SCIENTIFIC RESEARCH

EVALUATION OF 18FLUORIN-FLUOROTHYMIDINE UPTAKE IN BREAST AND LUNG-TUMOR BEARING MICE

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SUMMARY

Background: PET (positron emission tomography) techniques and radiopharmaceutical tracers are becoming increasingly common in clinical practice. Since 1991, 3'-deoxy-3'-18Fluorine-Fluorothymidine (18F-FLT) has been studied as a PET tracer for tumor proliferation assessment. The purpose of this study was to assess tracer uptake (%ID/g) in tumor-bearing mice and the relationship between uptake values and Ki-67 expression.

Materials and methods: two cell lines including breast cancer cell line 4T1 and lung cancer cell line LLC were inoculated into BALB/c mice. The radiotracer was then injected, and tumor resection was performed 20, 40, 60, and 90 minutes later. Gamma-ray spectrometer was used to calculate uptake values.

Results: The data show that the tumor's %ID/g were 5.52 ± 0.23 and 4.66 ± 0.49 for the 4T1 and LLC tumors, respectively, at 90 minutes after injection. The LLC tumors presented a higher Ki-67 index than the 4T1 tumors (90.16 ± 2.93% vs 71.83 ± 3.54%).

Conclusion: In our model experiment, accumulation of the tracer in the tumor samples correlates with its malignancy which indicates that 18F-FLT could be a feasible PET tracer.

Keywords: *3'-deoxy-3'-18Fluorine-Fluorothymidine, cell proliferation, Ki-67, positron emission tomography.*

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INTRODUCTION

^{18}F-FDG PET/CT is now a valuable tool in cancer diagnosis and treatment. The tumor-detectable radioactive agents, combined with imaging facilities such as CT (computed tomography) or MRI (magnetic resonance imaging), have provided an effective tool in tumor assessment, from staging to treatment response and restaging. However, due to its reliance on glucose metabolism, ^{18}F-FDG is significantly affected by physiological processes (i.e., a huge amount of glucose usage in the brain) and inflammation, resulting in a poor specificity of PET imaging [2,3]. Many new radiotracers based on different mechanisms such as DNA synthesis, hypoxia, or phospholipid synthesis, have been studied to overcome the disadvantages of ^{18}F-FDG, and ^{18}F-FLT is one of those compounds [4]. ^{18}F-FLT – a pyrimidine analog is phosphorylated by thymidine kinase 1 (TK1), which is a specific enzyme in the S phase. Hence, the presence of ^{18}F-FLT in the tumors reflects the rates of DNA synthesis and cell proliferation [5, 6]. The uptake of FLT also reflected the same cell-proliferative activity as Ki-67 immunostaining, forming an ideal combination for cancer research. The Ki-67 protein is present in all phases of cellular activity including G1, S, G2) but not in the resting phase G0, making it an important marker in the clinical evaluation of tumors (Ki-67 index) [7, 8]. In this study, we investigated the uptake of PET FLT radiotracer in mice induced with breast cancer cell line 4T1 and lung cancer cell line LLC and compared with obtained Ki-67 index.

MATERIALS AND METHODS

Radiopharmaceutical preparation. ^{18}F-FLT was synthesized at Cyclotron Center and the Nuclear Medicine department of 108 Military Central Hospital with an in-house developed synthesis module based on Synthera (IBA Molecular, Dulles, VA, USA). ^{18}F-FLT precursor using 3-*N*-Boc-5’-*O*-dimethoxytrityl-3’-*O*-nosyl-thymidine was purchased from ABX (Radeberg, Germany). The precursor reacted with the ^{18}F-fluoride ions in acetonitrile solvent with the presence of Kriptofix 2.2.2. catalyst at 100° C to 130° C. The protection groups were removed with 2M hydrochloric acid and 0.1M sodium hydroxide. The final product was purified on a C18 HPLC column and met the standards in the alovudine monograph of EP8 (European Pharmacopoeia 8).

Cell lines: 4T1 breast cancer cell line was purchased from ATCC (USA) and LLC lung cancer cell line was provided by Prof. J Meier, Milan University, Milan, Italy. LLC cells and 4T1 cells were cultured in DMEM medium and RPMI-1640 medium respectively, supplemented with 10% fetal bovine serum (FBS) and 1% PSF (Penicillin 10,000 U/mL - Streptomycin 10,000 µg/mL - Amphotericin B 250 µg/mL). All cells were maintained under a humidified incubator with 5% CO_2 at 37°C and harvested with trypsin-EDTA.

Animals: male and female BALB/c mice (n=48) aged 8 – 9 weeks were obtained from the Institute of Biotechnology, Vietnam Academy of Science and Technology. All mice were housed in a temperature-controlled room on a 12-h light/12-h dark cycle and provided with sterile water and food *ad libtium*. Mice were subcutaneously injected with 2 x 10^6 LLC cells (n=24) and 5 x 10^6 4T1 cells (n=24) to induce tumors in the thighs. After 7 – 10 days, tumor-bearing mice of each cell line were randomly distributed into 4 groups. A 0.2-mL (200 – 300 µCi) ^{18}F-FLT was administered intravenously to each mouse via tail vein. Mice were sacrificed at 20, 40, 60, and 90 minutes after radiotracer injection. Blood, tumor samples, and normal tissues including kidney, muscle, spleen, liver, and bone were excised. All collected samples were weighted and radioactivity uptake was measured in a gamma-ray spectrometer (Ortec, Canberra, USA) with decay correction applied. Accumulation of radiotracers in normal tissues and tumors was presented by the percentage of the injected dose per gram of tissues (%ID/g) [9,10].

Histology: after completion of the radioactivity measurement, tumor samples were fixed in 10% formalin, embedded in paraffin, and cut into 3.0 - 5.0 µm-thick slides. These slides were stained with hematoxylin and eosin. Immunohistochemical Ki-67 staining of the tissues was done by using an UtralView DAB Detection Kit (Ventana, Roche, Switzerland) following the instructions from the manufacturer in the Laboratory Department, 108 Central military hospital. Ki-67 expression was determined via light microscopy by experienced pathologists. If the tumor cell nuclei and occasionally cytoplasm were stained brown, it was considered Ki-67 positive. In case only cytoplasm but no nuclei were stained, the sample was deemed

negative. The positive Ki-67 scores were calculated by counting the nuclei in microscopic fields (magnification, x400) of each tumor section.

Statistical analysis

Data processing was analyzed by using SPSS 17.0 software for Windows (SPSS, Inc., Chicago, IL, USA). Measurement data were expressed as the mean ± SD (standard deviation). The significance of differences in radioactivity uptakes levels and ratios was evaluated by ANOVA. P values < 0.05 were considered significant.

RESULTS

Establishment of tumor-bearing mice: the average weight of each group of mice on the day of the experiment was 18.08 ± 1.93 g for the lung-tumor bearing group (n=24) and 18.93 ± 1.55 g for the breast-tumor bearing group (n=24). Tumors induced by the LLC cell line were significantly larger than those induced by the 4T1 cell line, with mean values of 1.19 ± 0.62 g and 0.19 ± 0.08 g respectively (P < 0.001).

^{18}F-FLT radioactivity distribution: The concentration of radioactivity in tumors increased gradually over time, with the highest %ID/g values obtained in the LLC tumors at 90 minutes after injection of 4.66 ± 0.49, meanwhile, the highest values in 4T1 tumors were 5.65 ± 0.40 at 60 minutes after injection (Figure 1 and 2). The radioactivity in other organs, such as blood, liver, and muscle, was quite low, ranging from 1 to 4 %ID/g. The kidneys are the organ for the elimination of ^{18}F-FLT, where the high radio-uptake was visualized from the first measurement at 20 minutes post-injection, with %ID/g values of 5.59 ± 0.80 and 10.41 ± 0.73 for lung-tumor bearing and breast-tumor bearing mice respectively. The tracer ^{18}F-FLT accumulated over time in two organs, the spleen and bone, resulting in increasing %ID/g values from 20 to 90 minutes post-injection. At the last time-point measurement in the LLC-tumor group, the uptake values for spleen and bone were 11.16 ± 7.50 and 10.74 ± 0.13, respectively, while the corresponding results in the 4T1-tumor group were 8.17 + 0.45 and 8.00 ± 2.47.

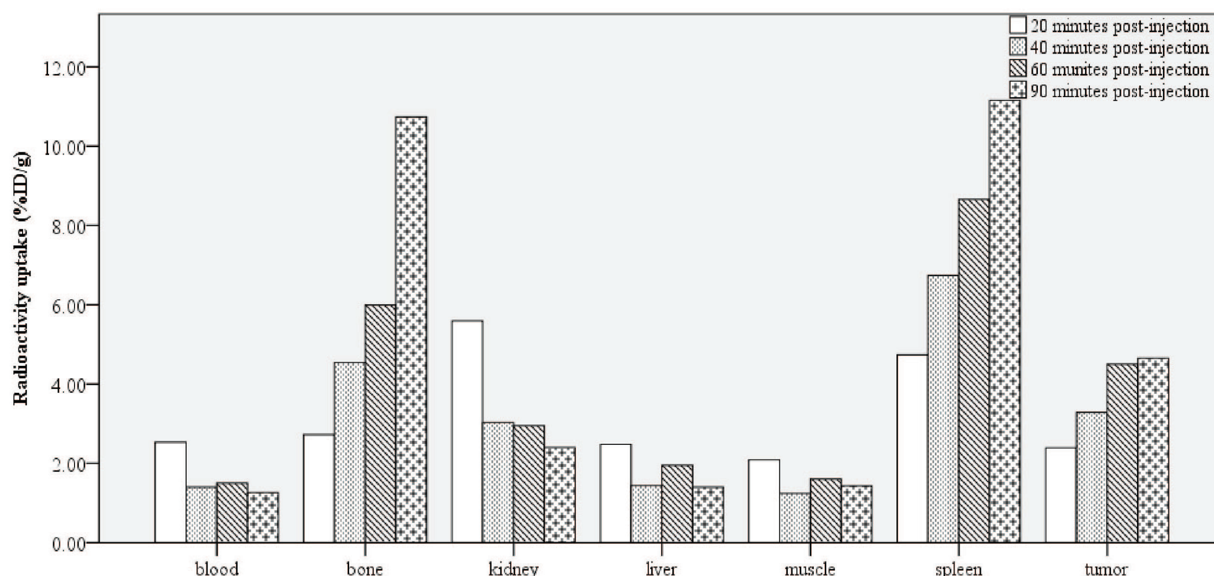

Figure 1. *Distribution of ^{18}F-FLT in the tumor and organs of lung-bearing mice at 20 minutes, 40 minutes, 60 minutes, and 90 minutes after radiotracer injection (n=24).*

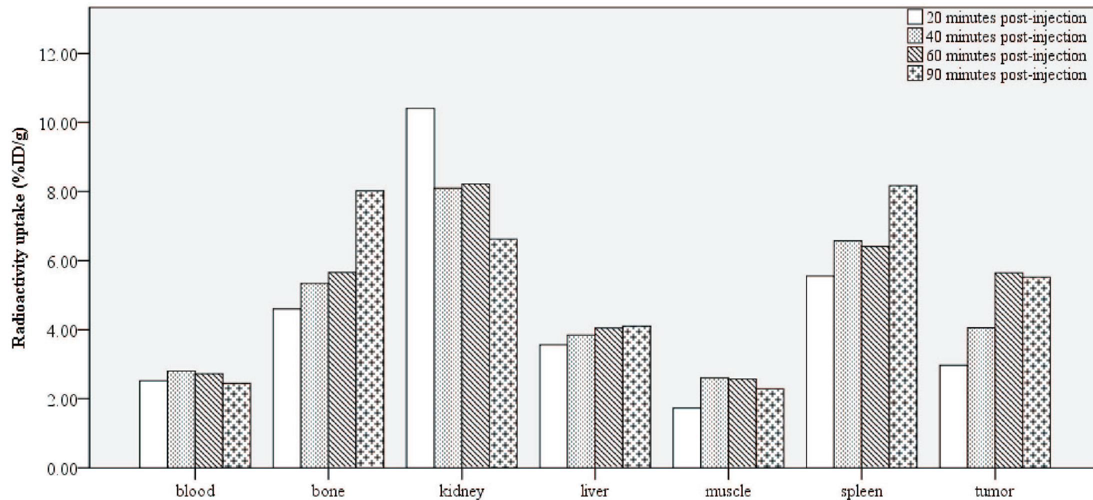

Figure 2. *Distribution of ^{18}F-FLT in the tumor and organs of breast-bearing mice at 20 minutes, 40 minutes, 60 minutes, and 90 minutes after radiotracer injection (n=24).*

Although the absolute concentration of the tracer at 90 minutes post-injection of the breast-tumor bearing mice was higher than that of lung-tumor bearing mice (4.66 ± 0.49 vs 5.65 ± 0.40), the %ID/g of the blood samples and muscle samples of the breast-tumor group was also higher than that of the lung-tumor group (2.45 ± 0.40 vs 1.26 ± 0.11 and 2.29 ± 0.06 vs 1.43 ± 0.02) and that leads to higher ratios of tumor and normal tissue (blood and muscle) (T/N ratios) of the lung-tumor group than that of the breast-tumor group with the mean T/N ratios of 3.38 ± 0.37; 3.25 ± 0.32 and 2.14 ± 0.40; 2.42 ± 0.16, respectively ($p < 0.001$) (Figure 3).

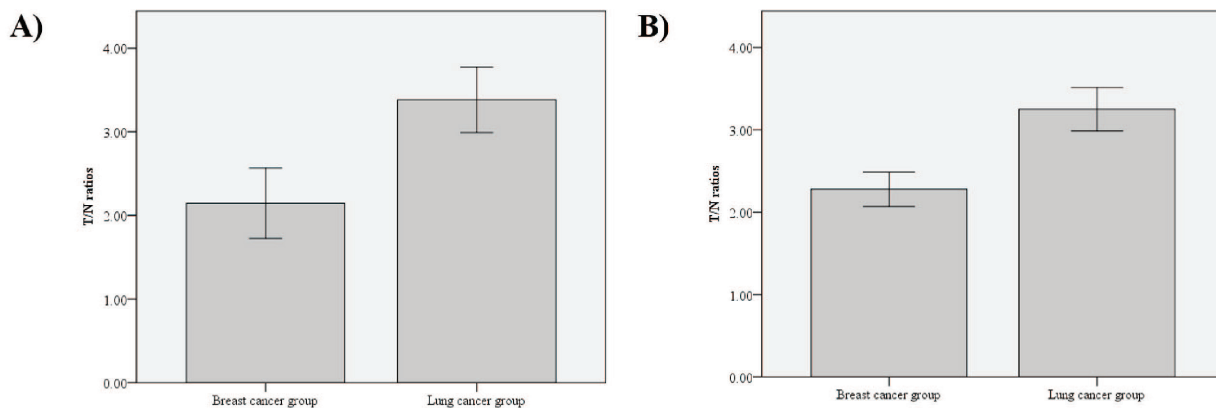

Figure 3. *T/N ratios between radioactivity uptake of tumor and blood (A); tumor and muscle (B) samples in the two groups.*

Ki-67 index: the measurement from the tumors showed that the positive percentage of Ki-67 protein in LLC tumor specimens was higher than in the breast cancer samples caused by the 4T1 cell line. The average values for the two cell line groups were 90.16 ± 2.93 % and 71.83 ± 3.54 %, respectively (Figure 4).

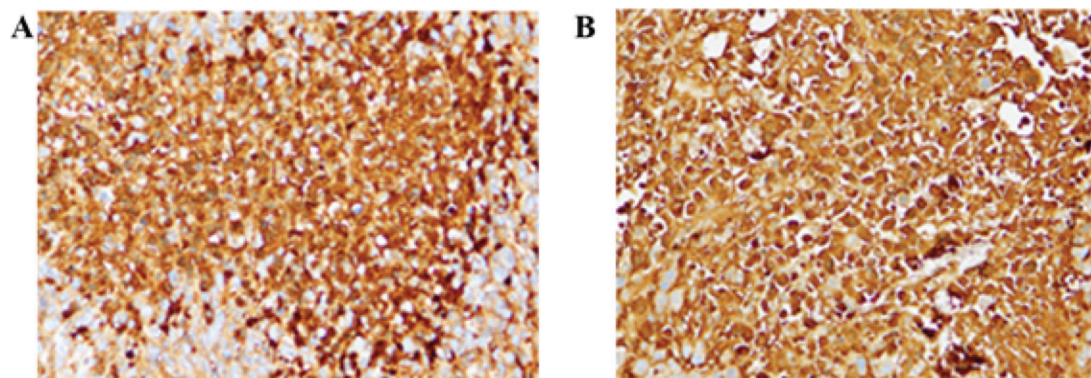

Figure 4. Ki-67 immunohistochemical staining of breast tumors (A) and lung tumors (B).

DISCUSSION

The ^{18}F-FLT tracer is primarily used to detect cancer cells as a surrogate marker for cell proliferation. In the salvage pathway, ^{18}F-FLT is transported into cells by nucleoside carrier proteins and phosphorylated to monophosphate form by TK-1, which is upregulated in the late G1-S phase of the cell cycle. This causes the tracer to be trapped in the cytosol as monophosphate without DNA incorporation, making it available as a substrate for cytoplasmic TK-1 [11]. The *de novo* pathway has been identified as a second mechanism for DNA synthesis in proliferating cells. The enzyme thymidylate synthase converts deoxyuridine monophosphate to thymidine monophosphate in this way. Both pathways are combined to supply sufficient thymidine for DNA synthesis [12].

In this study, the distribution of the radiopharmaceutical ^{18}F-FLT in mice inoculated with two cell lines revealed that the radiotracer uptake was highly concentrated on the tumors. Because of the primary elimination organ, ^{18}F-FLT also presented a high concentration in the kidneys. The tracer is also found predominantly in the liver, spleen, and bone marrow, which are involved in hematopoiesis with a high degree of cell proliferation. The tumors implanted in the two groups of mice also expressed a huge radioactivity accumulation over time, indicating the high proliferating rates of two cancer cell lines. Comparing the two groups, although the absolute uptake values were higher in the breast cancer tumors than in the lung cancer tumors (4.66 ± 0.49 vs 5.65 ± 0.40 %ID/g), the T/N ratios of the breast tumor group were lower than that of the lung cancer group (Figure 3). These ratios, however, were not as good as the ^{18}F-FDG ratios in some previous studies [13, 14]. As a result, when these two groups are given microPET/CT scan, the group with a better T/N provides good image contrast [15]. Moreover, after tumor dissection, the Ki-67 index reveals that the proliferation rates of the 4T1 cell line were lower than the rates of LLC cell lines. This finding is also consistent with the dissected tumor weights in which the LLC tumors were approximately 2.5 times larger than the 4T1 tumors when the tumor-bearing mice were housed under the same conditions and period.

In clinical practice and clinical trials, despite of the low uptake and sensitivity compared to ^{18}F-FDG, ^{18}F-FLT is being investigated as an imaging biomarker of proliferation in breast cancer and non-small cell lung cancer. It is more sensitive than ^{18}F-FDG in assessment of early treatment response in lung cancer and breast cancer [16-18].

Conclusion: In conclusion, we have succeeded in the synthesis of ^{18}F-FLT as a PET/CT tracer that showed a good accumulation of radioactivity in two groups of tumor-bearing mice. In other words, the difference in the uptake levels between two cancer cell lines reflects partially the proliferating rates of each tumor type. ^{18}F-FLT could be used as an alternative for ^{18}F-FDG PET/CT for evaluation of tumors.

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