



Screening of radiotracer for diagnosis of colorectal cancer liver metastasis based on MACC1-SPON2

Hao Jiang¹ · Wei Guo² · Kuan Huang³ · Huijie Jiang¹ · Rongjun Zhang⁴ · Hongbo Hu¹ · Xue Lin¹ · Song Wang⁵

Received: 4 August 2020 / Revised: 20 February 2021 / Accepted: 25 February 2021 / Published online: 13 March 2021
© The Author(s) 2021

Abstract

Background Metastasis-associated in colon cancer 1 (MACC1) and Spodin2 (SPON2) are newly discovered oncogenes, but little is known about their role in colorectal cancer (CRC) liver metastases. PET has become an important molecular imaging technology due to its high sensitivity and quantifiability. In particular, its targeted, specific molecular probes can detect biological behaviors. This study was designed to evaluate the different biological properties of ¹⁸F-FDG, ¹⁸F-FLT, and ¹⁸F-FMISO PET. The value of the CRC liver metastasis model explores the correlation and potential mechanisms of three tracers uptakes with tumor-related biological characteristics.

Methods Human CRC cell lines (LoVo and HCT8), were cultured for in vitro radionuclide uptake experiments to compare the molecular imaging features of colorectal cancer cells with different metastatic potentials. Two kinds of cells were injected into the spleen of nude mice to establish a liver metastasis model. After the tumor formation, three kinds of tracer PET images were performed to evaluate the characteristics of live PET imaging of high and low liver metastasis colorectal cancer models. The expression levels of MACC1 and SPON2 in tissues were detected by immunohistochemistry and Western blot. Correlation between tracer uptake and expression of MACC1 and SPON2 in liver metastases was assessed by linear regression analysis.

Results The uptake rate of in vitro three tracers uptake experiments was LoVo > HCT8. Micro-PET scan showed no significant difference between the ¹⁸F-FDG SUV values of the two cells ($P > 0.05$); there was significant difference between the ¹⁸F-FLT and ¹⁸F-FMISO SUV values ($P < 0.05$). All in vivo FLT and FMISO SUV values were significantly higher in LoVo tumors than in HCT8 tumors. The results of Western blot and immunohistochemistry showed that the expression levels of MACC1 and SPON2 in LoVo liver metastasis were higher than those in HCT8 ($P < 0.05$). The ¹⁸F-FLT SUVmax ratio was significantly correlated with the expression of MACC1 and SPON2 in hepatic metastases ($r = 0.737$, $P = 0.0026$; $r = 0.842$, $P = 0.0002$). The ¹⁸F-FMISO SUVmax ratio was only significantly correlated with the expression of MACC1 in hepatic metastasis ($r = 0.770$, $P = 0.0013$).

Conclusions Early screening with ¹⁸F-FLT and ¹⁸F-FMISO tracers has important clinical value for the efficient diagnosis and treatment of colorectal cancer liver metastases.

Keywords ¹⁸F-FLT · ¹⁸F-FMISO · ¹⁸F-FDG · PET · Colorectal cancer liver metastasis · Biological characteristics

Introduction

According to global cancer statistics in 2018, colorectal cancer (CRC) has become the fourth leading cause of death from cancer in the world, accounting for 10% of

the total number of cancers diagnosed and cancer-related deaths each year, and is the second most common cancer among women and men. It is the third most common cancer, causing nearly 900,000 deaths each year. The annual number of new incidences of colorectal cancer worldwide is expected to increase to 2.5 million in 2035 [1, 2]. The main cause of death of CRC is liver metastasis. From an anatomical point of view, the draining venous blood of the colorectal all converges into the portal vein and preferentially flows into the liver, the liver sinusoid is the part of the gastrointestinal tract where blood returns.

✉ Huijie Jiang
jhjemail@163.com

✉ Song Wang
songwangws@163.com

Extended author information available on the last page of the article

The liver has a high clearance rate for blood flow, so it is the organ where tumor cells are most likely to stay; the incidence of colorectal cancer invading veins is as high as 20% to 30%. Once tumor cells fall off the primary tumor and enter the blood circulation, they are easily Metastases formed in the liver. If effective treatment is not available, the probability of liver metastasis will eventually be at least 50% [3]. Therefore, to explore the genes that lead to the development of CRC, and to understand the clinical significance of these genes is crucial for the diagnosis and treatment of CRC. These key molecular characterizations are expected to help the development of new CRC therapeutic strategies. PET can display biological features within tumors in vivo at functional and molecular levels by means of targeted probes. We can use this characteristic of PET to evaluate the biological characteristics (invasion and metastasis) of tumors, and to identify the high invasion and metastasis characteristics of colorectal cancer in advance, which is helpful for the appropriate treatment of liver metastasis of colorectal cancer.

It is well known that the microenvironment of tumors leads to numerous physiological consequences, including invasion and metastasis. ^{18}F -FDG is currently the most widely used PET targeting probe. ^{18}F -FDG PET has become an indispensable means of examination for many types of tumors, including CRC. However, numerous studies have shown that ^{18}F -FDG reveals that the ability of many tumor and non-tumor biological processes is not completely reliable [4, 5]. At the same time, hypoxia can lead to an increase in tumor progression by promoting glycolysis, angiogenic factor activity, metastasis, mutation, and by inhibiting apoptosis [6]. The location and extent of hypoxia in solid tumors of different types/different stages are essential for clinical treatment of cancer. ^{18}F -FMISO is a common tracer for hypoxic imaging. In addition, the occurrence of malignant tumors is often due to rapid proliferation of cancer cells and formation of new blood vessels. 3'-deoxy-3'- ^{18}F -fluorothymidine (FLT) as a proliferation tracer [7], after the single enzyme thymidine kinase-1 (TK1) monophosphorylation in the DNA synthesis salvage pathway, the tracer is captured in the cytosol [8]. FLT has been found to non-invasively assess the proliferation rate of several types of tumors such as colorectal cancer, esophageal cancer, and lung tumors [8–11]. Therefore, the above three radiotracers were synthesized for PET imaging of tumors.

MACC1 is a differentially expressed gene discovered by Stein et al. [12] in 2009 through a genome-wide search for primary and metastatic colon cancer, which promotes metastasis of colon cancer, hence the name. MACC1 is a biomarker for a variety of solid tumors including colorectal cancer tumor progression, metastasis, and patient survival [13]. It has been found that MACC1 expression

in tumor lesions of CRC patients is significantly increased relative to their non-tumor adjacent tissues. High expression of MACC1 is significantly associated with tumor metastasis and poor clinical outcome, and is an early risk factor for cancer patients [14, 15]. Studies have shown that knockout MACC1 significantly inhibits proliferation, migration, invasion and tumorigenesis of colorectal cancer cells, and induces apoptosis [16]. Schmid et al. [17] showed for the first time that SPON2 is one of the main downstream effectors of MACC1, providing a new link between MACC1 and SPON2. Some scholars have identified through genome-wide expression analysis that SPON2, as one of the major downstream effectors of MACC1 gene, can be used as an independent prognostic indicator for CRC metastasis and metastasis-free survival (MSF) [12]. In addition, as early as 2013, it has been reported that SPON2 is highly expressed in prostate cancer cells, and serum SPON2 index has high sensitivity and specificity, which is suitable for tumor diagnosis [18]. In this paper, MACC1 and SPON2 were introduced to explore its mechanism of action in colorectal cancer metastasis. The correlation between multi-probe molecular imaging features of early liver metastases and MACC1 and SPON2 was determined by in vitro and in vivo experiments.

Based on the previous research [19], this study added new molecular probes (^{18}F -FLT) and biological indicators (MACC1, SPON2), which are intended to conduct in-depth research on the mechanism of liver metastasis of colorectal cancer, and select new organisms. The relationship between academic indicators and various molecular imaging features will further explore the relationship between the molecular mechanism of liver metastasis of colorectal cancer and tumor biology, and provide assistance for the early diagnosis, treatment and efficacy evaluation of CRC liver metastasis.

Materials and methods

Cell culture

The human colorectal cancer high metastatic potential cell line LoVo and the low metastatic potential cell line HCT8 were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. The LoVo cell line was cultured in F-12 k (Buddhist) medium, and the HCT8 cell line was cultured in 1640 (Buddhist) medium. All the medium was added with 1% cyan-streptomycin and 10% fetal bovine serum (Biological). Industries), cultured in a 5% CO_2 , 37 °C incubator.

A mouse model of hepatic metastases

Five-week-old BALA/C female nude mice were purchased from Changzhou Cavans Animal Experiment Company. The 40 nude mice purchased were housed in a sterile animal room with a temperature and humidity of 24 °C and 55 ± 10%, respectively, and the feeding conditions were carried out in accordance with the animal house feeding regulations. Animal research was approved by the Animal Management and Use Committee (IACUC) of the XXX of Atomic Medicine. Forty nude mice were divided into two groups, LoVo and HCT8, with 20 rats in each group. The nude mice were placed in the animal room for 2–3 days, and then modeled, and the spleen was inoculated to protect the spleen. The concentration of both cells was 5.0 × 10⁶/0.15 mL, and the whole operation was performed under anesthesia (2% isoflurane). There were 18 model rats remaining in the LoVo group due to surgical trauma. After the surgery, continue to be kept in the animal room. After micro-PET imaging, the model rats were sacrificed and specimens were taken.

Preparation of ¹⁸F-FDG, ¹⁸F-FLT and ¹⁸F-FMISO

¹⁸F-FDG, ¹⁸F-FLT and ¹⁸F-FMISO were synthesized with individual PET kits (XXX Industrial Technology and Trade Corporation, XXX) on a fluorine multifunctional synthesis module [PET MF-2 V, PET (Beijing) Technology Co. Ltd., PRC] with a computer interface. The radiochemical purity of the ¹⁸F-FDG, ¹⁸F-FLT and ¹⁸F-FMISO tracers was greater than 95% and the final specific activity was greater than 0.5 TBq/mmol. These ¹⁸F-labeled tracers were diluted with 0.9% saline and passed through a 0.22 μm Millipore filter (Millipore, Billerica, MA, USA) for administration to animals.

In vitro cellular uptake of radiotracers

Prior to ¹⁸F-FDG cell uptake, LoVo and HCT8 cells were cultured in serum-free medium and placed at 37 °C, 5% CO₂. Prior to the ¹⁸F-FMISO cell uptake assay, LoVo and HCT8 cells were pre-incubated at 37 °C in a 2% O₂ hypoxic environment. Cells were not treated specially before ¹⁸F-FLT ingestion. When the cells were grown to log phase, the cells were counted under the microscope. In the cell uptake experiment, LoVo or HCT8 cells, radiotracer and buffer were added to a glass test tube (2 ml) and incubated in a 37 °C water bath for 30, 60, 120, and 240 min, respectively, in which the O group: 100 μl was added. Radionuclide and 200 μl buffer (containing 0.2% BSA) as control group; T group: only 100 μl of radionuclide was added for measurement of radionuclide dose; X

group: 100 μl of radionuclide, 100 μl of cell suspension and 100 μl of buffer DMEM containing 0.2% bovine serum albumin. The number of cells in the cell uptake experiment was unified to 5 × 10⁵ cells/100 μl, and each experiment was repeated three times at the same time point. After incubation at different times, centrifugation (1000 rp/min, 5 min), aspirate all the liquid in the O and X group. The radioactivity in each tube was accurately measured using an automatic gamma counter (PerkinElmer, 2480, USA). The cell uptake rate was calculated as: X(cpm)—O(cpm) / T(cpm)%.

Micro-PET imaging

Eighteen weeks after the model rats were established, ¹⁸F-FDG, ¹⁸F-FLT, and ¹⁸F-FMISO PET imaging were performed. Micro-PET imaging was performed in the order of ¹⁸F-FDG, ¹⁸F-FLT, and ¹⁸F-FMISO for a specific imaging time point, with an interval of 24 h, in consideration of ¹⁸F attenuation. ¹⁸F-FDG, ¹⁸F-FLT, and ¹⁸F-FMISO were injected at 60 min, 120 min, and 240 min before scanning, respectively. The tail vein was injected with ¹⁸F-FDG (about 3.7 MBq, 100 μCi), ¹⁸F-FLT (about 7.4 MBq, 200 μCi), and ¹⁸F-FMISO (about 14.8 MBq, 400 μCi). Before ¹⁸F-FDG PET imaging, the model rats were starved for 12 h and fasted without water. The body temperature of the mice was maintained by a heat lamp throughout the process. All small animal PET imaging was performed under the protocol approved by the Animal Management and Use Committee of the Jiangsu Institute of Atomic Medicine. 10 min before the scan, anesthetized with isoflurane. Micro-PET images were acquired using a 3D Inveon Micro-PET scanner (Siemens). Image reconstruction based on ordered subset expectation maximization using Inveon Acquisition Workplace workstation (version 2.0, Siemens), matrix: 128 × 128 × 159; pixel size: 0.86 × 0.86 × 0.8 mm; truncation frequency 1.5, uniform resolution. For image analysis, ASIProVM 6.8.6.9 image processing software (Concorde Microsystems, LLC) was used to manually outline the region of interest (ROI) to cover the entire liver metastases on the image. The maximum normalized uptake ratio is defined as the tumor SUV_{max} divided by the liver SUV_{max}. The formula for SUV_{max} is:

$$\text{SUV}_{\text{max}} = \frac{\text{Max} \times 8000 \mu\text{Ci}/\text{ml} \times \text{weight}(\text{g})}{\text{injected dose} (\mu\text{Ci})}$$

Immunohistochemical staining

Liver metastasis tissue specimens were fixed in 10% formalin for 48 h, embedded in paraffin, and sliced to a thickness of 3 μm. After dewaxing, the tissue sections were boiled

in 10 mmol/L citrate buffer (pH6.0) for 10 min and then cooled at room temperature for 40 min for antigen retrieval. The endogenous enzyme was inactivated with 3% hydrogen peroxide, and the sections were incubated with the primary antibody overnight at 4 °C. The primary antibody was as follows: MACC1 (Abeam, ab106579, UK) primary antibody working concentration was 1:200, SPON2 (Abeam, ab215451, UK) primary antibody working fluid concentration was 1:20. On the next day, tumor sections were then incubated with HRP-labeled secondary antibody (Zhongshan Golden Bridge Biotechnology, Beijing, China) for 20 min at room temperature followed by counterstaining with hematoxylin. Slides were then rinsed with tap water and sealed with neutral resin. The staining was observed under a BX53 Olympus microscope (Olympus) at magnification 200×. A brown-yellow staining was defined as positive. Staining intensity was defined as follows: –, no staining; +, stained areas < 25% or weak staining; ++, stained area within 25–75% or moderate staining; + + +, stained area > 75% or strong staining. The slides were examined by two independent researchers in a double-blinded manner.

Western blot analysis

Western blot analysis can quantitatively analyze the protein expression of colorectal cancer liver metastases with different metastatic potentials. The operation method refers to the previous literature [20]. The samples were placed in 4 °C RIPA buffer for 30 min. The protein concentration of the samples was determined using a Protein Concentration Assay (BCA) kit (Beyotime Biotech). An equal amount of sample protein was dissolved in a 10% or 12% SDS-PAGE gel and blotted onto a PVDF membrane (Millipore, Billerica, MA, USA). The cells were then sealed with TBS buffer containing 10% skim milk powder (containing 0.1% Tween-20) for 1 h, followed by incubation with different primary antibodies overnight at 4 °C. The primary antibody used was the same as the immunocytochemistry experiment described above, MACC1, SPON2 (1:1000 dilution), GAPDH (1:5000 dilution, Zhongshan Jinqiao Biotechnology Co., Ltd.), and then with the peroxidase-labeled secondary antibody (Diluted 1:5000) Incubate for 1 h at room temperature. Band images were acquired using a Bio-Rad chemiDoc XRS + imaging system, normalized to GAPDH expression levels, and four protein expression levels were quantified by Image-J (NIH, Bethesda, MD, USA) software.

Statistical analysis

All data were expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS software version 19.0 (SPSS, Chicago, IL, USA). The difference between

Table 1 Comparison of ¹⁸F-FDG cell uptake by LoVo and HCT8 cells in vitro (%)

	LoVo	HCT8	P值
30 min	11.56 ± 0.09	13.57 ± 0.82	0.013
60 min	17.42 ± 1.05	19.59 ± 3.64	0.377
120 min	25.36 ± 0.33	26.30 ± 0.73	0.114
240 min	33.77 ± 1.86	23.81 ± 0.96	0.001

Table 2 Comparison of ¹⁸F-FLT cell uptake by LoVo and HCT8 cells in vitro (%)

	LoVo	HCT8	P值
30 min	6.34 ± 0.20	2.13 ± 0.31	0.000
60 min	9.52 ± 0.06	3.66 ± 0.24	0.000
120 min	13.35 ± 0.47	4.85 ± 0.05	0.000
240 min	21.90 ± 0.76	12.14 ± 0.77	0.000

two cell lines was assessed using Student's unpaired *t*-test. The correlation between Western blotting results and PET SUV was analyzed using linear regression. *P* < 0.05 was considered statistically significant.

Results

In vitro radiotracer uptake analysis

The in vitro cellular uptake differences of the ¹⁸F-FDG, ¹⁸F-FLT and ¹⁸F-FMISO tracers were compared between LoVo and HCT8 cells (Tables 1, 2, 3). According to the results of repeated measurement analysis, the cell uptake rates of both cells of ¹⁸F-FLT and ¹⁸F-FMISO increased with time. The difference in uptake between LoVo and HCT8 cells was analyzed by independent sample *t*-test. The results showed that the uptake rate of three tracers was lower in HCT8 cells than in LoVo cells at 240 min. The difference was statistically significant (*P* values were 0.001, 0.000, 0.020). Moreover, the uptake rates of the two cells to ¹⁸F-FDG were significantly higher than those of ¹⁸F-FMISO and ¹⁸F-FLT.

Micro-PET imaging analysis

The most important issue in the establishment of a liver metastasis xenograft model is to determine the optimal time for PET imaging. The survival time of the liver metastasis mouse model is generally shorter than that of the traditional subcutaneous tumor model. Considering the time of imaging of multiple tracers, we determined the optimal imaging time as 8 weeks after tumor cell inoculation. Figure 1 shows LoVo and HCT8 models

Table 3 Comparison of ^{18}F -FMISO cell uptake by LoVo and HCT8 cells in vitro (%)

	LoVo	HCT8	<i>P</i> 值
30 min	1.31 ± 0.32	1.22 ± 0.11	0.672
60 min	1.36 ± 0.06	1.24 ± 0.09	0.093
120 min	1.74 ± 0.12	1.55 ± 0.34	0.398
240 min	2.91 ± 0.19	2.11 ± 0.32	0.020

Tables 1, 2, 3: Comparison of in vitro ^{18}F -FDG, ^{18}F -FLT, and ^{18}F -FMISO uptake rates for different metastatic potential colorectal cancer cell lines over four time periods. Values are expressed as ^{18}F -FDG (Table 1), ^{18}F -FLT (Table 2), and ^{18}F -FMISO (Table 3) uptake dose percentages (% ID) per 5×10^5 cells. Each cell was repeated three times at the same time point. Table 1,2,3: The cell uptake rates of the ^{18}F -FLT and ^{18}F -FMISO of the two cells increased with time. The uptake rate of the three tracers by HCT8 cells was only lower than that of LoVo cells at 240 min. The uptake rates of the two cells to ^{18}F -FDG were significantly higher than those of ^{18}F -FMISO and ^{18}F -FLT.

^{18}F -FDG, ^{18}F -FLT, and ^{18}F -FMISO PET images and their corresponding liver anatomy. The LoVo model rat liver metastasis rate is 50% (9/18), HCT8 model rat liver The metastatic rate was 35% (7/20). The difference between

the three tracer SUVmax values between LoVo and HCT8 liver metastases was analyzed using an independent sample t-test. The results showed that there was no significant difference between the ^{18}F -FDG SUVmax value and the SUVmax ratio of LoVo and HCT8 liver metastasis ($P = 0.057$ and 0.286) (Table 4, Fig. 2). However, there was a significant difference between the ^{18}F -FMISO and ^{18}F -FLT SUVmax and SUVmax ratio values of LoVo and HCT8 liver metastasis tissues, which was statistically significant ($P < 0.05$). And the ^{18}F -FDG SUVmax values of the two cells were significantly higher than the SUVmax values of ^{18}F -FLT and ^{18}F -FMISO, which was consistent with the results of in vitro cell uptake experiments. It can be seen that ^{18}F -FDG is more sensitive to liver metastases, but its specificity is poor.

Immunohistochemical results analysis

Figure 3 shows the expression of both MACC1 and SPON2 proteins in LoVo and HCT8 liver metastases. Immunohistochemical results showed that two models (LoVo and HCT8) had two proteins in liver metastasis,

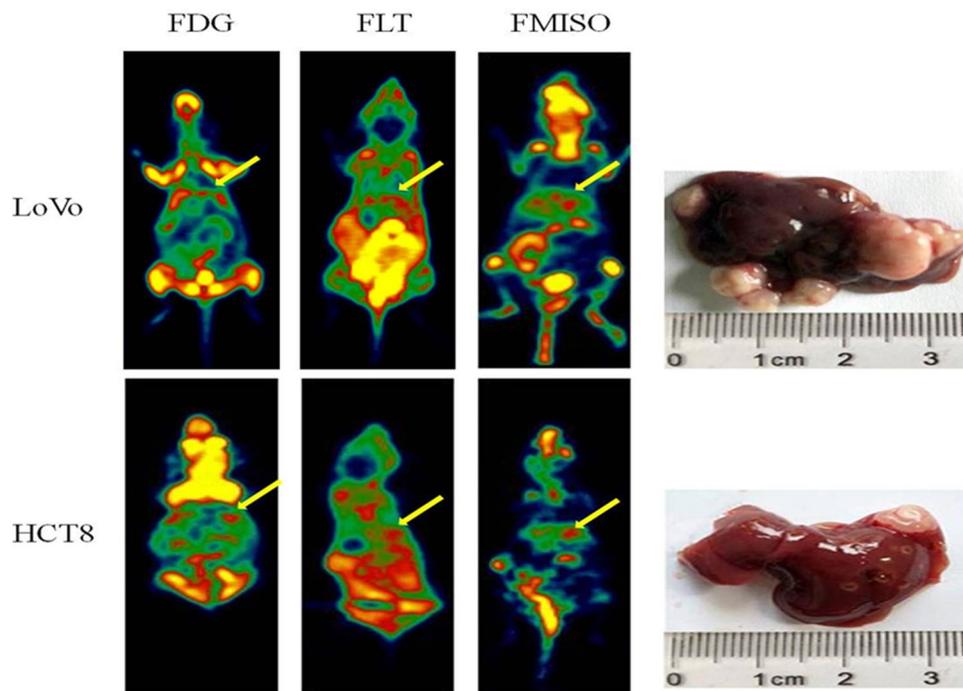


Fig. 1 ^{18}F -FDG, ^{18}F -FLT, and ^{18}F -FMISO PET images of LoVo and HCT8 liver metastasis models and their corresponding liver anatomical images after 8 weeks of spleen injection of tumor cells. Yellow arrows indicate uptake of ^{18}F -FDG, ^{18}F -FLT, and ^{18}F -FMISO in LoVo and HCT8 liver metastasis lesions; white nodules indicate liver metastases. It can be seen that the edge of liver metastasis lesions in ^{18}F -FMISO Micro-PET imaging is clear, and the boundary between

the liver and the surrounding organs is clear, and the degree of recognition is high. The edge of liver metastasis lesions in ^{18}F -FLT Micro-PET imaging is blurred, intestinal tube, bladder, etc. The organ intake was higher and the recognition was relatively low. In the ^{18}F -FDG Micro-PET imaging, the liver and metastases were relatively insignificant due to the high intake of the mouse head and heart

Table 4 Comparison of FDG, FLT, and FMISO indices between LoVo and HCT8 metastatic tumors

Index	LoVo(n=9)	HCT8(n=7)	P value
FDG PET			
FDG SUVmax	2.62 ± 0.07	2.45 ± 0.04	0.057
FDG SUVmax ratio*	2.24 ± 0.03	2.20 ± 0.03	0.286
FLT PET			
FLT SUVmax	1.20 ± 0.04	0.70 ± 0.04	0.000
FLT SUVmax ratio*	2.11 ± 0.07	1.62 ± 0.11	0.002
FMISO PET			
FMISO SUVmax	0.39 ± 0.02	0.23 ± 0.02	0.000
FMISO SUVmax ratio*	1.98 ± 0.12	1.49 ± 0.13	0.014

*Metastatic tumor SUVmax divided by the liver SUVmax

and the level of yellow staining reflected the level of protein expression. MACC1 is mainly expressed in the cytoplasm of two cell liver metastasis tissues; SPON2 is expressed in both its cytoplasm and cell membrane. The expression of both proteins in LoVo liver metastasis was higher than that in HCT8 liver metastasis (×400).

Western blot results analysis

The protein expression levels of MACC1 (0.457 ± 0.017 and 0.206 ± 0.016) and SPON2 (0.419 ± 0.015 and 0.267 ± 0.026) in LoVo liver metastasis were higher than those in HCT8 ($P < 0.0001$, $P = 0.0003$). Our results indicate that in vivo experiments, the expression of MACC1 and SPON2 in high metastatic potential LoVo cells is higher than that of low metastatic potential HCT8 cells (Fig. 4).

Linear regression analysis

Figure 5 shows the correlation between MACC1 and SPON2 protein expression and PET SUVmax ratio in different cell

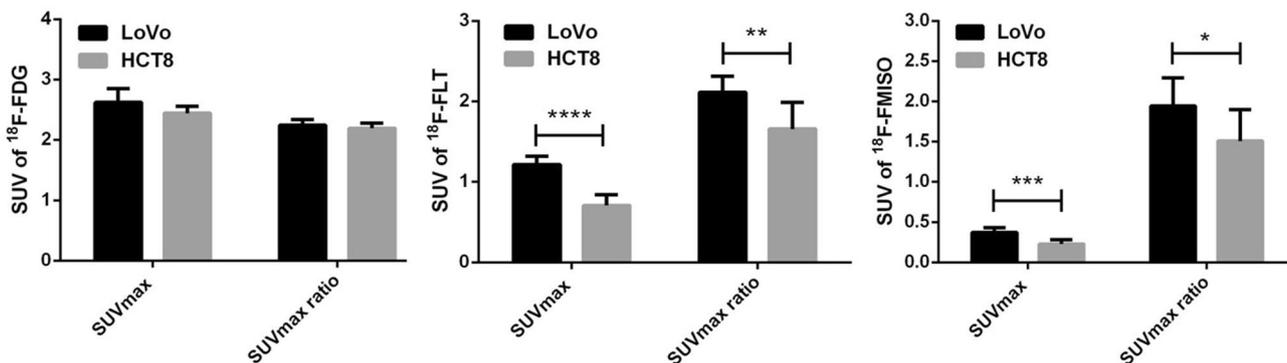


Fig. 2 Between three tracer (¹⁸F-FDG, ¹⁸F-FLT, ¹⁸F-FMISO) PET parameter values (SUVmax, SUVmax ratio) in the LoVo (n=9) and HCT8 (n=7) liver metastasis models Compare, **** $P < 0.0001$

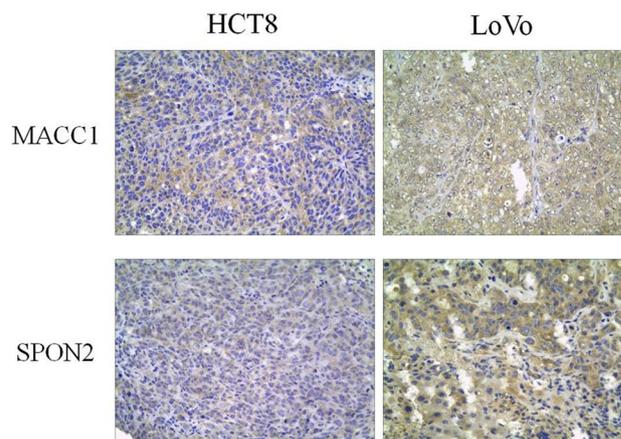


Fig. 3 Immunohistochemical staining of human colorectal cancer LoVo and HCT8 liver metastases reflecting the level of protein expression as a result of yellow staining. The results showed that MACC1 was mainly expressed in the cytoplasm of two kinds of cellular liver metastasis tissues; SPON2 was expressed in both cytoplasm and cell membrane. The expressions of MACC1 and SPON2 in LoVo liver metastasis were significantly higher than those in HCT8 (×400)

liver metastases; Fig. 6 shows the correlation between MACC1 and SPON2 protein expression. Linear regression analysis showed a significant positive correlation between MACC1 and SPON2 ($r = 0.763$, $P = 0.0015$). Correlation analysis of ¹⁸F-FLT SUVmax ratio showed that the ¹⁸F-FLT SUVmax ratio was significantly positively correlated with the expression of MACC1 and SPON2 in hepatic metastasis ($r = 0.737$, $P = 0.0026$; $r = 0.842$, $P = 0.0002$). Correlation analysis of ¹⁸F-FMISO SUVmax ratio showed that the ¹⁸F-FMISO SUVmax ratio was significantly positively correlated with the expression of MACC1 in hepatic metastasis ($r = 0.770$, $P = 0.0013$).

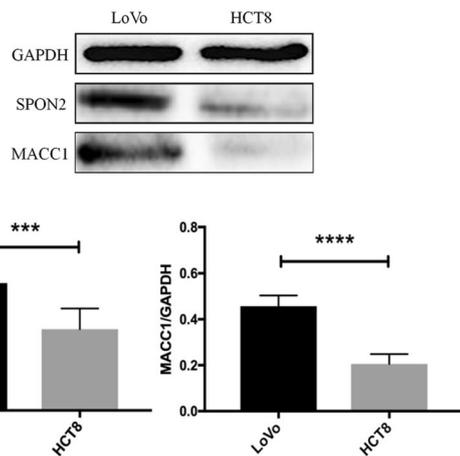


Fig. 4 Western blot quantitative analysis of SPON2 and MACC1 in LoVo and HCT8 liver metastases, using independent sample t-test to analyze the differences between the two proteins (*** $P=0.0003$, **** $P<0.0001$)

Discussion

The most central aspect of the biological behavior of colorectal cancer is invasion and metastasis, which is mediated by a series of ordered steps. Molecular imaging can visualize the metastatic process, and the living body shows changes in physiological functions at the cellular level. PET, as the main imaging device for molecular imaging, displays specific protein expression changes at the molecular level by means of specific targeting probes, which provides an effective method for visualization of tumor invasion and metastasis. In the early stage, our group established a correlation between GLUT-1 and HIF-1 α by establishing molecular imaging features of liver metastasis nodules (^{18}F -FMISO and ^{18}F -FDG), in order to achieve early diagnosis of colorectal cancer liver metastasis [19]. However, due to the initial stage of the experiment, the selected molecular imaging indicators and tumor-related biological characteristics indicators are limited, and it is impossible to detect the biological behaviors related to tumor metastasis in many aspects. Therefore, based on this study, the new tumor metastasis

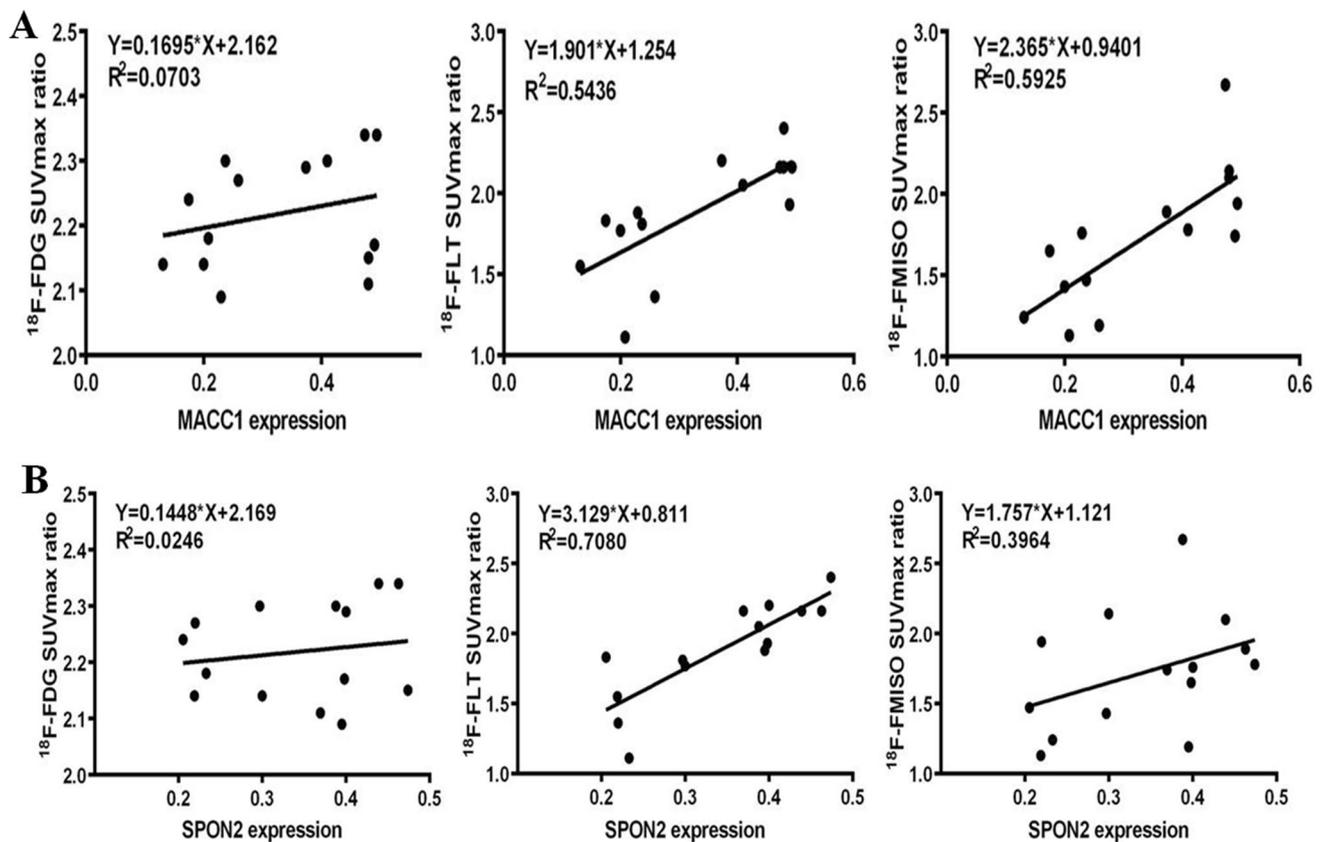


Fig. 5 Correlation analysis between MACC1 and SPON2 and three tracers in LoVo and HCT8 liver metastases (n=14). **a** There was a significant positive correlation between MACC1 expression and expression of ^{18}F -FLT SUVmax ratio and ^{18}F -FMISO SUVmax ratio

in liver metastasis. **b** There was a significant positive correlation between the expression of SPON2 in liver metastases and the expression of ^{18}F -FLT SUVmax ratio

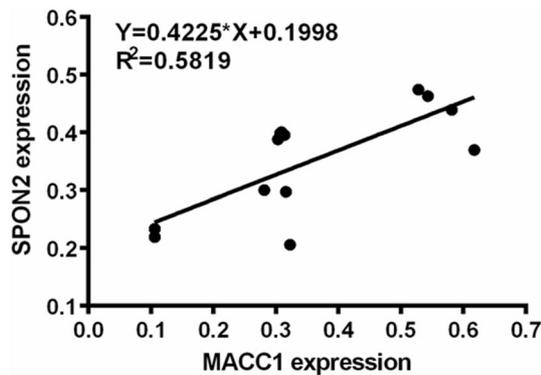


Fig. 6 Correlation between MACC1 and SPON2 protein expression. Linear regression analysis showed a significant positive correlation between MACC1 and SPON2 ($r=0.763$, $P=0.0015$)

markers MACC1 and SPON2 were combined to detect the physiological function of liver metastasis of colorectal cancer. The results showed that ^{18}F -FLT and ^{18}F -FMISO uptake finally reflected the difference in expression of tumor biomarkers (MACC1, SPON2), predicted the metastatic potential of the two tumor cells, and provided early diagnosis and metastatic potential prediction for clinical colorectal cancer liver metastasis.

In recent years, many literatures have reported on the abnormally high expression of MACC1 and SPON2 in various cancers and their potential applications in the development, progression and prognosis of cancer. In hepatocellular carcinoma, MACC1 is considered to be a biomarker of survival prognosis [21]. In lung adenocarcinoma, high levels of MACC1 expression have been shown to be associated with postoperative recurrence of tumors [23]. SPON2 has been proposed as a diagnostic biomarker for ovarian cancer and prostate cancer [23, 24]. In addition, the SPON2 gene is significantly upregulated in CRC compared to colorectal adenomas [25]. These findings of MACC1 and SPON2 effects in different malignancies support the results of this study, suggesting that they may ubiquitously promote carcinogenesis.

First, we carried out in vitro cellular uptake experiments of LoVo and HCT8 on three tracers, which laid the foundation for the detection of different biological behaviors of PET in vitro. The results showed that LoVo had higher rates of uptake of the three tracers at 240 min than HCT8, with statistically significant differences ($P=0.001$, 0.000 , 0.020 , respectively). Moreover, the uptake of ^{18}F -FDG is always higher than the intake of the other two tracers, regardless of cell type. PET imaging results of small animals showed that the SUVmax and SUVmax ratio values of the three tracers in LoVo cells were higher than those of HCT8, which was consistent with the results of in vitro cell uptake experiments, and further confirmed the diagnostic value of different tracer PET imaging on tumor tissues/cells. Compared with the

other two tracer PET images, the FDG SUVmax and FDG SUVmax ratio values of all liver metastases were not statistically different between the two cells, which was consistent with the ^{18}F -FDG in vitro uptake experiment. We speculate that this difference may be related to the internal environment, indicating that the glucose consumption of these two liver metastases is more consistent, and ^{18}F -FDG cannot distinguish the difference between the two cell metastases. Most cancer cells are metabolically active, with relatively little cell proliferation and hypoxia. The net absorption of FDG is higher than FLT and FMISO in tumors. Moreover, in addition to tumor cells, many inflammatory cells are usually present in malignant lesions, resulting in higher FDG uptake [26]. The FLT SUVmax and SUVmax ratio values were both higher than FMISO, consistent with the results of the cellular uptake experiments. This suggests that FLT imaging is more sensitive to CRC liver metastasis than FMISO.

Tumor invasiveness is a key factor affecting the prognosis of colorectal cancer, including differentiation, growth rate, and metastatic potential. In this manuscript, the new biological characteristics of liver metastasis related to liver metastasis, MACC1 and SPON2, were combined to analyze the feasibility, specificity and effectiveness of multi-probe uptake on biological characteristics of metastasis from both in vivo and external aspects.

As a biological marker for the progression, metastasis and survival of many solid tumors, MACC1 has considerable basic and clinical research value [27, 28]. However, it has not been reported in the pathological mechanism of liver metastasis of colorectal cancer and clinical marker research. Western blot results showed that there was a significant difference in the expression of MACC1 between LoVo and HCT8 liver metastases ($P<0.0001$), which was consistent with the results of immunochemical experiments. This indicates that the stronger the cell's ability to metastasize, the higher its MACC1 expression, and MACC1 can also reflect the metastatic ability of colorectal cancer cells. In this study, we found that MACC1 was significantly correlated with ^{18}F -FLT SUVmax ratio and ^{18}F -FMISO SUVmax ratio ($r=0.737$, $P=0.0026$; $r=0.770$, $P=0.0013$). It can be seen that MACC1, a novel tumor metastasis marker protein, can not only reflect the malignant degree of tumor, but also closely related to tumor proliferation and hypoxia metabolism. That is, the uptake characteristics of ^{18}F -FLT and ^{18}F -FMISO tracers can reflect the invasion and metastasis ability of colorectal cancer, and can be detected by MACC1 expression level, which has not been reported in previous studies.

SPON2 has been observed to increase SPON2 gene and protein expression in liver cancer [29], gastric cancer [30], ovarian cancer [23], and prostate cancer [18, 24, 31, 32]. Studies have shown that the SPON2 gene is upregulated in colorectal cancer compared to colorectal adenomas [25,

33]. Western blot analysis showed that there was a significant difference in the expression of SPON2 between LoVo and HCT8 liver metastases ($P=0.0003$), which was consistent with the results of immunochemistry experiments. The stronger the cell's ability to metastasize, the higher its SPON2 expression, and SPON2 also reflects the metastatic ability of colorectal cancer cells. In this study, we found that SPON2 was significantly correlated with ^{18}F -FLT SUVmax ratio ($r=0.842$, $P=0.0002$). It can be seen that the new tumor metastasis marker protein of SPON2 can not only reflect the malignant degree of tumor, but also closely related to tumor proliferation and metabolism. That is, the uptake characteristics of ^{18}F -FLT can reflect the invasion and metastasis ability of colorectal cancer, and can be detected by SPON2 expression level. It can be seen that overexpression of SPON2 can enhance the proliferation of colon cancer cells. This is consistent with previous reports that SPON2 may be a biomarker for CRC diagnosis and prognosis [34].

This study shows that ^{18}F -FLT and ^{18}F -FMISO are more favorable for the detection of liver metastases from colorectal cancer than ^{18}F -FDG. These three tracers have different metabolic pathways and reflect different physiological processes. The difference in imaging reflects differences in the levels of metabolic substrates (thymine, nitroimidazole and glucose), which may be due to microenvironment, blood. The effects of circulation, metabolism or related pathways. This study showed that the level of glucose metabolism was significantly higher than that of thymine and nitroimidazole at the cellular or tissue level, but there was no significant difference in the expression of ^{18}F -FDG between cells with different metastatic potential and corresponding liver metastases. It is indicated that malignant tumors have high glucose metabolism, but glucose consumption is similar. ^{18}F -FDG itself cannot distinguish the difference between different metastatic potential colorectal cancer liver metastases, which indirectly indicates that ^{18}F -FDG is highly sensitive. Poor specificity. The ^{18}F -FLT SUV values in both colorectal cancer liver metastasis models were higher than ^{18}F -FMISO, which was consistent with the results of in vitro cell uptake experiments. It indicates that the proliferation and metabolism of tumors are higher than their hypoxia metabolism at the tissue or cell level, that is, the proliferation level can better reflect the invasion and metastasis ability of tumors. In addition, ^{18}F -FMISO has lower sensitivity to ^{18}F -FLT than liver metastases, but it has different degrees of correlation with tumor metastasis-related proteins. By comparing the characteristics of in vitro and in vivo nuclide uptake and the comprehensive analysis of MACC1 and SPON2 protein levels in colorectal cancer, we found that the higher the ^{18}F -FLT uptake, the stronger the cell transfer ability, the higher the protein levels of MACC1 and SPON2, ^{18}F -FLT The level of

uptake was significantly correlated with the expression of MACC1 and SPON2. The higher the ^{18}F -FMISO uptake, the stronger the cell transfer ability, and the higher the MACC1 protein level, the ^{18}F -FMISO uptake level was significantly correlated with MACC1 expression. Individual differences between tumor phenotypes complicate the choice of tumor treatment options [35, 36]. As the results of this study show, ^{18}F -FLT and ^{18}F -FMISO PET can not only show the heterogeneity between tumors, but also have the ability to distinguish the biological characteristics of tumors. Therefore, the combination of ^{18}F -FLT and ^{18}F -FMISO PET imaging is also of great value for the choice of tumor treatment options.

This study has certain limitations. The experiment used ROI technology to semi-quantitatively analyze the lesions. Through visual analysis, the ROI region was drawn in the abnormal uptake area of the liver. Due to the small lesions of liver metastases in some nude mice, there was a certain error in the outline of the ROI region, and the liver metastasis model Less in quantity. In the future, it is necessary to expand the sample size and improve the experimental methods to provide greater clinical applicability for early detection, metastatic potential evaluation, and prognosis evaluation of colorectal cancer liver metastasis.

In summary, although ^{18}F -FDG has higher sensitivity than the other two tracers, it lacks specificity, and the inability to distinguish the diagnosis is still a necessary disadvantage. Both ^{18}F -FLT and ^{18}F -FMISO imaging can evaluate the invasion and metastasis ability of colorectal cancer and can be detected by detecting the expression level of new tumor markers and multi-probe metabolic markers. It is a preferred PET tracer for the diagnosis of colorectal cancer liver metastasis.

Author Contributions Hao Jiang and Rongjun Zhang carried out the studies, participated in collecting data, and drafted the manuscript. Hao Jiang and Wei Guo performed the statistical analysis and participated in its design. Kuan Huang and Hongbo Hu participated in acquisition and analysis of data. Huijie Jiang designed the study and carefully revised the manuscript. Song Wang supervised the study and contributed to data interpretation. Xue Lin has contributed to the experiments. They also carefully revised the manuscript. All authors read and approved the final manuscript.

Funding This study was supported by grants from the National Key Research and Development Program of China (2019YFC0118100), the National Natural Science Foundation of China (81671760 and 81873910), Scientific Research Transformation Special Fund of Heilongjiang Academy of Medical Sciences (2018415), Scientific Research Project of Health and Family Planning Commission of Heilongjiang Province (201812 and 201622), and Heilongjiang Province Postdoctoral Special Funding Project (LBH-Z20078).

Compliance with ethical standards

Conflict of interest: All authors declare that they have no any conflict of interests.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries[J]. *CA Cancer J Clin.* 2018;68(6):394-424.
- Arnold M, Sierra MS, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global patterns and trends in colorectal cancer incidence and mortality[J]. *Gut.* 2017;66(4):683–91.
- Bae SH, Moon SK, Kim YH, Cho KH, Shin EJ, Lee MS, et al. Feasibility and response of helical tomotherapy in patients with metastatic colorectal cancer[J]. *Radiat Oncol J.* 2015;33(4):320-7.
- Kawai K, Tamura K, Sakata I, Ishida J, Nagata M, Tsukada H, et al. A new in vivo model to analyze hepatic metastasis of the human colon cancer cell line HCT116 in NOD/Shi-scid/IL-2Rgamma(null) (NOG) mice by (18)F-FDG PET/CT[J]. *Oncol Rep.* 2013;29(2):464-8.
- Cheng Z, Wei R, Wu C, Qing H, Jiang X, Lu H, et al. Ex-vivo biodistribution and micro-PET/CT imaging of ¹⁸F-FDG, ¹⁸F-FLT, ¹⁸F-FMISO, and ¹⁸F-AIF-NOTA-PRGD2 in a prostate tumor-bearing nude mouse model[J]. *Nucl Med Commun.* 2015;36(9):914-21.
- Bejot R, Kersemans V, Kelly C, Carroll L, King RC, Gouverneur V, et al. Pre-clinical evaluation of a 3-nitro-1,2,4-triazole analogue of [18F]FMISO as hypoxia-selective tracer for PET[J]. *Nucl Med Biol.* 2010;37(5):565-75.
- Shen G, Ma H, Pang F, Ren P, Kuang A. Correlations of ¹⁸F-FDG and ¹⁸F-FLT uptake on PET with Ki-67 expression in patients with lung cancer: a meta-analysis[J]. *Acta Radiol.* 2018;59:188195.
- Rasey JS, Grierson JR, Wiens LW, Kolb PD, Schwartz JL. Validation of FLT uptake as a measure of thymidine kinase-1 activity in A549 carcinoma cells[J]. *J Nucl Med.* 2002;43(9):1210-7.
- Buck AK, Halter G, Schirrmeister H, Kotzerke J, Wurzigler I, Glatting G, et al. Imaging proliferation in lung tumors with PET: ¹⁸F-FLT versus ¹⁸F-FDG[J]. *J Nucl Med.* 2003;44(9):1426-31.
- Francis DL, Freeman A, Visvikis D, Costa DC, Luthra SK, Novelli M, et al. In vivo imaging of cellular proliferation in colorectal cancer using positron emission tomography[J]. *Gut.* 2003;52(11):1602-6.
- van Westreenen HL, Cobben DC, Jager PL, van Dullemen HM, Wesseling J, Elsinga PH, et al. Comparison of ¹⁸F-FLT PET and ¹⁸F-FDG PET in esophageal cancer[J]. *J Nucl Med.* 2005;46(3):400-4.
- Stein U, Walther W, Arlt F, Schwabe H, Smith J, Fichtner I, et al. MACC1, a newly identified key regulator of HGF-MET signaling, predicts colon cancer metastasis[J]. *Nat Med.* 2009;15(1):59-67.
- Koelzer VH, Herrmann P, Zlobec I, Karamitopoulou E, Lugli A, Stein U. Heterogeneity analysis of Metastasis Associated in Colon Cancer 1 (MACC1) for survival prognosis of colorectal cancer patients: a retrospective cohort study[J]. *BMC Cancer.* 2015;15:160.
- Chen S, Zong ZH, Wu DD, Sun KX, Liu BL, Zhao Y. The role of metastasis-associated in colon cancer 1 (MACC1) in endometrial carcinoma tumorigenesis and progression[J]. *Mol Carcinog.* 2017;56:1361-71.
- Stein U, Burock S, Herrmann P, Wendler I, Niederstrasser M, Wernecke KD, et al. Circulating MACC1 transcripts in colorectal cancer patient plasma predict metastasis and prognosis[J]. *PLoS One.* 2012;7(11):e49249.
- Zhen T, Dai S, Li H, Yang Y, Kang L, Shi H, et al. MACC1 promotes carcinogenesis of colorectal cancer via beta-catenin signaling pathway[J]. *Oncotarget.* 2014;5(11):3756-69.
- Schmid F, Wang Q, Huska MR, Andrade-Navarro MA, Lemm M, Fichtner I, et al. SPON2, a newly identified target gene of MACC1, drives colorectal cancer metastasis in mice and is prognostic for colorectal cancer patient survival[J]. *Oncogene.* 2016;35(46):5942-52.
- Lucarelli G, Rutigliano M, Bettocchi C, Palazzo S, Vavallo A, Galleggiante V, et al. Spondin-2, a secreted extracellular matrix protein, is a novel diagnostic biomarker for prostate cancer[J]. *J Urol.* 2013; 190(6):2271-7.
- Zhang MY, Jiang HJ, Zhang RJ, Xu HL, Jiang H, Pan WB, et al. Noninvasive evaluation of ¹⁸F-FDG/¹⁸F-FMISO-based Micro PET in monitoring hepatic metastasis of colorectal cancer[J]. *Sci Rep.* 2018;8(1):17832.
- Sato M, Hirose K, Kashiwakura I, Aoki M, Kawaguchi H, Hatayama Y, et al. LW6, a hypoxia-inducible factor 1 inhibitor, selectively induces apoptosis in hypoxic cells through depolarization of mitochondria in A549 human lung cancer cells[J]. *Mol Med Rep.* 2015;12(3):3462-8.
- Qiu J, Huang P, Liu Q, Hong J, Li B, Lu C, et al. Identification of MACC1 as a novel prognostic marker in hepatocellular carcinoma[J]. *J Transl Med.* 2011;9:166.
- Shimokawa H, Uramoto H, Onitsuka T, Chundong G, Hanagiri T, Oyama T, Y et al. Overexpression of MACC1 mRNA in lung adenocarcinoma is associated with postoperative recurrence[J]. *J Thorac Cardiovasc Surg.* 2011;141:895–8.
- Anderson GL, McIntosh M, Wu L, Barnett M, Goodman G, Thorpe JD, et al. Assessing lead time of selected ovarian cancer biomarkers: a nested case-control study[J]. *J Natl Cancer Inst.* 2010; 102(1):26-38.
- Barbieri CE. Evolution of novel biomarkers for detection of prostate cancer[J]. *J Urol.* 2013; 190(6):1970-1.
- Pesson M, Volant A, Uguen A, Trillet K, De La Grange P, Aubry M, et al. A gene expression and pre-mRNA splicing signature that marks the adenomaadenocarcinoma progression in colorectal cancer[J]. *PLoS One.* 2014; 9(2):e87761.
- Kirchner J, Schaarschmidt BM, Kour F, Sawicki LM, Martin O, Bode J, et al. Incidental ¹⁸F-FDG uptake in the colon: value of contrast-enhanced CT correlation with colonoscopic findings[J]. *Eur J Nucl Med Mol Imaging.* 2020;47(4):778–786.
- Wang L, Wu Y, Lin L, Liu P, Huang H, Liao W, et al. Metastasis-associated in colon cancer-1 upregulation predicts a poor prognosis of gastric cancer, and promotes tumor cell proliferation and invasion[J]. *Int J Cancer.* 2013;133(6):1419-30.
- Yamamoto H, Miyoshi N, Mimori K, Hitora T, Tokuoaka M, Fujino S, et al. MACC1 expression levels as a novel prognostic marker for colorectal cancer[J]. *Oncol Lett.* 2014;8(5):2305-9.

29. Liao CH, Yeh SC, Huang YH, Chen RN, Tsai MM, Chen WJ, et al. Positive regulation of spondin 2 by thyroid hormone is associated with cell migration and invasion[J]. *Endocr Relat Cancer*. 2010; 17(1):99-111.
30. Lu H, Feng Y, Hu Y, Guo Y, Liu Y, Mao Q, et al. Spondin 2 promotes the proliferation, migration and invasion of gastric cancer cells[J]. *J Cell Mol Med*. 2020;24(1):98–113.
31. Qian X, Li C, Pang B, Xue M, Wang J, Zhou J. Spondin-2 (SPON2), a more prostate-cancer-specific diagnostic biomarker[J]. *PLoS One*. 2012; 7(5):e37225.
32. Carvalho B, Sillars-Hardebol AH, Postma C, Mongera S, Terhaar Sive Droste J, Obulkasim A, et al. Colorectal adenoma to carcinoma progression is accompanied by changes in gene expression associated with ageing, chromosomal instability, and fatty acid metabolism[J]. *Cell Oncol*. 2012; 35(1):53–63.
33. Zhang Q, Wang XQ, Wang J, Cui SJ, Lou XM, Yan B, et al. Upregulation of spondin-2 predicts poor survival of colorectal carcinoma patients[J]. *Oncotarget*. 2015;6(17):15095-110.
34. Gu G, Dustin D, Fuqua SA. Targeted therapy for breast cancer and molecular mechanisms of resistance to treatment[J]. *Curr Opin Pharmacol*. 2016;31:97-103.
35. de Castro J, Tagliaferri P, de Lima VCC, Ng S, Thomas M, Arunachalam A, et al. Systemic therapy treatment patterns in patients with advanced non-small cell lung cancer (NSCLC): PivOTAL study[J]. *Eur J Cancer Care (Engl)*. 2017;26(6).

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Authors and Affiliations

Hao Jiang¹ · Wei Guo² · Kuan Huang³ · Huijie Jiang¹ · Rongjun Zhang⁴ · Hongbo Hu¹ · Xue Lin¹ · Song Wang⁵

Hao Jiang
hydeyjh@163.com

Wei Guo
guowei1966@163.com

Kuan Huang
784115236@qq.com

Rongjun Zhang
zhangrongjun@jsinm.org

Hongbo Hu
huboshi@tom.com

Xue Lin
1151700815@qq.com

¹ Department of Radiology, The Second Affiliated Hospital of Harbin Medical University, Harbin, China

² Department of Ultrasound, Harbin the First Hospital, Harbin, China

³ Department of Radiology, Ningbo Yinzhou No.2 Hospital, Ningbo, China

⁴ Jiangsu Institute of Nuclear Medicine, Wuxi, China

⁵ Department of Radiology, Longhua Hospital Shanghai University of Traditional Chinese Medicine, Shanghai, China