. This model was generated by CRISPR/Cas9 system in C57BL/6 J mouse background. Briefly, pCAG-loxp-Neo-loxp-Myc-polyA fragment was inserted into the well-defined Igls2 locus (Hipp11 or H11 locus by homology recombination). The LNL-Myc targeting vector was created with the CMV enhancer/chicken beta-actin core promoter (CAG), a loxp-NEO-loxp cassette (LNL), a myc gene, a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), and the SV40 polyA signal. Cas9 mRNA was in vitro transcribed with mMESSAGE mMACHINE7 Ultra Kit (Ambion, TX,
USA) according to the manufacturer’s instructions and subsequently purified using the MEGAclear™ Kit (Thermo Fisher, USA). 5'-ATGATGGCCATCTAATTGAGCT-3' was chosen as Cas9-targeted guide RNA (sgRNA) and in vitro transcribed using the MEGAshortscript Kit (Thermo Fisher, USA) and subsequently purified using MEGAclear™ Kit. The donor vector with sgRNA and Cas9 mRNA was microinjected into C57BL/6 J fertilized eggs. F0 generation mice positive for homologous recombination were identified by long PCR. The PCR products were further confirmed by sequencing. F0 mice were crossed with C57BL/6 J mice to gain H11LNL−Myc heterozygous mice. H11LNL−Myc; Alb-Cre double-positive mice used in this study were generated by crossing H11LNL−Myc heterozygous mice with Alb-Cre transgenic mice. Additional technical details are the same as described in the previous literature [21].

**Preparation of 18F-S3**

NOTA-CSISSLTHC (NOTA-S3) were synthesized by the Chinese Peptide Company (Hangzhou, China) in the following procedure: NOTA bis(tBu)ester and full covered peptide was once dissolved in N,N-dimethylformamide (DMF). Then, 2-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HATU) and N-methylmorpholine (NMM) were introduced to the reaction mixture. The response mixture was stirred at room temperature for 3 h then workup to supply the NOTA-modified peptide which was cleavage with the aid of tallow fatty acid to provide the crude peptide. Last, it was purified via HPLC to provide the final product. The peptide is available as a freeze-dried powder freely soluble in saline solution. Then, the radiolabeling reaction procedure was as follows: the vials containing 8 nmol of NOTA-S3 and 6 nmol of AlCl3·6H2O was added with 65 μl of (NMM) were introduced to the reaction mixture. The response mixture was stirred at room temperature for 3 h then workup to supply the NOTA-modified peptide which was cleavage with the aid of tallow fatty acid to provide the crude peptide. Last, it was purified via HPLC to provide the final product. The peptide is available as a freeze-dried powder freely soluble in saline solution. Then, the radiolabeling reaction procedure was as follows: the vials containing 8 nmol of NOTA-S3 and 6 nmol of AlCl3·6H2O was added with 65 μl of (NMM) were introduced to the reaction mixture. The response mixture was stirred at room temperature for 3 h then workup to supply the NOTA-modified peptide which was cleavage with the aid of tallow fatty acid to provide the crude peptide. Last, it was purified via HPLC to provide the final product. The peptide is available as a freeze-dried powder freely soluble in saline solution.

**PET/CT Imaging**

The mice were fasted from food but not water for 6 h prior to the imaging experiments. Mice were anesthetized with 1.2% tribromethanol solution at a dose of 250 mg/kg and then intravenously injected with approximately 60–100 μCi (2.22–3.70 MBq) of 18F-S3. PET/CT scans were performed after 1–1.5 h of circulation. While waiting, the mice were kept warm by an electric blanket. After acquiring the images, regions of interest (ROIs) were drawn on the tumor and normal organs. The maximum uptake value was calculated automatically, and the tumor boundary was differentiated by a low threshold of 30% of the maximum uptake. The radiation intensity of tumor and normal organs was calculated as the percentage of the injected dose per gram (%ID/g).

**Biodistribution Study**

To investigate the biodistribution of 18F-S3 in vivo, the DEN-induced mice bearing tumors were euthanized 60 min after intravenous injection of 18F-S3. Tumor and normal organs were collected, and the radioactivity was measured by a γ counter (Wallac Wizard 1470, PerkinElmer Inc.). The organs were weighed to calculate their mean organ distribution. Radioactivity was expressed as a percentage of the injected dose per gram [% ID/g, mean ± standard deviation (SD), n = 3].

**Immunohistochemistry**

Samples were fixed with formalin, embedded in paraffin, sectioned, and stained with H&E in accordance with the standard histopathological techniques. Paraffin sections were de-paraffinized with xylene and rehydrated. The sections were processed in citrate buffer (pH 6) and microwaved for antigen retrieval. To block non-specific binding, the sections were incubated with 1% bovine serum albumin after a treatment with 3% hydrogen peroxide in methanol and quenching the endogenous peroxidase activity. The sections were then stained with an NKAα1 antibody (Abcam, M7-PB-E9, 1:150) for 12 h at 4 °C. After washing, the sections were incubated with an HRP-conjugated polyclonal secondary antibody (1:200). The sections were immersed in 3-amino-9-ethyl carbazole, counterstained with 10% Mayer’s hematoxylin, dehydrated, and mounted in crystal mount.

**Statistical Analysis**

Statistical analyses were performed using R 4.0.3. Data were presented as means ± SD. The means were compared using Student’s t test. The significance is given as < 0.05 (*p < 0.05, **p < 0.01, ***p < 0.001).

**Results**

**Overexpression and Prognostic Value of NKAα1 in HCC**

To investigate the overexpression of NKAα1 and its positive rate in HCC, we first analyzed the GEPIA database (http://gepia.cancer-pku.cn/), a website for cancer and normal gene expression profiling and interactive analyses. Retrieval results showed that NKAα1 expression is significantly elevated in liver hepatocellular carcinoma (LiHC) compared to normal liver tissue (Fig. 1a). In a recent research by Jiang et al., paired tumors and adjacent normal liver tissues of...
HBV-related early-stage HCC were characterized [22]. According to the original gene expression profiles of HCC tumor and non-tumor quantified, in which NKAα1 expression was included in 35 pairs of tumor/non-tumor samples, we observed approximately 94% (33 out of 35) of early-stage HCC with NKAα1 overexpression compared with paired normal liver tissues at the RNA level (Fig. 1b). Finally, we linked NKAα1 to the prognosis of HCC by analyzing the GEPIA database. In accordance with a previous study by Zhuang et al. [18], we observed that high NKAα1 correlates with poor overall survival (Fig. 1c) and disease-free survival (Fig. 1d). To sum up, we confirmed the extreme high positive rate of NKAα1 overexpression in clinical early-stage HCC. NKAα1 may serve as a potential diagnostic biomarker for targeted molecular imaging of early-stage HCC.

*Fig. 1. Overexpression and prognostic value of NKAα1 in HCC. a Boxplot of NKAα1 expression in LIHC tumor compared with normal tissue. Red: tumor tissues; blue: normal liver tissues. Student’s t-test, ***p<0.001. b Ninety-four percent of HCC samples with ATP1A1 overexpression. Paired Student’s t-test. ***p<0.001. High NKAα1 expression correlates with poor overall survival (c) and disease-free survival (d) in HCC by analyzing the GEPIA database (http://gepia.cancer-pku.cn/). Gene: NKAα1; datasets: LIHC; group cutoff: median; cutoff high (%): 50%; cutoff low (%): 50%.*

**PET Imaging with ¹⁸F-S3 in Genetically Engineered, DEN-Induced, and Orthotopic HCC Mouse Models and Biodistribution of ¹⁸F-S3 In Vivo**

We radiolabeled the NKAα1-targeted S3 peptide with a radionuclide fluorine-18 (¹⁸F) to form a PET tracer ¹⁸F-S3 (Fig. 2) according to our previous report [19], in which the stability and time activity curves of this tracer were also characterized. The radiochemical yield of ¹⁸F-S3 was about 35% without decay correction. We then performed PET/CT imaging with ¹⁸F-S3 in orthotopic, DEN-induced, and genetically engineered HCC mice.

Seven days after the orthotopic implantation surgery, bioluminescence imaging (Fig. 3a) was performed for
monitoring HCC tumorigenesis. The diameters of the orthotopically implanted HCC-LM3 tumors were in the range of 2 to 5 mm, which were then confirmed by autopsy (Fig. 3b) after the PET imaging experiment. In an orthotopic HCC-LM3-luciferase liver tumor-bearing mouse, representative PET/CT imaging and maximum intensity projection (MIP) image (Fig. 3c) showed high and specific tumor uptake of 18F-S3. In whole-body PET imaging, tumor uptake was second only to the urinary system, and the lesion can be finely distinguished. Quantification indicated that tumor uptake of 18F-S3 was 3.02 ± 0.59%ID/g and background liver uptake was 1.28 ± 0.34%ID/g (Fig. 3d), leading to a tumor-to-liver ratio of 2.56 ± 1.02.

In a DEN-induced HCC mouse (Fig. 4a), representative PET/CT imaging with maximum intensity projection (Fig. 4b) showed 18F-S3 was highly accumulated in the tumor. We quantitatively calculated the tumor uptake of 18F-S3 was 4.61 ± 0.44%ID/g and normal liver uptake was 1.08 ± 0.24%ID/g (Fig. 4c), resulting in a tumor-to-liver ratio of 4.14 ± 1.09. Immunohistochemistry showed significant overexpression of NKAα1 in tumor tissues compared to the adjacent normal liver in DEN-induced HCC (Fig. 4d). Quantitative radioactivity measurement of the main organs and tumors in sacrificed mice (Fig. 4e) indicated the highest radioactive accumulation of 18F-S3 in the kidneys, suggesting it was predominantly cleared by the urinary system. Tracer uptake in tumor tissues was 5.53 ± 0.58, higher than in other normal tissues and organs (including the intestine, bone, liver, heart, lung, muscle, and brain) except for the kidneys. The tumor-to-liver ratio in this analysis was 2.78 ± 0.02 (Fig. 4f). This ratio was lower than that in the PET imaging experiment because of the difference in the measurement time points (60 min vs 90 min). This could suggest that 18F-S3 was cleared more rapidly in the liver than in the tumor. The tumor-to-other-organs ratios ranged from 2.25 ± 0.08 (tumor-to-intestine) to 4.87 ± 0.12 (tumor-to-muscle).

Before we used H11LNL−Myc genetically engineered mice for imaging, the tumorigenesis of these mice had been monitored by primovist (Gd-EOB-DTPA)-enhanced MRI (Fig. 5a), which had shown an approximate 3 mm × 6 mm tumor near the right-lower margin of the liver in a representative mouse. PET/CT and MIP imaging showed that 18F-S3 was highly accumulated in the tumor (Fig. 5b). In quantitative calculation, the tumor and liver uptake of 18F-S3 were 4.45 ± 0.97%ID/g and 0.86 ± 0.09%ID/g, respectively (Fig. 5c). The tumor-to-liver ratio is 4.59 ± 0.65. We dissected the liver of this mouse intact and obtained tumor tissue (Fig. 5d). Immunohistochemistry revealed that in genetically engineered mice of liver cancer, NKAα1 was significantly overexpressed in tumor tissue compared to adjacent normal liver tissue (Fig. 5e).

**Discussion**

Difficulty in the early diagnosis of HCC is one of the major obstacles to improving the prognosis of liver cancer. PET imaging modalities are widely used in the field of oncology for improving the diagnosis and management of cancer patients, because of their unlimited penetration depth, high sensitivity, and quantitative imaging capabilities. In the field of HCC, for example, 18F FDG/11C acetate dual tracer PET imaging emerged as a sensitive tool for diagnosis of HCC and could guide further therapy [23, 24]. However, the efficient use of PET in the early diagnosis of liver cancer has been hampered by the lack of appropriate tracers. Prior to this
In this study, several antibody-based PET imaging agents specifically targeting HCC had been proposed, such as $^{89}$Zr-conjugated monoclonal antibody against GPC3 [25] and CD146 [26]. However, peptide-based tracers may have an advantage over them in PET imaging because of their small size, low immunogenicity, sufficient capillary permeability, and rapid clearance from non-target tissues. Evidence from established literature in the past decade suggests that NKAα1 may be an important target in the treatment of HCC [12, 18, 27]. In this study, we furtherly demonstrated the overexpression of NKAα1 in HCC. As it was observed that high NKAα1 expression was related to poor survival of HCC patients, NKAα1 may also have prognostic value. Since there have been evidence that the cardiac glycosides (CGs) digoxin, digitoxin, and ouabain, which directly inhibit NKAα1 function, could exhibit anticancer effects [28], it is promising that this study may help integrate the diagnosis and treatment of HCC in the future.

As far as we know, none of the currently available mouse models of HCC meet all the criteria of the ideal animal model, which include biologic, genetic, etiologic, and therapeutic criteria [29]. Different animal models have their advantages and disadvantages. Therefore, multiple animal models were used in this study to leverage their strengths. The implantation models are the most accessible and definitive HCC mouse model and are widely used in PET targeting.
Fig. 4. PET/CT imaging with $^{18}$F-S3 in DEN-induced HCC mice. Representative MR (a) and PET/CT (b) imaging in the same mouse. c Quantification of tumor and liver uptakes of $^{18}$F-S3. Student’s $t$ test, **$p < .01$, $n = 3$, mean ± SD. d NKA$\alpha 1$ expression was analyzed using immunohistochemistry (scale bar, 100 μm). e Quantification of $^{18}$F-S3 uptake in normal organs and tumor tissues was calculated as percent ID per gram (mean ± SD, $n = 3$) at 60 min after tracer injection. f Ratio of the tumor to the main organs in DEN-induced mice at 60 min after $^{18}$F-AlF-NOTA-S3 tracer injection ($n = 3$).
research. In this class of models, the orthotopic implantation model has advantages over the subcutaneous implantation model because they mimic human HCCs in a better way with respect to tumor morphology, microenvironment, metastatic potential, and the response to anticancer agents [30, 31]. Our study first adopted this model. Various kinds of external agents contribute to human HCC, which were referred to as hepatocarcinogens, such as aflatoxins, ethanol, and combined oral contraceptives. Some of these are also external carcinogens in mice (e.g., aflatoxin B1 and oral contraceptives) [32]. Although DEN is not considered a carcinogen for humans, it is a genotoxic carcinogen, and its induction in mouse HCC models may share some genetic alteration with human HCC. Our use of the DEN-induced model helps to cautiously extrapolate our findings to human HCC caused by hepatocarcinogens. On the other hand, the accessibility of this model is between orthotopic implantation and transgenic mouse model. So, this non-invasive model was also used in the study of biodistribution, which can avoid bias caused by surgery-related tissue damage and inflammation. As with other types of cancer, the development of HCC requires genetic alterations in various cellular pathways, including those involved in cell growth, apoptosis, proliferation, and angiogenesis. Genetically engineered HCC mouse models are excellent for studies concerning the molecular mechanism of liver cancer development. Although the exact genetic events underlying hepatocarcinogenesis are not fully understood, c-Myc is frequently overexpressed in HCC. In particular, c-Myc is overexpressed in up to 70% of patients with viral and alcoholic HCC [33]. Therefore, our study also includes H11LNL-Myc knock-in HCC mouse model in order to make our conclusions closer to the characteristics of human HCC development from the genetic level.

In all three of the above animal models used in this study, we obtained favorable PET imaging results with the tracer 18F-S3. Good contrast between the tumor and the liver background was observed. The difference is that the tumor uptake of 18F-S3 and the tumor-to-liver ratio in the orthotopic model was lower than that in DEN-induced and genetically engineered mouse models. This may be mainly due to the generally smaller size (diameters of 2 to 5 mm) and more irregular shape of tumors in the orthotopic mice. The latter two HCC models having a tumorigenic process and tumor microenvironment closer to the real one may also contribute to this as well. Another different finding is that the expression of NKAα1 is upregulated to a different extent in DEN-induced and genetically engineered mouse models. More significant overexpression was observed in the genetically engineered HCC mice. The good tumor specificity of this 18F-S3 and its low toxicity to other organs are supported by the biodistribution experiments performed in DEN mice. Of particular note, the detection of lesions with the longest diameter of less than 5–6 mm was achieved with 18F-S3 in multiple mouse models.

Nevertheless, this study has several limitations. More experiments are needed to verify the S3 peptide’s binding affinity of mouse NKAα1 was similar to that of human NKAα1. Besides, we have not succeeded in measuring the binding affinity value of S3 to NKAα1 as the protein is difficult to purify due to its multiple transmembrane and complex structure. In our subsequent research, we plan to measure and compare the binding affinity of S3 with mouse and human NKAα1 using microscale thermophoresis. Quantitative analysis in this study shows that the affinity of 18F-S3 is relatively low compared to the integrin-α6-targeted RWY peptide we previously reported in PET imaging of HCC [21]. This peptide may also be less well prepared than other widely used peptides such as RGD. This is partly because the abovementioned peptides have been intensively

Fig. 5. PET imaging with 18F-S3 in a genetically engineered HCC mouse model. MR (a) and PET/CT (b) imaging with 18F-S3 in the same mouse. c Quantification of tumor and liver uptakes of 18F-S3. Student’s t test, *p < .05, n = 3, mean ± SD. d Photograph of the liver dissected. e NKAα1 expression was analyzed using immunohistochemistry (scale bar, 100 μm and 50 μm).
studied in the PET and MR imaging of a variety of cancers [34–36], and their structures and affinities have been optimized several times. In this experiment, little morphologic changes can be identified by the simultaneous CT images, and we had to rely on MR, bioluminescence imaging, and autopsy to confirm the tumor before or after PET imaging. This may lead to mild inaccuracies in locating the lesion and the assessment of tumor size in PET images. Although these limitations do not undermine the basic conclusions of this study, they will be improved in our future research.

To sum up, we constructed various types of mouse models and used them to validate the feasibility of 18F-S3 in PET imaging of HCC for the first time. Although the imaging results are promising, some limitations exist in this research. In order to promote the further application of this tracer in PET imaging, it needs to be modified and optimized for better affinity and specificity in future research. The safety and the proper radiation dose for imaging of 18F-S3 in HCC should also be further explored.

**Conclusion**

In conclusion, NKAα1 may serve as a suitable diagnostic biomarker for HCC. We reported the use of an NKAα1-targeted peptide-based PET tracer 18F-S3 for the detection of HCC in three kinds of HCC mouse models. The high sensitivity of 18F-S3 and its well-defined feasibility of detecting small HCCs have been demonstrated. 18F-S3 shows good potential for the imaging of HCC, and it may serve as a new approach for the diagnosis of early and small HCC.

**Author Contribution** All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by ST, XCY, and YM. ST, JCY, XFZ, and GKF performed the experiments. The whole experiment was conducted under the guidance of WGZ, XZ, and WF. The first draft of the manuscript was written by ST and XCY. All authors commented on the previous versions of the manuscript and approved the final manuscript.

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**Declarations**

**Ethics Approval and Consent to Participate** All animal studies were approved by the Animal Care at Sun Yat-sen University Cancer Center and in accordance with its guidelines.

**Conflict of Interest** The authors declare that they have no conflict of interest.

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