Chemicals and Reagents

All chemicals were purchased from Energy Chemical Co. and Nanchang Tanzhen Biological Technology Co., Ltd.; FAPI-46 was purchased from C.S. Bio. Anti-FAP mAb was purchased from Abcam (Cat. No. ab207178) and Abclonal (Cat. No. A6349). The human hepatocellular carcinoma (HCC) cell line Huh7 was purchased from the China National Infrastructure of Cell Line Resource. The CAFs from surgical specimens of HCC patients were kindly provided by Dr. Dongyan Shen (The First Affiliated Hospital of Xiamen University, Xiamen University, China). The labeling efficiency and radiochemical purity were tested using a radio-TLC scanner (MSFC1-00220, Eckert & Ziegler) Dionex Ulti-Mate 3000 high-performance liquid chromatography (HPLC; Thermo Scientific), an SPD-20A UV detector (λ = 254 nm), and an Elysia Raytest Gabi Star γ-radiation detector. Radioactivity (counts per minute) was measured using a γ-counter (WIZARD 2480; Perkin-Elmer) and CRC-25R dose calibrators (CAPIN-TEC Inc.).

Procedure of DOTA-2P(FAPI)$_2$ synthesis

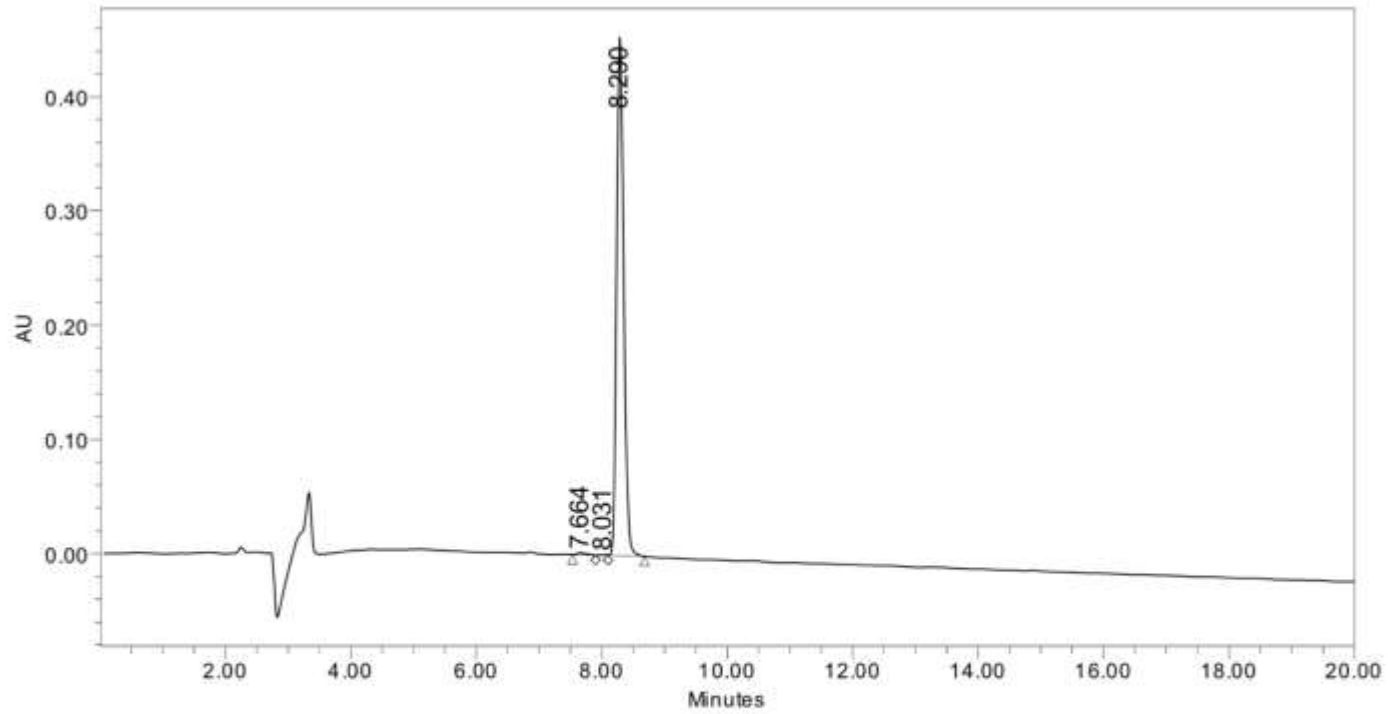
Compound 2 (500 mg, 1 mmol) and Boc-PEG3-OH (338 mg, 1.1 mmol) in DMF (3 mL) were treated with HATU (570 mg, 1.5 mmol) and DIPEA (710 mg, 5.5 mmol). The reaction mixture was stirred for 16 h at 80°C. After completion of the reaction, the mixture was transferred into an ice bath and TFA (1.15 mL, 15 mmol) was added dropwise. After another 3 h of stirring at room temperature, the mixture was adjusted to pH=5.5 by NaOH solution and purified by prep-HPLC to afford 200 mg of compound 3. LC-MS (ESI+): m/z 689.6 [M+H]+, 345.4 [M+2H]+/2.

Compound 3 (68.9 mg, 0.1 mmol) and compound 4 (56 mg, 1.1 mmol) in DMF (1 mL) were treated with HATU (57 mg, 0.15 mmol) and DIPEA (71 mg, 0.55 mmol). The reaction mixture was stirred for 8 h at 80°C. After completion of the reaction, the mixture was transferred into an ice bath and TFA (0.2 mL, 1.8 mmol) was added dropwise. After another 1 h of stirring at room temperature, the mixture was adjusted to pH=5.5 by NaOH solution and purified by prep-HPLC to afford compound 5 (50 mg). LC-MS (ESI+): m/z 1039.9 [M+H]+, 520.5 [M+2H]+/2.

Compound 5 (30 mg, 0.028 mmol) and compound 6 (20 mg, 0.035 mmol) were dissolved in DMF (1.5 mL) and treated with HATU (16 mg, 0.042 mmol) and

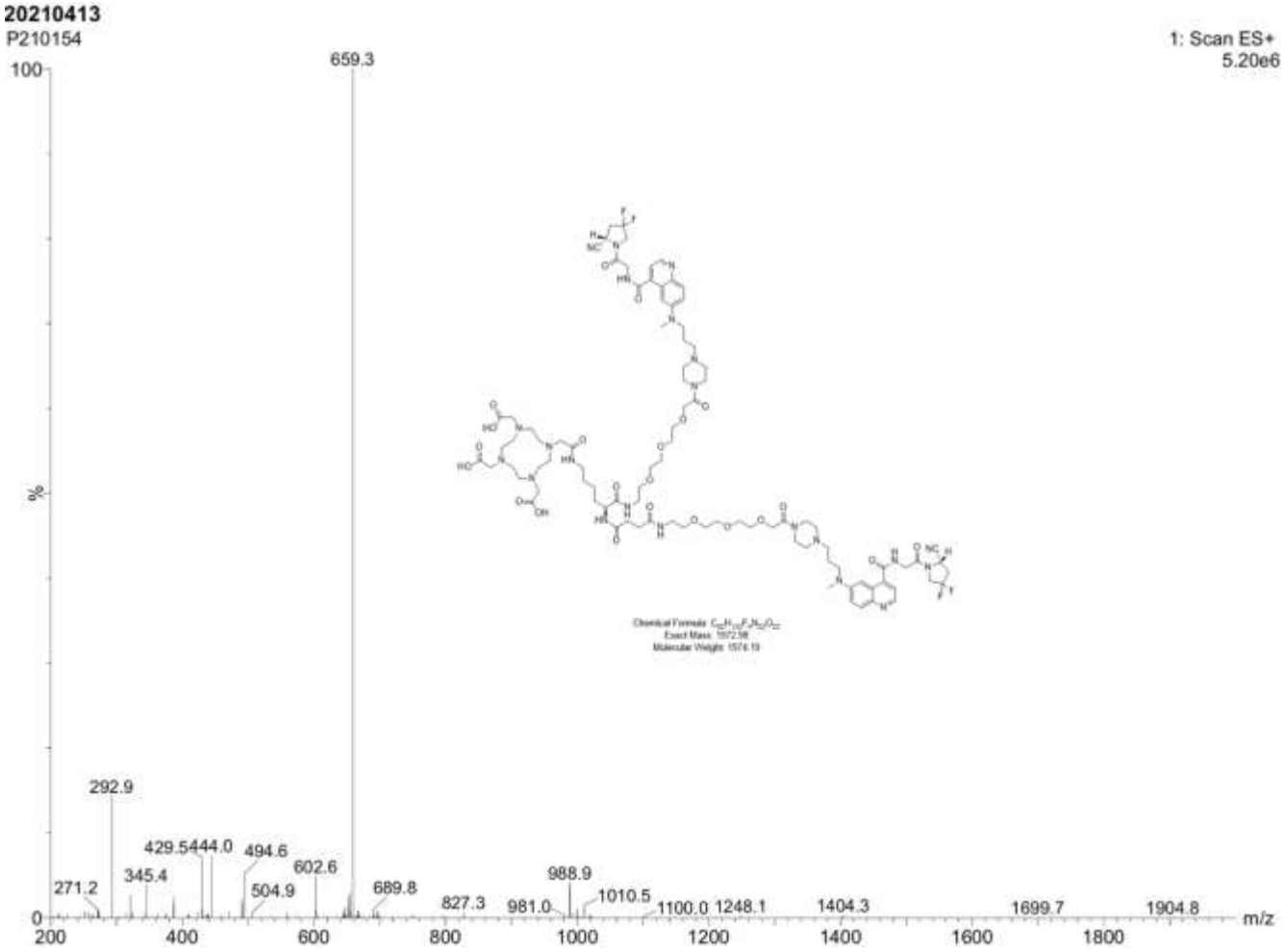
DIPEA (20 mg, 0.154 mmol). The reaction mixture was stirred for 6 h at room temperature. After completion of the reaction, DMF was removed under reduced pressure, and 25% DEA/THF (1 mL) was added dropwise. After another 1 h of stirring at room temperature, the mixture was purified by prep-HPLC to afford compound 7 (10 mg). LC-MS (ESI+): m/z 686.8 $[M+2H]^+/2$

Compound 7 (16 mg, 0.01 mmol) and Compound 8 (9.5 mg, 0.011 mmol) in DMF (1 mL) were treated with HATU (5.7 mg, 0.015 mmol) followed by DIPEA (7.1 mg, 0.055 mmol). The reaction mixture was stirred for 4 h at 80°C. After completion of the reaction, the mixture was transferred into an ice bath and TFA (0.02 mL, 0.18 mmol) dropwise. After another 1 h of stirring at room temperature, the mixture was adjusted to pH5.5 by NaOH solution and purified by prep-HPLC to afford compound 9 (5 mg). LC-MS (ESI+): m/z 659.3 $[M+3H]^+/3$.



	RT	Area	% Height	Width (sec)	% Area
1	7.664	16974	0.36	22.000	0.48
2	8.031	3271	0.10	12.000	0.09
3	8.290	3545868	99.54	35.000	99.43

The high-performance liquid chromatography (HPLC) of DOTA-2P(FAPI)$_2$



The liquid chromatography–mass spectrometry of DOTA-2P(FAPI)$_2$.

Radio-HPLC

HPLC chromatogram analysis was performed using the analytic C-18 reversed-phase column (4.6 × 250 mm, 5 μm, 120Å, Thermo).

HPLC conditions were as follows: trifluoroacetic acid (TFA) (0.1%) and CH_3CN (0.1%TFA) flow rate = 1 mL/min; λ = 254 nm; A = 0.1% trifluoroacetic acid (TFA)/H_2O; B = 0.1% TFA/acetonitrile. B gradient: 0–16 min, from 5% to 75%; 17–20 min, from 75% to 95%; 21-25 min, 95%; 26–30 min, 5%. The retention time was 10.66 min for ^{68}Ga-DOTA-2P(FAPI)$_2$ and 9.41 min for ^{68}Ga-FAPI-46.

PDX-models production

The tumor specimens were obtained from surgical resection, and the hepatocellular carcinoma (HCC) specimens were immediately placed in DMEM (Cat. #C11995599BT, Gibco, USA) supplemented with 2% antibiotics (penicillin and streptomycin) and stored in an ice box. Immunodeficient BALB/c nude mice were bred under specific pathogen free (SPF) conditions at Xiamen University Laboratory Animal Center from founders originally obtained from Shanghai SLAC Laboratory Animal Co., Ltd (China). In brief, the necrotic tissue was removed from the fresh tumor specimen (less than 2 h after the surgery). Then the specimen were cut into approximately 30 mm^3 pieces with scissors and washed

three times by DMEM supplemented with 2% antibiotics before subcutaneous implantation into the mice right side of the trunk. All procedures were performed in super-clean benches. Tumor growth was monitored until it reached 1500 mm^3, then the mice were sacrificed, and the tumor was minced for passaging to the next generation BALB/c nude mice. The remaining fragments were used for the histological verification, including western blot of FAP, immunohistochemistry staining of FAP and Ki67, and Hematoxylin and eosin (H&E) staining.

Western blot and histopathological staining

Proteins were extracted with lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 1mM EDTA and 1% Triton X-100). Approximately 20 μg of total protein per sample was separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). The membranes were pre-incubated with 5% skimmed milk in TBST for 1 h, followed by incubation with human FAP antibody (ab207178; Abcam). Membranes were washed with TBST three times and incubated with horseradish peroxidase-labelled secondary antibody, which was detected using an enhanced chemiluminescence detection system (C280, Azure).

Anti-FAP mAb (ab207178, Abcam and A6349, Abclonal) was used to label surgical specimens of HCC patients and mice tumors during

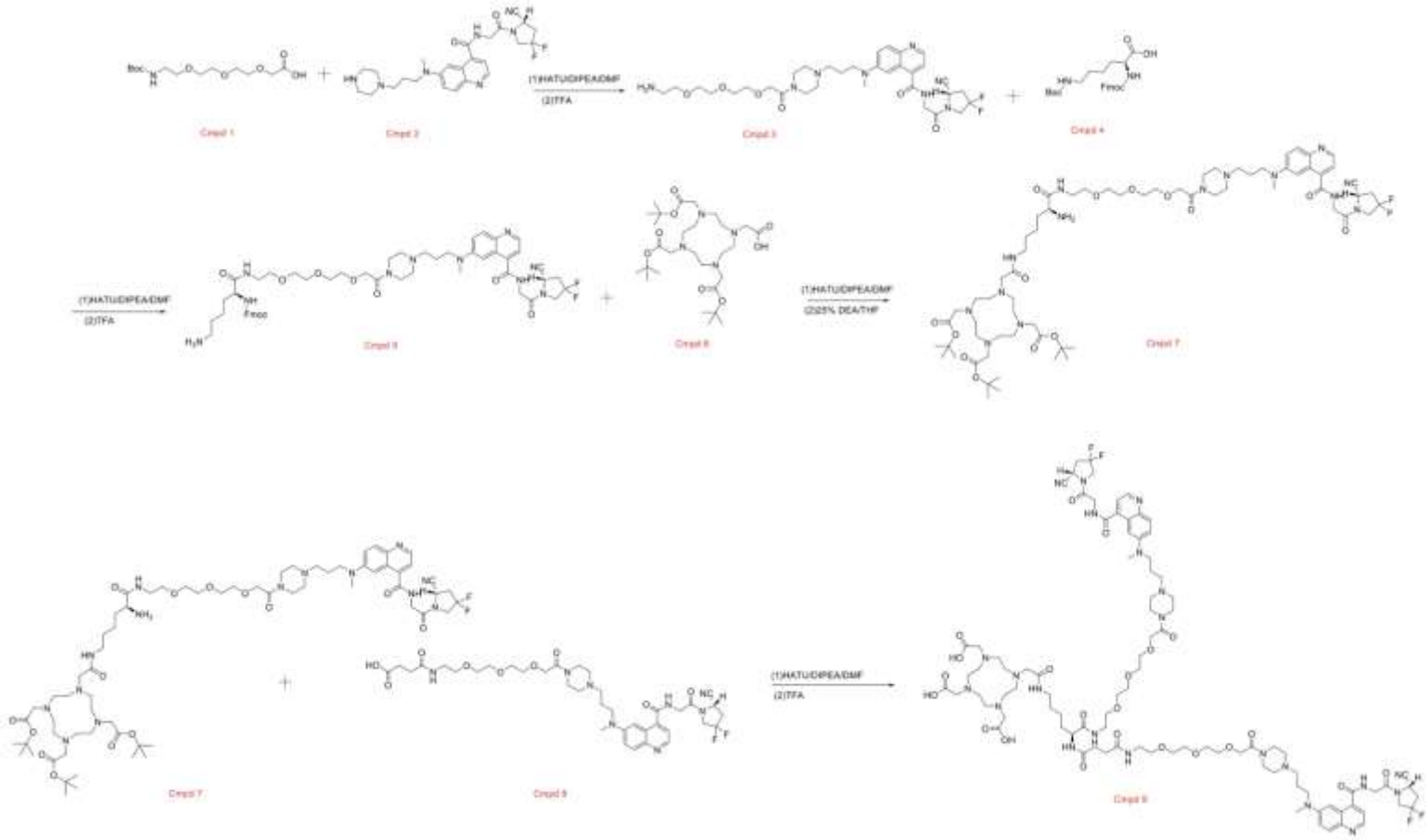
immunohistochemistry (IHC). FAP expression IHC staining was conducted according to our previous protocol using the above mAb (*1*).

Dynamic PET imaging and static PET imaging for mice

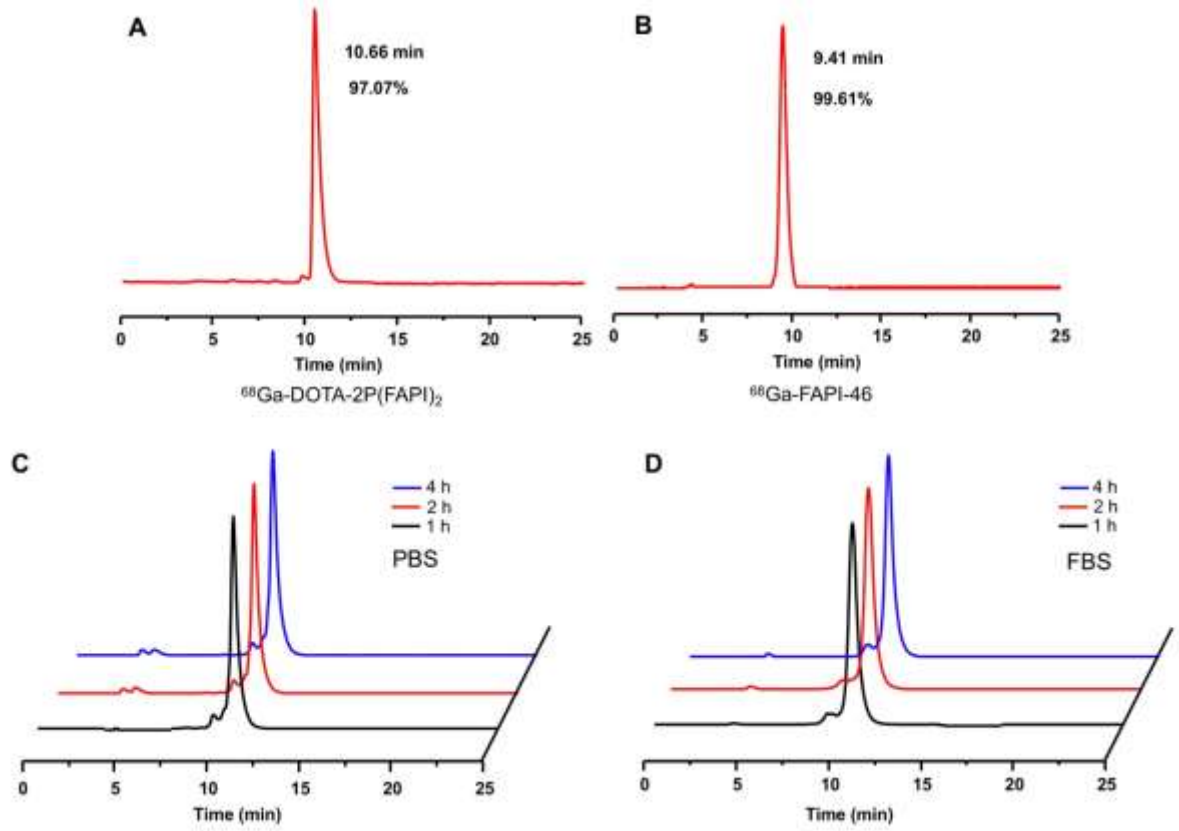
For dynamic PET imaging, the duration of the scan was 60 min, and the reconstruction frames were 10×30 s, 10×60 s, 10×120 s, and 9×160 s. For 10-min static PET imaging, the acquisition times were 0.5, 1, 2, and 4 h post injection (p.i.). For the blocking experiment, 30 nmol of unlabelled FAPI-46 was added to the solution of ^{68}Ga-DOTA-2P(FAPI)$_2$ before injection. Images were reconstructed iteratively using a 3D OPMAP 256.pPetRcn (Siemens), and converted to % ID/g images. Regions of interest (ROIs) in the tumor, liver, heart, kidney, and muscle were counted on the PET images to quantify the radioactive signals.

The dose of intravenously injected ^{68}Ga-FAPI-46 and ^{68}Ga-DOTA-2P(FAPI)$_2$ was calculated according to the patient's weight (1.8-2.2 MBq [0.05-0.06 mCi]/kg for FAPI). The time interval in cancer patients between ^{68}Ga-DOTA-2P(FAPI)2 and ^{68}Ga-FAPI-46 was three days. For healthy volunteers, data were acquired using a hybrid PET/CT scanner (Discovery MI, GE Healthcare, Milwaukee, WI, USA)

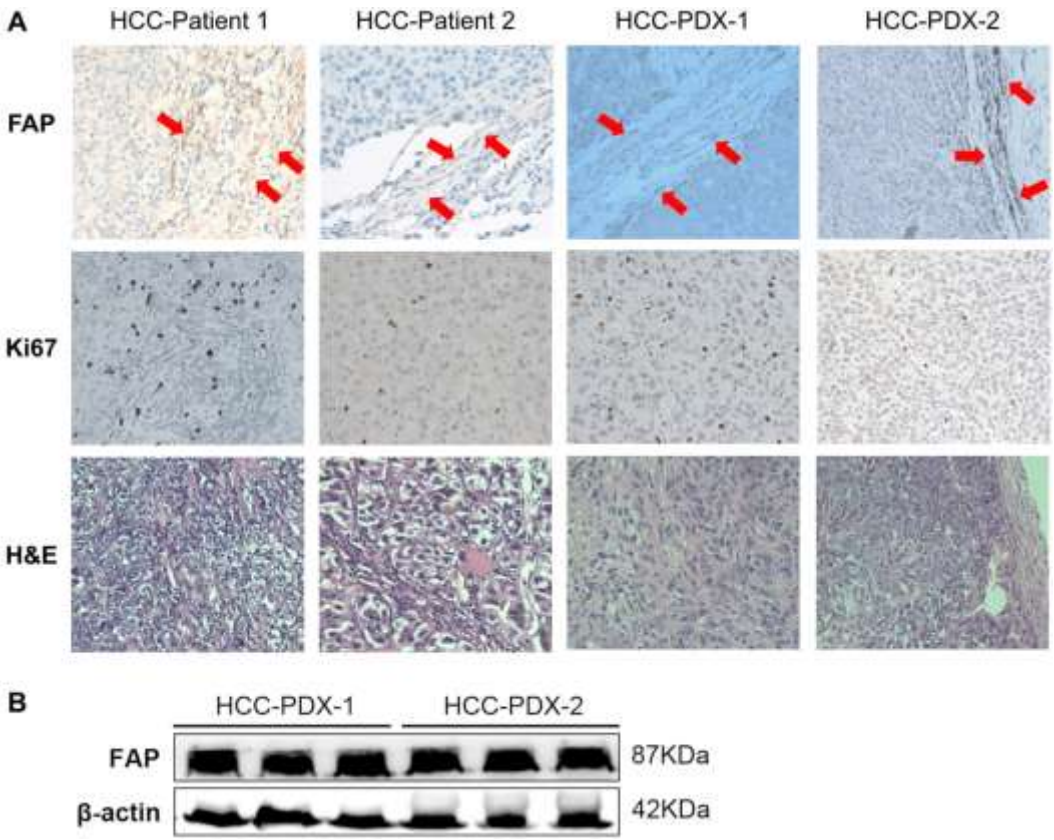
after 10 min, 30 min, 60 min, and 180 min of intravenous injection. For cancer patients, PET/CT images were acquired after 60 min of intravenous injection (one patient was scanned twice at 60 min and 240 min p.i.). All scans and reconstruction were performed according to a previously described protocol (*2*). The maximum standard uptake values (SUVmax) were automatically calculated using a region of interest (ROI) drawn on the transaxial images.



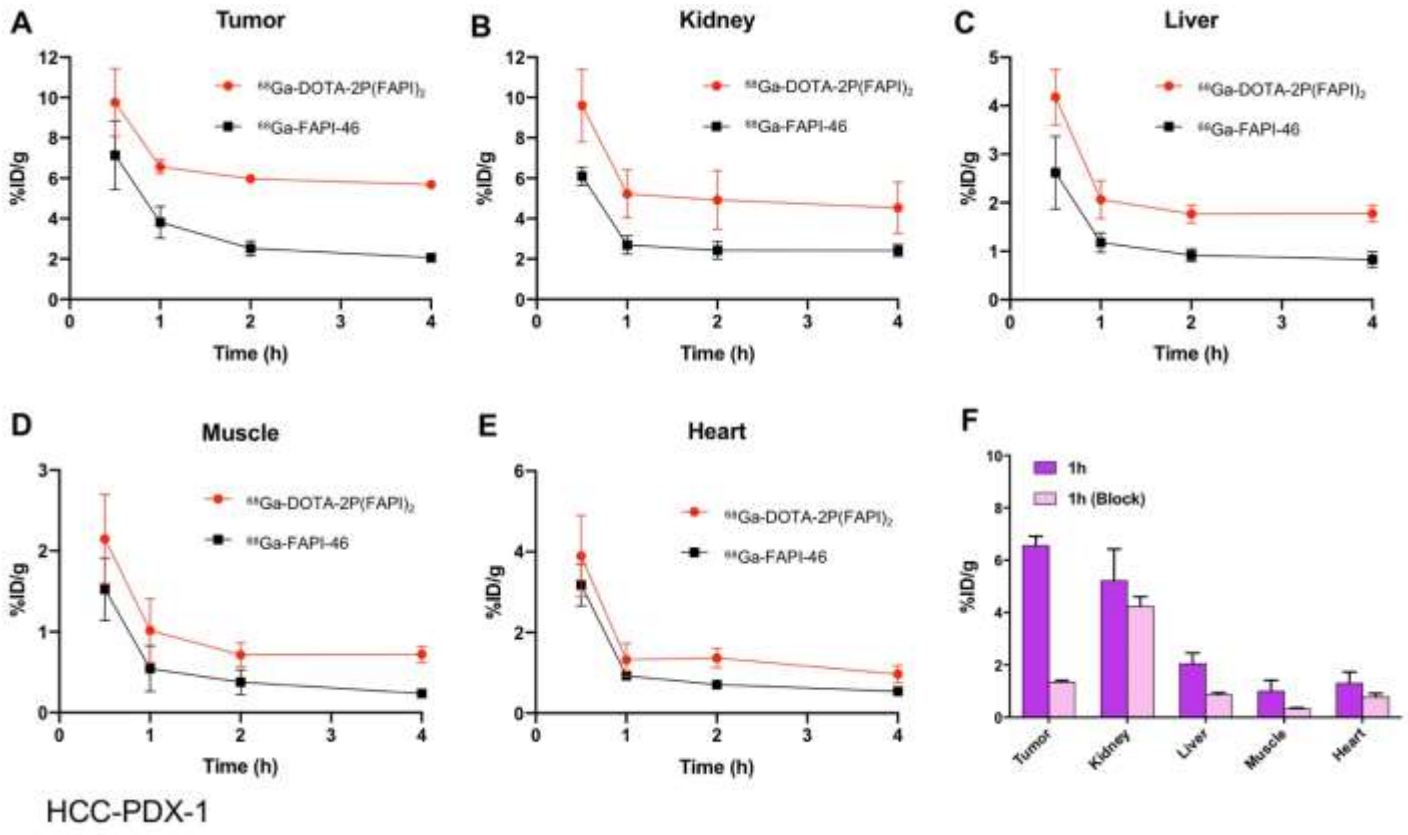
Supplemental Figure 1. Flow diagram of DOTA-2P(FAPI)$_2$ synthesis



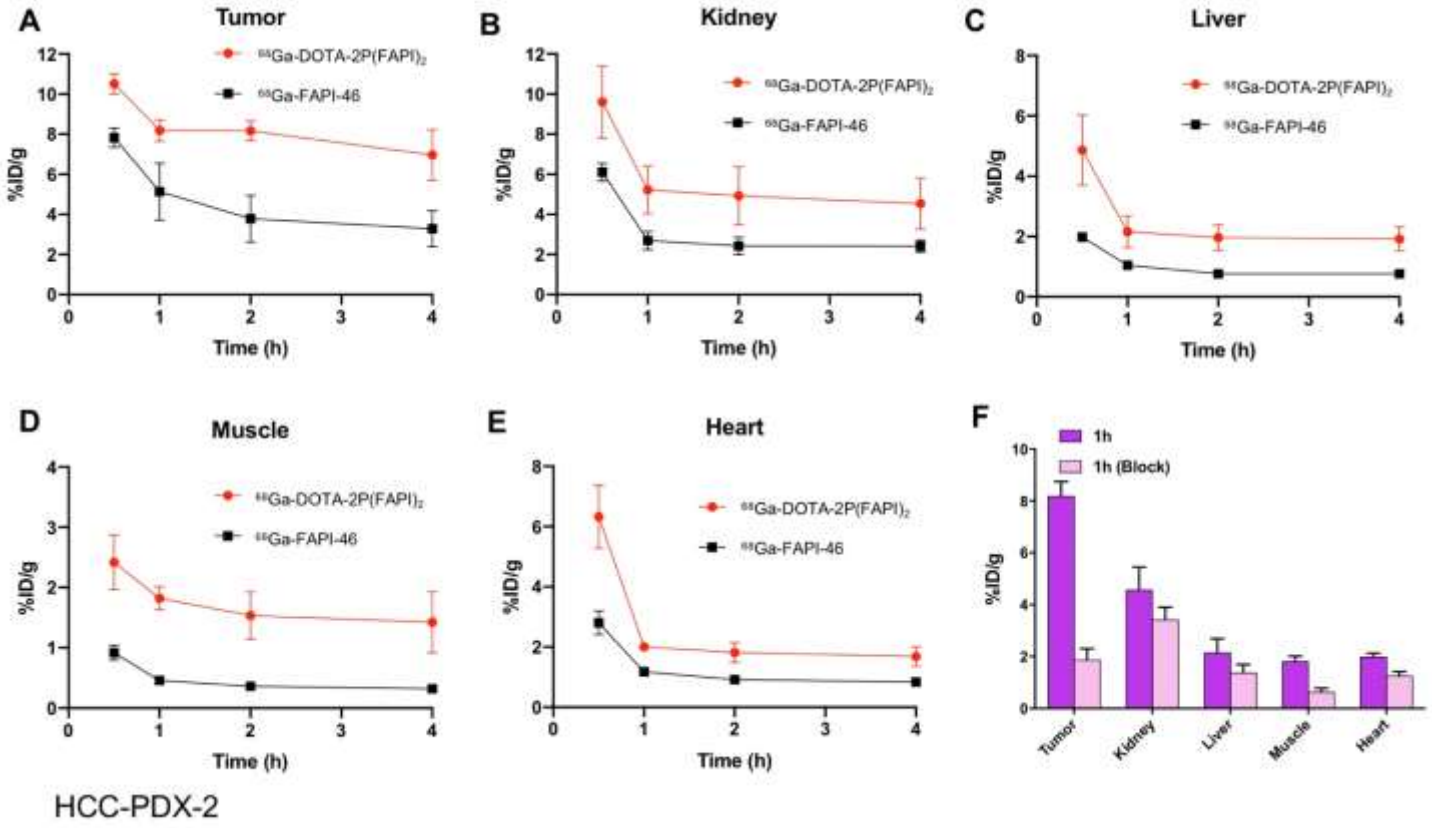
Supplemental Figure 2. (A, B) HPLC profiles of pure ^{68}Ga-DOTA-2P(FAPI)$_2$ and ^{68}Ga-FAPI-46, respectively; (C, D) Stability of ^{68}Ga-DOTA-2P(FAPI)$_2$ in phosphate buffer saline (PBS) and fetal bovine serum (FBS), respectively, 1, 2, and 4 h after incubation.



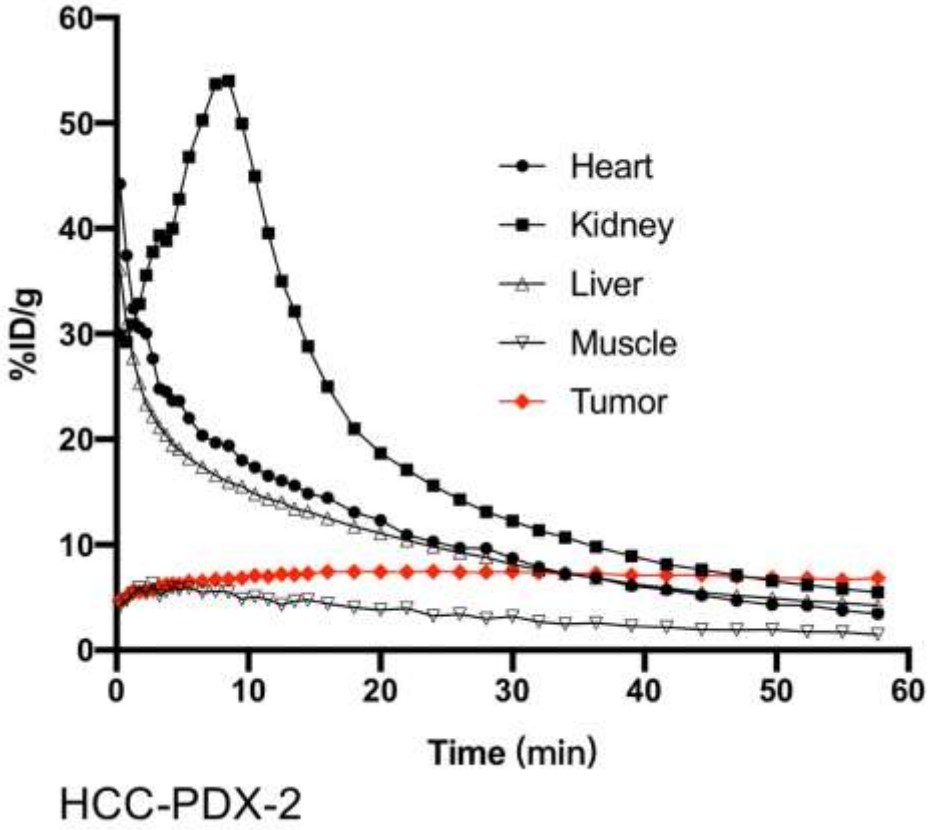
Supplemental Figure 3. (A) Immunohistochemistry staining of FAP and Ki67, and Hematoxylin and eosin (H&E) staining in human hepatocellular carcinoma and corresponding PDXs (positive FAP in CAFs are indicated by arrows). (B) Western blot of FAP in PDXs



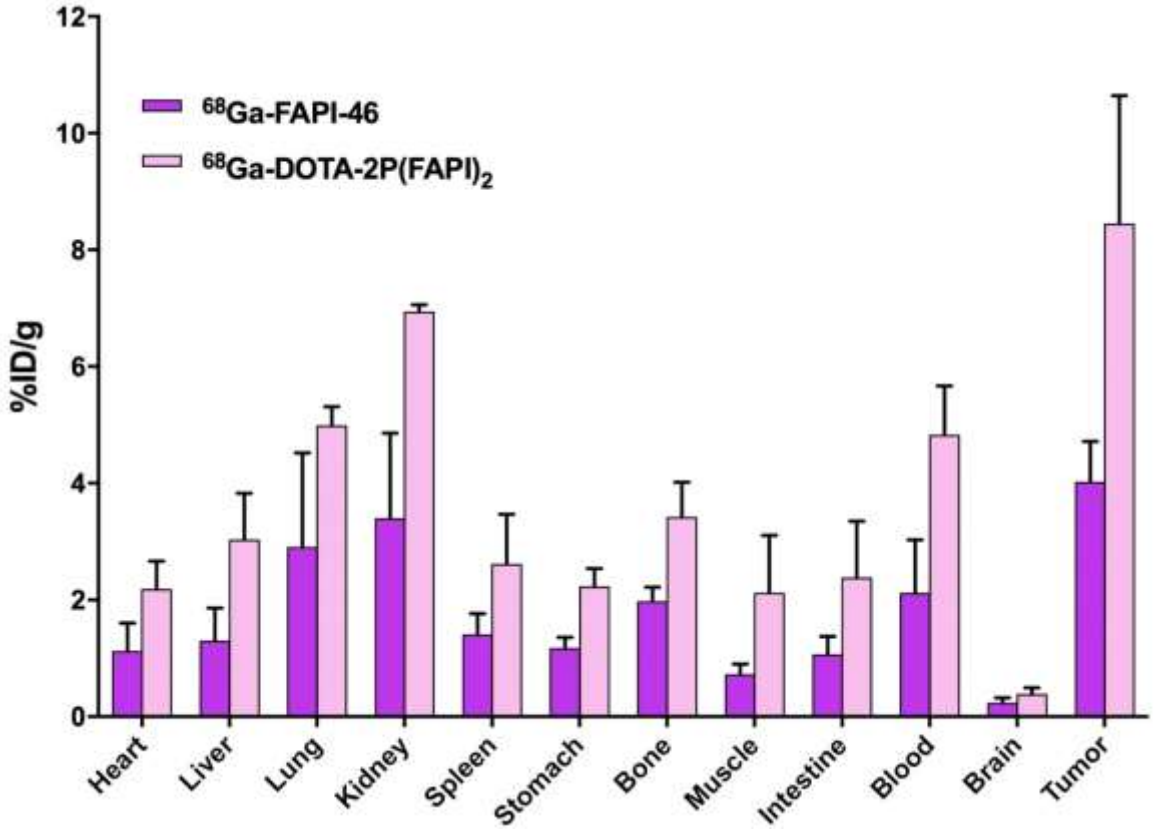
Supplemental Figure 4 (A-E) Comparison of tumor and organ uptake of ^{68}Ga-FAPI-46 and ^{68}Ga-DOTA-2P(FAPI)$_2$ in HCC-PDX-1 0.5, 1, 2, and 4 h post-injection (n = 3/group). (F) Tumor and organ uptake of ^{68}Ga-DOTA-2P(FAPI)$_2$ in HCC-PDX-1 1 h post-injection with and without co-administration of unlabeled FAPI-46 as a blocking agent (n = 3/group).



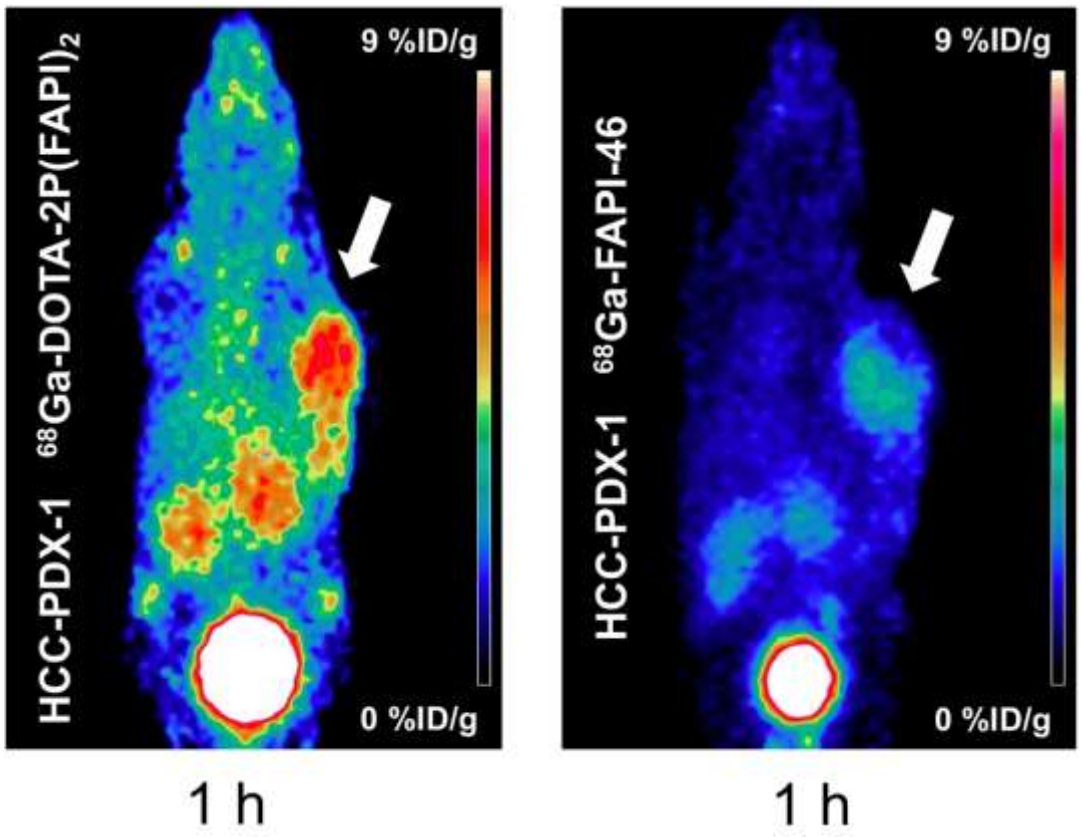
Supplemental Figure 5 (A-E) Comparison of tumor and organ uptake of ^{68}Ga-FAPI-46 and ^{68}Ga-DOTA-2P(FAPI)$_2$ in HCC-PDX-2 0.5, 1, 2, and 4 h post-injection (n = 3/group); (F) Tumor and organ uptake of ^{68}Ga-DOTA-2P(FAPI)$_2$ in HCC-PDX-2 1 h post-injection with and without co-administration of unlabeled FAPI-46 as a blocking agent (n = 3/group).



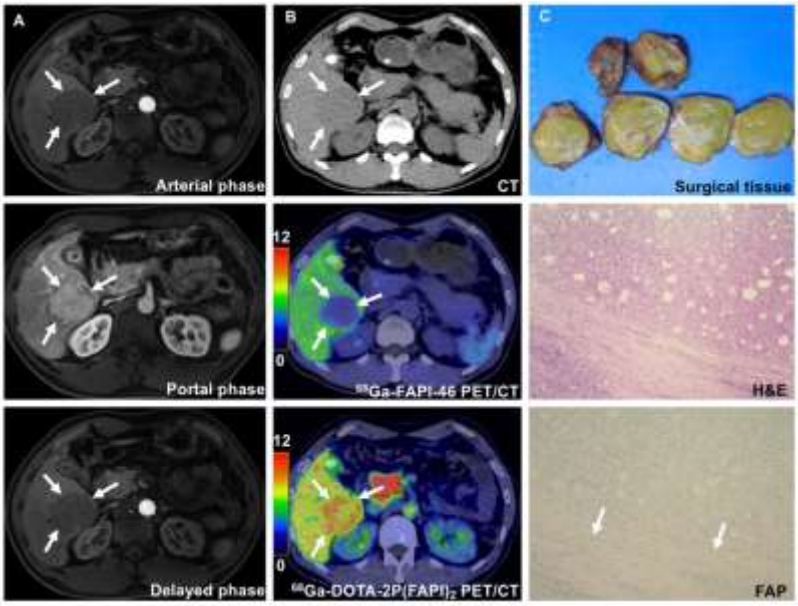
Supplemental Figure 6 Dynamic time-activity curves of ^{68}Ga-DOTA-2P(FAPI)$_2$ in the heart, kidney, liver, muscle, and tumor in HCC-PDX-2.



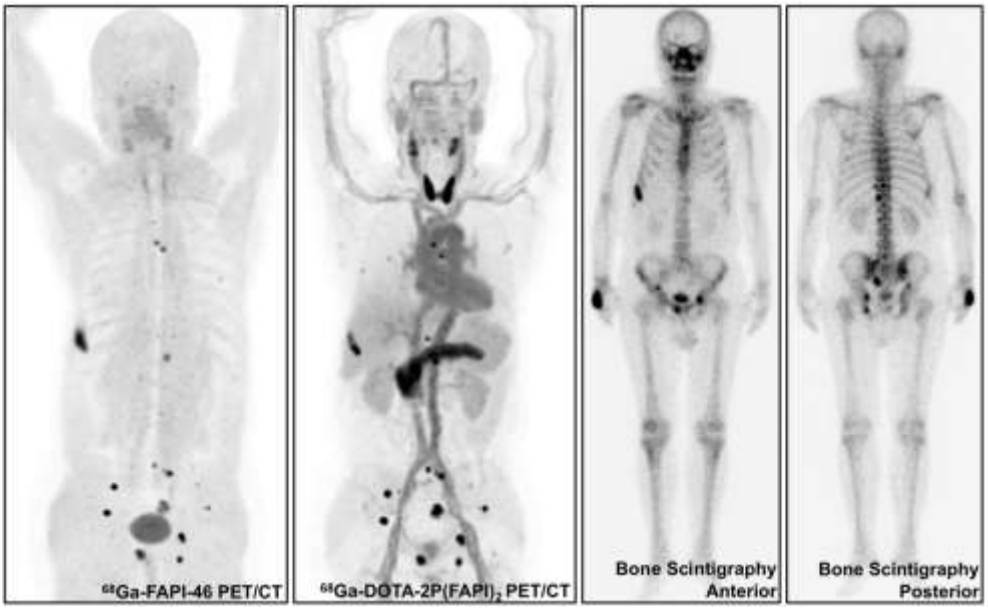
Supplemental Figure 7. Ex-vivo biodistribution of ^{68}Ga-FAPI-46 and ^{68}Ga-DOTA-2P(FAPI)$_2$ (with the same specific activity) in HCC-PDX-1 at 1 h post-injection (n = 3/group).



Supplemental Figure 8. Representative PET imaging of ^{68}Ga-DOTA-2P(FAPI)$_2$ and ^{68}Ga-FAPI-46 (with the same specific activity) in HCC-PDX-1 at 1 h after administration.



Supplemental Figure 9 (A). Contrast-enhanced MRI images in a patient with hepatocellular carcinoma (HCC, lesions indicated by arrows); (B). ^{68}Ga-DOTA-2P(FAPI)$_2$ PET/CT showed higher tracer uptake than ^{68}Ga-FAPI-46 in the HCC lesion (images acquired at 1 h post-injection). The increased background FAPI uptake in liver parenchyma was due to liver cirrhosis; (C). Histopathological results after surgery confirmed the diagnosis of HCC (Edmondson-Steiner grading system, grade II), and immunohistochemistry staining showed low FAP expression in the tumor stroma.



Supplemental Figure 10. ^{68}Ga-FAPI-46 and ^{68}Ga-DOTA-2P(FAPI)$_2$ PET/CT showed multiple bone metastases in a patient with metastatic nasopharyngeal carcinoma (non-keratinized undifferentiated carcinoma, who presented disease progression after chemoradiotherapy and immunotherapy). The uptake of ^{68}Ga-DOTA-2P(FAPI)$_2$ was higher than ^{68}Ga-FAPI-46 in most of the bone metastases (images acquired at 1 h post-injection). The bone metastases were further observed in the radionuclide bone scan.

Supplemental Table 1: Lesion-by-lesion comparison of ^{68}Ga-FAPI-46 and ^{68}Ga-DOTA-2P(FAPI)$_2$ uptake in three cancer patients.

Patient No.	Age	Sex	Status	No. of lesions	Site of lesions	^{68}Ga-FAPI-46 SUVmax	^{68}Ga-DOTA-2P(FAPI)$_2$ SUVmax	*P*
Patient1 (NPC)	71	Male	Recurrence	>10	Bone 1	16.3	17.8	0.005
					Bone 2	24.7	27.5	
					Bone 3	23.4	27.4	
					Bone 4	14.4	25.1	
					Bone 5	22.0	29.8	
					Bone 6	9.8	20.8	
					Bone 7	8.1	17.2	
					Bone 8	13.3	22.1	
					Bone 9	17.4	27.6	
					Bone 10	11.6	23.3	
Patient 2 (Thyroid cancer)	34	Male	Recurrence	>10	Cervical node 1	20.0	32.8	0.005
					Cervical node 2	1.7*	8.1	
					Supraclavicular node 1	24.0	39.0	
					Supraclavicular node 2	12.8	24.1	
					Axillary node 1	17.8	23.2	
					Axillary node 2	17.7	33.2	
					Axillary node 3	16.7	28.9	
					Bone 1	11.4	26.4	
					Mediastinal node 1	23.4	24.6	
					Hilar node 1	11.5	18.1	
Patient 3 (HCC)	46	Male	Initial stage	1	Primary tumor	2.7	9.8	NA

Note: HCC, hepatocellular carcinoma; NPC, nasopharyngeal carcinoma; No., number; SUVmax, maximum standardized uptake value; *, negative in ^{68}Ga-FAPI-46 PET/CT

References

1. Chen H, Zhao L, Fu K, et al. Integrin alphavbeta3-targeted radionuclide therapy combined with immune checkpoint blockade immunotherapy synergistically enhances anti-tumor efficacy. *Theranostics.* 2019;9:7948-7960.

2. Chen H, Pang Y, Wu J, et al. Comparison of [(68)Ga]Ga-DOTA-FAPI-04 and [(18)F] FDG PET/CT for the diagnosis of primary and metastatic lesions in patients with various types of cancer. *Eur J Nucl Med Mol Imaging.* 2020.