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Application of molecular imaging technology in evaluating the inhibiting effect of apigenin *in vivo* on subcutaneous hepatocellular carcinoma

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ABSTRACT

The aim of this study was to evaluate the inhibiting effect of apigenin on liver cancer *in vivo* based on the optical molecular imaging method. Subcutaneous liver tumor models were established using respective 1×10^6 firefly luciferase (fLuc) and green fluorescent protein (GFP) labeled human hepatocellular carcinoma cells (HepG2-fLuc and HepG2-GFP cells) in 20 BALB/c nude mice which were randomly divided into two groups, 10 in each group. After the tumor cells were implanted 15 days, apigenin was administered through intraperitoneal injection in group B, the other ten mice as control group A. Bioluminescence imaging (BLI) and fluorescence molecular imaging (FMI) were carried out for the follow-up of subcutaneous tumor model. As time goes on, intensity and distribution of bioluminescence and fluorescence of tumours increased gradually with the growth of tumours little by little. The whole process of observation was in accordance with known activities of HCC in the human liver. The tumor volume and tumor weight were significant lower in group B than in group A ($p < 0.05$), Subcutaneous tumours in the apigenin treatment group B based on BLI and FMI were significantly inhibited compared to the control group A ($p < 0.05$). Apigenin could be expected as a new drug to treat hepatocellular carcinoma. Optical molecular imaging technology enabled the non-invasive and reliable assessment of anti-tumor drug efficacy on liver cancer.

1. Introduction

Hepatocellular carcinoma (HCC) remains the fifth most common cancer as well as the third leading cause of cancer mortality worldwide [1]. Current therapeutic options, including surgical resection, radiotherapy, and chemotherapy, have been unsatisfactory in most patients. Patients with underlying liver disease, unsuitability for resection, or little organ availability for transplantation are not candidates for surgery [2]. Therefore new therapies and new detection methods for this aggressive disease are extremely needed. Apigenin has gained particular interest in recent years as a beneficial and health promoting agent because of its low intrinsic toxicity and striking effects on normal versus cancer cells, compared with other structurally-related flavonoids [3]. Interest in the possible cancer preventive of apigenin has increased owing to reports of potent antioxidant and anti-inflammatory activities [4]. Indirect support for this assumption is correlated with a study where consumption of apigenin free diets by healthy human volunteers has been reported to lead to a decrease in markers of oxidative stress in blood viz. plasma antioxidant vitamins, erythrocyte superoxide dismutase (SOD) activity and lymphocyte DNA damage commonly associated with enhanced disease risk, suggesting the beneficial effects of apigenin [5]. However, there is very little evidence to date to suggest that apigenin promotes adverse metabolic reactions *in vivo* as a cancer chemopreventive agent [6].

The traditional approach for anti-neoplastic research such as histopathological analysis is accurate but time-consuming and cannot provide 3-dimensional (3D) structural information. Therefore, the evaluation of therapeutic response only through tumor

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volume measurement is no longer comprehensive [7], and it is an urgent need to develop a more sensitive and effective detection method. Molecular imaging, which allows for sensitive, longitudinal observation, has become an invaluable tool for early lesion detection, monitoring therapeutic efficacy, and facilitating drug development. Noninvasive imaging of tumor allows for much earlier diagnosis and better prognosis, which will eventually lead to effective therapy [8,9].

Although the liver tumor model is the suitable way to represent the disease process in human, animal models of human cancer are the optimal tool for preclinical evaluation of novel therapies and has been studied extensively [10,11]. Its application in optical molecular imaging for longitudinal monitoring and drug response on liver cancer has seldom been tried [12]. In this study, we comprehensively evaluated the inhibiting effect of apigenin on liver cancer *in vivo* using molecular imaging technology.

2. Materials and methods

2.1. Animals and reagent

Twenty 4–6 weeks old Balb/c nude mice (male, 20–22 g) used in this study were obtained from the Academy of Military Medical Science (Beijing, China). Apigenin was purchased from the Bei Na Chuang Lian Biotechnology Research Institute (Beijing, China), which was injected through intraperitoneal at a dose of 0.5 mg/kg [13,14]. D-Luciferin was bought from Biotium (CA, Fremont, USA). The labeled human hepatocellular carcinoma cell line HepG2-fLuc and HepG2-GFP were kindly provided by Prof. Lin-Sheng Zhan of the Academy of Military Medical Science.

2.2. Cell culture

HepG2-fLuc and HepG2-GFP cells were cultured in Dulbecco's modified eagle medium (DMEM; Thermo Scientific) and supplemented with 10% fetal calf serum (FCS) (HyClone; Thermo Scientific). They were maintained at 37°Cincubator with 5% CO_2.

2.3. Animal models

Mice were routinely bred in a specific pathogen-free (SPF) laboratory in the animal center of Institute of Automation, Chinese Academy of Sciences, with controlled temperature at 23 ± 2 °C and relative humidity of 50% with 12 h light/dark cycle. All animal experiments were carried out according to the standards of animal care as outlined in the NIH guide for the Care and Use of Laboratory Animals. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. The subcutaneous tumor models were established by respectively injecting 1×10^6 HepG2-fLuc and HepG2-GFP cells suspension into the left upper flanks (using HepG2-fLuc cells) and the right upper flanks (using HepG2-GFP cells) of BALB/c nude mice (N = 20) respectively. After tumor cell implantation, animals were kept in a warm cage, observed for 1~2 h, and subsequently returned to the animal room after full recovery from the anesthesia was documented. The body weight and general physical status of mice were recorded daily. The mice all had euthanasia on the 45th day and the tumours were removed, weighed and fixed in neutral buffered 10% formalin.

2.4. Apigenin treatment

The subcutaneous tumor mice were randomly divided into the control group (group A) and the experimental group (group B) (N = 10 per group). After the tumor cells were implanted 15 days, the experimental group and control group for the subcutaneous tumor-bearing mice were given intraperitoneal injections with apigenin (0.5 mg/kg/2days) and an equal amount of 0.9% saline for the rest of days.

2.5. Bioluminescence imaging (BLI) and fluorescence molecular imaging (FMI) tumour evaluation

For our experiment, we used the IVIS Spectrum (PerkinElmer Life Sciences, Hopkinton, MA, USA) *in vivo* imaging system to measure the average fluorescence intensity and distribution using a region of interest (ROI) centered of liver via Living Image 4.4 software. IVIS is calibrated to enable absolute quantitation of the bioluminescent signal and longitudinal studies can be performed over many months and over several orders of signal magnitude without compromising the quantitative result. Measurement data are displayed in the table together with all experimental parameters relating to the image capture which can be saved or exported for analysis. Multiple images can be acquired and compared in longitudinal studies covering seconds or months depending on the nature of the experiment.

The mice were fasted overnight prior to the experiment to prevent food from interfering with the bioluminescence results. The mice were kept in lie supine and affixed on the mouse rack and anesthetized with 2% isoflurane through a respiratory mask which was attached to the mouse rack. BLI and FMI were scanned on the IVIS Spectrum imaging system. The mice received D-luciferin solution (150 mg/kg body weight) 3 min before BLI was started. The bioluminescent and fluorescent signals of the subcutaneous tumor were obtained daily and their intensity and distribution of tumours were observed and compared between two groups. The body weight and general physical status of the animal were recorded daily. The tumor volume was measured by caliper every two days. At the endpoint of the study, the tumours were removed and weighed.

2.6. Three-dimensional (3D) reconstruction and volume measurement

3D-Modality provides a histogram-based method for classifying 3D volumetric data. The histogram represents the distribution of voxel intensities in the 3D volumetric data and their color opacity values. The goal is to set color and opacity values for different intensity ranges so that the color-opacity map shows the volume regions that are interested in (opaque in the map) and hides unimportant regions (transparent in the map). 3D-Modality also enable classify the volumetric data by specifying color and opacity values for different intensity ranges so that researchers can view or hide certain parts of the data as needed.

2.7. Statistical analysis

Results of the quantitative studies were expressed as mean ± SD. Means were compared using the independent-samples T test and one-way analysis of variance (ANOVA) by using the SPSS Statistical Software Package, version 19.0 (SPSS, Inc), and p value < 0.05 were considered statistically significant.

3. Results

3.1. General physical status of tumours in two groups

In the first three days, tumor cells became fewer because of the phagocytosis and decomposition of the macrophage and the trypsins. At about the five day, the tumor cells were equal with the

number of the cells on the first day. In the following days, tumours began to mount up exponentially. Thereafter, these tumours grew rapidly in the injected lobe and began to spread to other lobes. Three (15%) out of 20 mice died during the surgery (1 in each group A and 2 in group B), 2 (10%) mice did not develop to tumours (1 in each group) and the corresponding number mice were added. No mice succumbed to perioperative complications.

Compared with mice in the control group (group A), those in the experimental group (group B) had a significantly lower tumor volume, tumor weight, BLI/FMI light intensity (all $p < 0.05$) and no significant dominance was found respect to mouse weight ($p > 0.05$) (Fig. 1).

3.2. Neoplastic inhibition was monitored by BLI during apigenin treatment

In order to track tumor progression and regression more accurate, we not only measured tumor volume, but also performed BLI to record the dynamic light intensity changes. The results showed that the subcutaneous tumor BLI signal of the experiment group was significantly inhibited since day 20 ($p < 0.05$) (Fig. 2b), which appeared regularly all long. The most obvious difference between the two groups was obtained on day 30. The control group light intensity was 4.15 $\times$ 10^6 Photons/cm^2/s on day 12, whereas the experiment group was only1.04 $\times$ 10^6 Photons/cm^2/s (Figs. 2b and 3).

3.3. Monitoring of neoplastic inhibition by FMI during apigenin treatment

Based on the above results, we further used FMI to detect the dynamic fluorescent changes in the subcutaneous tumor models. Firstly, a significant light intensity difference was also detected as early as the 20th day (the FMI light intensity of the experiment group was 0.42 $\times$ 10^9 Photons/cm^2/s, whereas the control group was as high as 1.21 $\times$ 10^9 Photons/cm^2/s) ($p < 0.05$) (Fig. 2d), which appeared regularly all long. ($P < 0.05$) (Figs. 2d and 4).

4. Discussion

In recent years, optical molecular imaging has emerged as an important tool of technologies to advance our understanding of disease mechanisms and accelerate drug discovery [15,16]. It can especially enhance the visualization, characterization and quantification of biological processes in living subjects [17,18]. These features are highly valuable for preclinical tumor research especially for the assessment of new anti-tumor drugs. Apigenin, a naturally occurring plant flavone, abundantly present in common

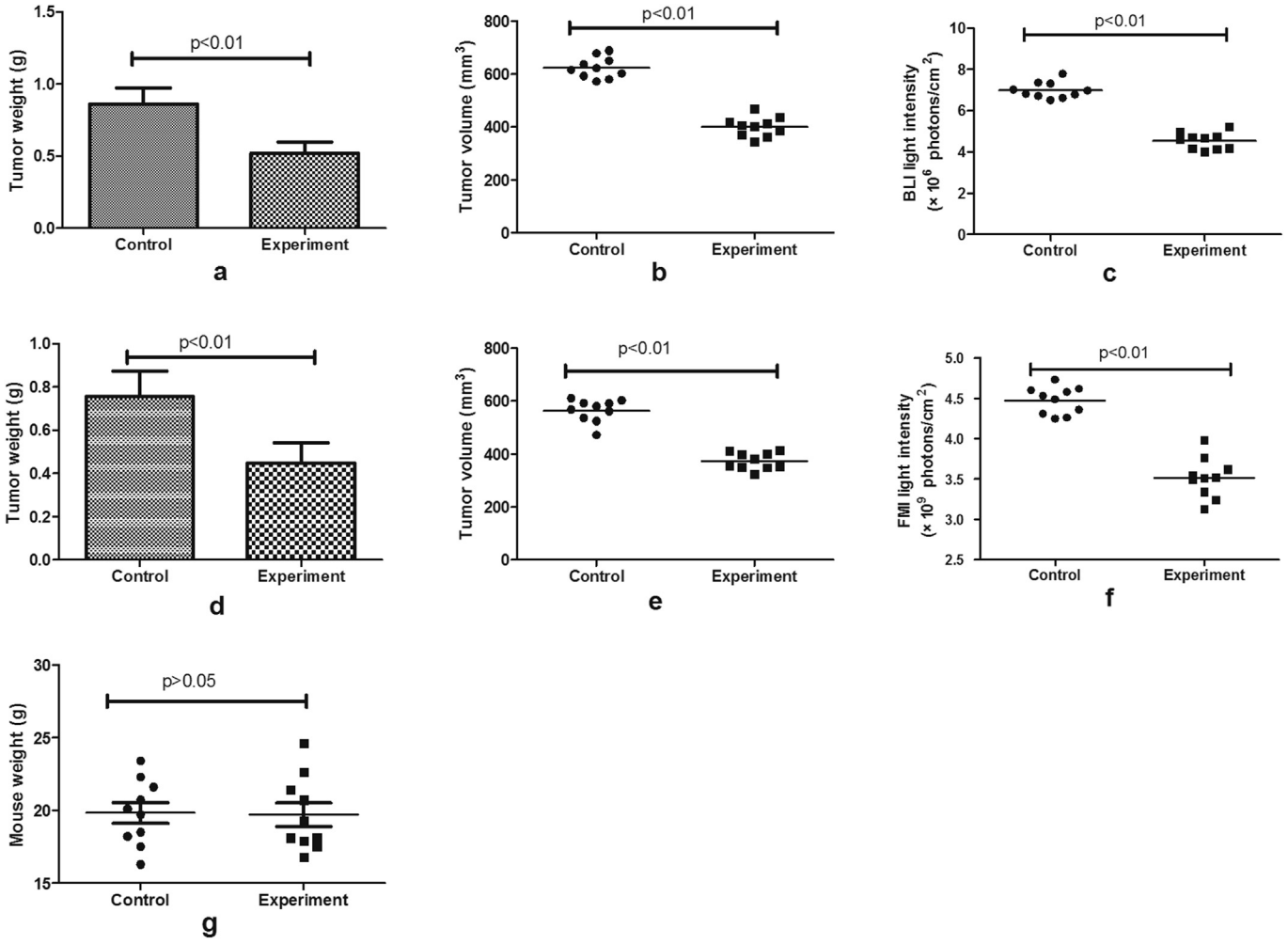

Fig. 1. Effects of apigenin treatment on the inhibition of tumor weight, tumor volume and the quantitative light intensity based on HepG2-fLuc cells line (a, b, c) and HepG2-GFP cells line (d, e, f) at the end 45 days point. There were statistically significant differences between two groups in tumor volume, tumor weight, BLI/FMI light intensity (all $p < 0.05$). There was no statistically significant change in body weight between animal groups (g, $p > 0.05$), which indicated that the dosing regimens were well tolerated.

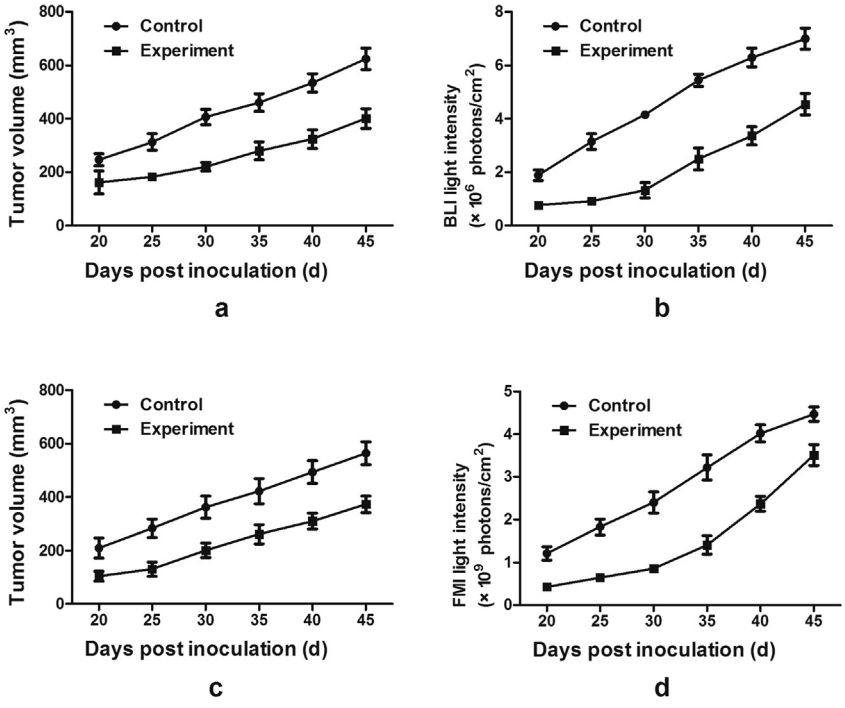

Fig. 2. The growth curve of the tumor volume and the quantitative light intensity based on HepG2-fLuc cells line (a, b) and HepG2-GFP cells line (c, d).

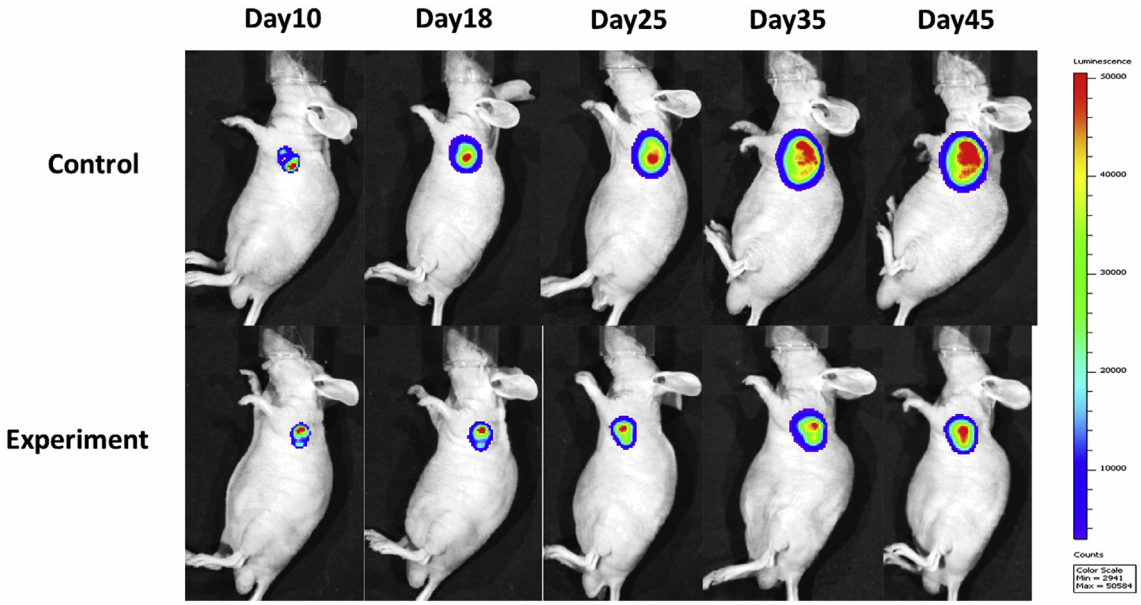

Fig. 3. The BLI of subcutaneous tumor model. Continuous BLI light intensity observation of the subcutaneous tumor model from day 10 to day 45. The bioluminescence intensity and distribution of experiment group were obviously lower than that of control group at all five time points.

fruits and vegetables is recognized exert a broad range of molecular signaling effects and involve in a variety of material metabolism in the body [19]. It has been shown to possess anti-tumor activity in a variety of tumor cells [4]. In this study, firstly, we consistently demonstrated that apigenin treatment causes significant inhibits of liver tumour compared to the control treatment. Secondly, we combined BLI and FMI to overcome the imaging depths and resolution limits, which allowed researchers to pinpoint the tumor progression and location, which also showed that using molecular imaging technology to observe the progression of tumours was feasible and reliable. The IVIS Spectrum system permitted facile and early detection of tumor progression and therapeutic responses, and we presented a comprehensive evaluation of the anti-tumor effect of apigenin.

We found that the differences of the light intensity detected by BLI between control and experiment groups were more dramatic than the tumor volume measured by calipers (Fig. 1). The reason could be that the bioluminescent light signal emitted from the tumor cells was derived from living tumor cells, which provided a quantitative surrogate measurement of the number of living tumor

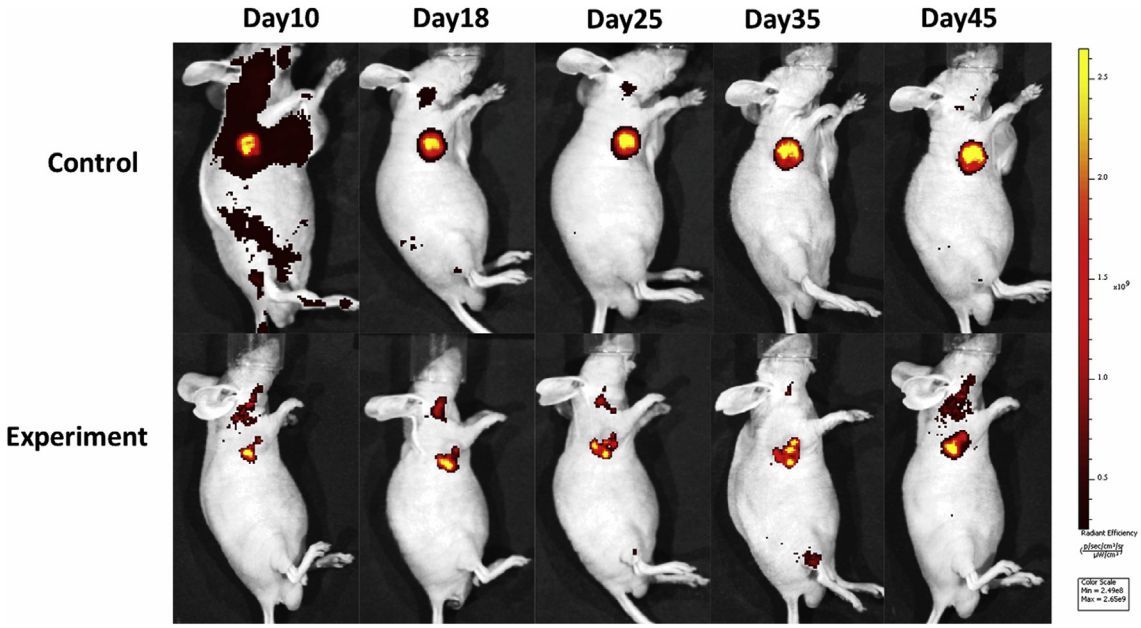

Fig. 4. The FMI of subcutaneous tumor model. Continuous FMI light intensity observation of the subcutaneous tumor model from day 10 to day 45. It could be saw that the fluorescence intensity and distribution of experiment group were obviously lower than that of control group at all five time points.

cells [20]. Therefore, BLI was not only more sensitive than traditional measurement when the tumor inhibition was not fully reflected in the tumor volume shrinkage but also more suitable for the imaging of subcutaneous tumor model due to the minimal background. At the meantime, volume measurement is a very important aspect to detect and follow liver tumours. Unfortunately, in liver volumetry is not always available, because it is usually need to kill animal or linked to the CT scanner system. The running hardware was too expensive and requires an experienced radiologist. User-friendly and easily accessible instruments are needed to predict liver volume [21].

Imaging modalities can be broadly divided into primarily morphological/anatomical and functional imaging techniques [22]. Primarily morphological/anatomical imaging technologies, such as CT, MRI (with contrast agents injected at millimolar blood concentrations) and ultrasound, are characterized by high spatial resolution. However, they also share the limitation of not being able to detect diseases until tissue structural changes (for example, growth of a tumour) are large enough to be detected by the imaging modality [23,24]. PET and SPECT (with radiotracers injected at nanomolar blood concentrations) belong to molecular imaging modalities, offering the potential to detect molecular and cellular changes of diseases. However, these modalities suffer from a relatively weaker spatial resolution with currently available technology and a high radiation exposure [25,26]. In this study, on the one hand, we combined the strengths of morphological/anatomical and functional imaging, double-modality imaging allowed the detection of pathophysiological changes in early disease phases at high structural resolution. On the other hand, we measured tumours volume using 3D construction technology via the IVIS Spectrum System, allowing the stereoscopic identification of tumor location. It took only approximately 10 min to reconstruct the integrated 3D images. The roles BLI and FMI are complementary to each other, through the IVIS Spectrum, FMI could be carried out to fluorescence molecular tomography (FMT) to analyze vascular 3D morphological changes and give the quantitative assessment. So, the tumor location can be reconstructed, which was less depth dependent and was able to reconstruct the 3D fluorescence distribution and provide accurate quantification [27]. Therefore the combination of BLI and FMI enabled us to improve the accuracy of apigenin anticancer efficacy.

In conclusion: (1) The results confirmed the effects of apigenin on HCC suppression and apigenin could be expected as a new drug to treat HCC; (2) The integration of BLI and FMI meets the requirements of being noninvasive, accurate, and capable of continuously monitoring apigenin anti-cancer research and drug evaluation.

Author contributions

Conceived and designed the experiments: Chi-Hua Fang, Xian-Fang Shao.

Performed the experiments: Gang Li, Chong-Wei Chi.

Analyzed the data: Gang Li.

Contributed reagents/materials/analysis tools: Xian-Fang Shao.

Wrote the paper: Gang Li.

Conflict of interest statement

None declared.

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I certify that none of the material in this manuscript has been published previously in any form and that none of this material is currently under consideration for publication elsewhere. This includes symposia and proceedings of meetings and preliminary publications of any kind. All of the listed authors contributed to the article and are aware of and agree to its submission for publication. There has been no industry or pharmaceutical support.

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