

Original Article

Proliferation PET image to characterize pathological spatial features in patients with non-small cell lung cancer: a pilot study

Xiao-Ting Chen^{1,2*}, Xin Zhao^{3*}, Zhen-Hua Gao¹, Yong-Sheng Gao⁴, Bai-Jiang Zhang⁵, Zheng Fu⁶, Dian-Bin Mu⁴, Jin-Ming Yu¹, Xue Meng¹

¹Department of Radiation Oncology, Shandong Cancer Hospital and Institute, Shandong Academy of Medical Sciences, Jinan, China; ²Department of Oncology, Affiliated Hospital of Taishan Medical University, Taian, China; ³Department of Cardiothoracic Surgery, Qilu Hospital, Shandong University, Jinan, China; ⁴Department of Pathology, Shandong Cancer Hospital and Institute, Jinan, China; ⁵Department of Thoracic Surgery, Shandong Cancer Hospital and Institute, Jinan, China; ⁶Department of Nuclear Medicine, Shandong Cancer Hospital and Institute, Jinan, China. *Equal contributors.

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Abstract: Purpose: ¹⁸F-FLT-PET imaging was proposed as a tool for measuring in vivo tumor cell proliferation and detecting sub-volumes to propose escalation in radiotherapy. The aim of this study was to validate whether high FLT uptake areas in ¹⁸F-FLT PET/CT are coincident with tumor cell proliferation distribution indicated by Ki-67 staining in non-small cell lung cancer, thus provide theoretical support for the application of dose painting guided by ¹⁸F-FLT PET/CT. Materials and methods: Twelve treatment naive patients with biopsy proven NSCLC underwent ¹⁸F-FLT PET/CT scans followed by lobectomy were enrolled. The surgical specimen was dissected into 4-7 μ m sections at approximately 4-mm intervals. The best slice was sort out to complete Ki-67 staining. Maximum Ki-67 labelling Index and SUV_{max} of the corresponding PET image was calculated. The correlation between Ki-67 Labelling Index and SUV_{max} of FLT was determined using Spearman Correlation analysis. High uptake areas and high proliferating areas were delineated on the two images, respectively, and their location was compared. Results: The maximal SUV was 3.26 ± 0.97 (1.96-5.05), maximal Ki-67 labeling index was $49\% \pm 27.56\%$ (5%-90%). Statistical analysis didn't reveal a significant correlation between them ($r = -0.157$, $P = 0.627$, > 0.05). 9 patients can contour high proliferating area on Ki-67 staining slice, and eight can contour the high uptake areas. In 4 patients, we can observe a generally close distribution of high uptake areas and high proliferating areas, in one patient, both the uptake level and proliferation status was low, while the others didn't not find a significant co-localization. Conclusion: Noninvasive ¹⁸F-FLT PET assessing the proliferative status may be a valuable aid to guide dose painting in NSCLC, but it needs to be confirmed further.

Keywords: ¹⁸F-FLT PET, pathological spatial validation, non-small-cell lung cancer

Introduction

Traditionally, radiotherapy plans are designed to deliver uniform doses to target volumes defined on anatomical images obtained by computer tomography (CT). Previous studies have shown that escalation beyond current prescribed doses could increase tumor control in non-small cell lung cancer (NSCLC) patients treated with radiotherapy [1-5]. However, the interim analysis of Radiation Therapy Oncology 0617 protocols showed that the higher radiation dose of 74 Gy could not produce a long-

term survival benefit compared with the lower standard dose of 60 Gy [6]. Though reported adverse events were similar, the injury of high-dose radiotherapy on normal lungs and heart may lead to more deaths than the standard dose, which is the most likely explanation for the unexpected results [7]. Recently, non-uniform radiation boosting of biological target volumes, often referred to as dose-painting, has been proposed to improve tumor control [8, 9]. For example, in order to protect normal tissue from higher doses, treatment adaptation based on PET imaging has been proposed where only

tumor sub-volumes that are characterized by active cell proliferation and are at high risk for recurrence are receiving escalated doses [9, 10]. Therefore, biological imaging has been uniquely suited to define biological target volumes due to its non-invasive, volumetric, and quantitative properties [8, 11].

3'-deoxy-3'- ^{18}F -fluorothymidine (^{18}F -FLT) is currently under consideration as a diagnostic non-invasive imaging marker of tumor cell proliferation. During S phase, FLT is phosphorescent by TK-1 and is trapped inside the cell, but it can neither be incorporated into DNA or return to the tissue fluid through the cell membrane, thus it serves as an indirect measurement of proliferation by reflecting TK-1 activity [12, 13]. However, before the use of PET for tumor proliferation is accepted and introduced into radiotherapy planning, we must demonstrate that it accurately reflects the underlying biological information. Histopathological validation is one of the ways to demonstrate that the investigated PET images correctly identify these regions of interest, e.g. characterized by active cell proliferation. Previous studies have demonstrated that ^{18}F -FLT standardized uptake value (maximum or average SUV) has a significant correlation with the labelling index of specific Ki-67 (Ki-67 LI), the gold standard for assessing tumor proliferation status in clinical practice [14-16]. However, the methodology utilized in these validation studies does not evaluate the spatial co-localization of the ^{18}F -FLT PET tracer uptake with that of biological markers of cell proliferation. Up to date, there are no published data verifying the spatial concordance between the intratumoral distribution of ^{18}F -FLT uptake and the pattern of tumor cell proliferation in patients with NSCLC.

Then the goal of our study was to investigate the spatial accordance between the pattern of intratumoral ^{18}F -FLT uptake and the spatial distribution of cell proliferation marker Ki-67 obtained in patients with NSCLC, thus providing theoretical support for the application of dose painting and biological intensity modulated radiation therapy (BIMRT) guided by proliferation PET/CT image.

Methods and materials

Patient selection

This prospective study was carried out in Shandong Cancer Hospital and institute

between March, 2013 and January 2014. People who were initially diagnosed with NSCLC and considered radically operable were included. All patients underwent routine staging procedures, including CT scan of chest and upper abdomen, bone scanning, brain magnetic resonance imaging, et al. All patients were histologically confirmed to be NSCLC and had not received either chemotherapy or radiotherapy before surgery. Tumor stages were classified IA to IIIA according to the proposals of the American Joint Committee on Cancer (AJCC version 7). To insure the intra-tumor heterogeneity can be positive on PET, the diameter of the tumor should be at least 2 cm. The age of our selected patients ranged from 18 and 75 years old. The ECOG score was 0-2, patients should have sufficient caloric intake, adequate haematological, renal, and hepatic function. The protocol for this study was approved by the institutional review board of our hospital and all patients were given informed consent.

^{18}F -FLT synthesis and PET/CT image acquisition

^{18}F -FLT was obtained from PET/CT Center in Shandong Cancer Hospital using the cyclotron GE Minitracer. Synthesis and quality control was performed strictly according to standard operating procedures and was suspected of inspection. The products had to meet certain criteria (e.g., the radiochemical yield had to be > 10% and the radiochemical purity had to be > 95%) to be used for imaging.

FLT PET/CT scans were performed with a Philips, GEMINI TF Big Bore PET/CT scanner (Philips Healthcare; Chalfont St. Giles, The Netherlands) with a well-defined protocol. All patients had been fasting for at least 6h and rest for 15 minutes before the injection of ^{18}F -FLT. After intravenous injection of 300-400 MBq radioactive tracers, the patients were kept at rest in a quiet room for at least 60 minutes. Then emission scans of the thorax were generated for 5 min/field of view, at an axial sampling thickness of 4 mm/slice from head to thigh, each covering about 50 cm. Transmission imaging was obtained for 3 min/bed position for attenuation correction. The images were reconstructed using an iterative reconstruction technique and were read from workstation (Xeleris, GE Healthcare, Milwaukee, WI) computer monitors.

Spatial pathological validation of ¹⁸F-FLT PET in NSCLC

Table 1. Patient characteristics

No	Sex	Age	Site	Pathologic	Size	Stage
1	M	71	right, superior	ADC	4.5 × 4.0 × 3.5	I B
2	M	56	left, superior	ADC	2.0 × 2.5 × 2.5	I A
3	M	57	left, inferior	ADC	4.5 × 3.0 × 3.0	II B
4	F	43	left, inferior	SCC	3.5 × 3.5 × 2.5	II A
5	F	65	left, superior	SCC	3.0 × 2.0 × 3.0	I B
6	F	54	right, superior	ADC	2.0 × 2.5 × 2.0	I A
7	F	67	left, superior	ADC	3.0 × 3.0 × 2.5	II B
8	F	45	right, superior	ADC	5.0 × 4.0 × 5.0	III A
9	F	54	right, superior	ADC	3.0 × 1.5 × 1.5	I B
10	M	69	right, superior	SCC	3.0 × 2.5 × 3.0	II A
11	M	69	right, superior	ADC	2.2 × 1.6 × 1.5	II A
12	M	45	right, inferior	SCC	5.0 × 4.0 × 5.0	III A

M (male); F (Female); ADC (adenocarcinoma); SCC (squamous cell carcinoma).

PET/CT imaging processing

The attenuation-corrected PET images, CT images, and fused PET/CT images were reviewed in axial, coronal, and sagittal planes, as was a cine display of maximum-intensity projections of the PET data, using the manufacturer's review station (Xeleris; GE Healthcare). Maximal standardized uptake values (SUV_{max}) of FLT within the tumor were calculated, and then, the region of interest was determined at various percentage thresholds of SUV_{max} , from 45% to 70% at 5% intervals, represents PET images at different cut-off percentage thresholds of the maximal SUV-45%, 50%, 55%, 60%, 65%, or 70%. And contours were delineated for regions above 80% SUV_{max} , representing high uptake regions. The above was performed by two experienced nuclear medicine physicians, unaware of surgical or pathologic findings or any clinical information except the patients with NSCLC, prospectively interpreted PET/CT images, and only one final decision was made by these two physicians.

Pathological measurement

All patients underwent lobectomy and mediastinal lymph node dissection within 3 days after PET/CT scan. A rigid protocol was developed for pathologic examinations. Once the tumor was resected, it was oriented to the in vivo geometry as best as possible, bisected in the transverse plan in the operating room by the surgeon and the pathologist, and fixed in 10% formalin (≥ 24 h). The dimensions of the tumor samples

were documented by digital photography and then cut into slices at 4 mm intervals (the same layer thickness as PET/CT) parallel to the level mentioned before, we think these slices have one to one correspondence with PET images, and all the analyses below were done under this hypothesis. All the slices were manufactured into whole-mount paraffin sections according to standard protocol. The specimen was sectioned into 4~7 μ m-thick slices with a microtome (Microme HM 450). All these slices were stained with hematoxylin and eosin (H-E). An experienced pathologist who was unaware of the clinical data and PET findings overviews these pathological sections using light microscopy at $\times 100$ magnifications

and outlined the tumor margin (including tumor, intratumoral stroma, and areas of necrosis but excluding surrounding normal tissue). The optimal slice was sort out to accomplish Ki-67 immunohistochemical staining using the same whole -mount Paraffin section.

The Ki-67_{max} labelling index was calculated by counting the percentage of Ki-67-positive cell nuclei per 500-1,000 tumor cells in the areas of the tumor with the highest proliferation.

With reference to previous reports, the Ki-67 LI was defined as high if $> 20\%$ of the tumor nuclei stained positively with the MoAb, and was considered low if staining was $< 20\%$ [17, 18]. After carefully screening, high proliferation areas were contoured with areas of optical staining intensity above 20%.

Spatial concordance of FLT uptake and cell proliferation

Before the spatial accordance validation between PET and pathological images, there should be co-registration between them. As the accurate co-registration for in vitro tumor was almost impossible, we can roughly make the co-registration. The ki-67 staining images, H-E staining images and its corresponding PET image were registered approximately on Adobe Photoshop 8 according to tumor 3-dimensional directions, the ratio of their actual size, surrounding vessels and so on.

Spatial pathological validation of ^{18}F -FLT PET in NSCLC

Table 2. Optimal cut-off % SUV_{max} and distribution of high uptake and high LI regions

NO	SUV_{max}	Diameter (cm)	Ki-67 _{max}	Optimal cut-off % SUV	High uptake	High ki-67
1	3.18	3.0	80%	60%	Bottom left	Whole image
2	2.56	1.5	90%	65%	Bottom right	Whole image
3	2.80	3.5	80%	60%	—	Bottom right
4	3.20	2.5	25%	50%	—	Bottom (left + right)
5	3.53	2.0	40%	65%	Center	Center
6	3.52	2.5	30%	50%	Top right	Bottom (left + right)
7	1.96	3.0	40%	60%	—	Left (top + bottom)
8	5.02	3.0	15%	45%	Top right	—
9	2.01	1.5	5%	50%	—	—
10	3.15	2.5	70%	55%	Left (top + bottom)	Bottom left
11	3.15	5.0	60%	60%	Top left	Bottom right
12	5.05	3.6	50%	50%	Top right	—

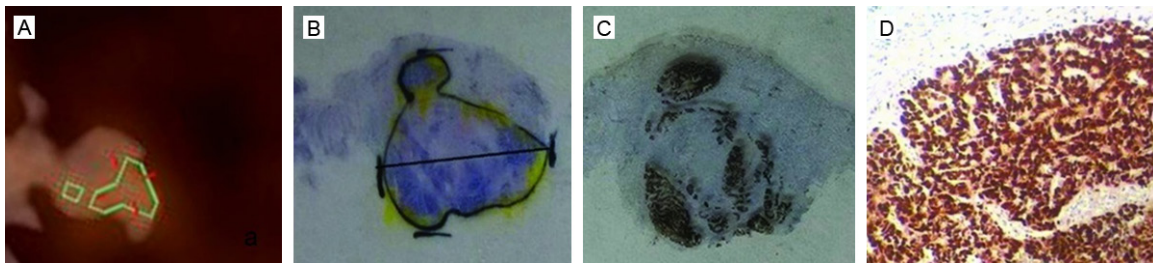


Figure 1. Imaging processing. (A) Delineation of high uptake of FLT with regions with 80% on the bottom left and bottom right quadrant, (B). H-E staining Image (B, C). Ki-67 staining microscopy image with high proliferation on the whole image, (D) Image with Ki-67 LI > 20%.

The contours of interest for different % SUV_{max} were compared with HE image case by case to see which % SUV_{max} can match the tumor bestly, then we get the optimal SUV cut-off value for this slice.

Four-grid method was used to describe the location of high uptake and high proliferation areas as the bottom left quadrant, bottom right quadrant, top left quadrant and top right quadrant (details were shown in **Table 2**). Then we compared the distribution of high uptake areas and high proliferation areas to see whether they are in the same or close quadrant on the whole image.

An example of the localization comparison is depicted in **Figure 1**.

Statistics

SPSS software (SPSS for Windows, version 17.0, SPSS Inc. Chicago, IL) was utilized to perform statistical analysis. FLT SUV_{max} and Ki-67_{max} were both reported as the means \pm SD.

The correlation between SUV_{max} and Ki-67 LI was assessed using spearman correlation analysis. Two-tailed *p*-values are provided and a *P*-value of less than 0.05 was considered statistically significant.

Results

Patient and tumor characteristics

A total of twelve consecutive patients with an average of 58 years old (including 6 male and 6 female) met the eligibility criteria and were enrolled in this study. Patient and tumor characteristics are given in **Table 1**. Most patients were clinically and pathologically as stage IA to IIIA. All patients underwent ^{18}F -FLT PET/CT on the hybrid scanner. All the patients underwent surgery within 3 days after PET/CT scan.

Diagnosis with FLT-PET/CT

Good-quality images could be available for all 12 patients, and all primary tumor was found with abnormal FLT uptake. The average SUV_{max}

was 3.26 ± 0.97 (range, 1.96-5.05). All cases had well-defined FLT distributions inside the lesions, showing intra-tumor heterogeneity of FLT uptake inside the lesions. The optimal SUV cut-off value for each patient was $55.83\% \pm 6.69\%$ (range, 45%-65%), further showing that all tumors have an abnormal FLT uptake (details are shown in **Table 2**). 8 patients can get the high uptake area on the corresponding PET/CT image. With one patient in the center, 3 in the top right, 1 in the top left, 1 in the bottom left, 1 in the bottom right, 1 in the left (top + bottom).

Histological examination

All tumors staining positively for Ki-67 staining. 10 out of 12 patients showed a high maximal Ki-67 LI of more than 20%, the other 2 were less than 5%. Maximal Ki-67 LI was $49.6\% \pm 7.9\%$ (range, 5%-90%). In most of the 12 tumor microscopy images, high proliferating tumor cells were located very sparse, and only 9 can contour the area of Ki-67 LI $\geq 20\%$. In two patients, the whole image can we observe a high ki-67 LI of more than 20%. While for the other 7 patients, 1 was distributed in the center, 2 on the bottom right, 2 on the bottom (left + right), 1 on the bottom left, 1 on the left (bottom + top). Distribution details were shown in **Table 2**.

Correlation between Ki-67 labelling index and ¹⁸F-FLT PET

In all tumors, Spearman correlation analysis didn't indicate a statistically significant correlation between the maximal Ki-67 labeling index and the SUV_{max} of ¹⁸F-FLT PET ($r = -0.157$, $P = 0.627 > 0.05$).

Only in 4 patients we can see a co-localization of high uptake and high proliferation areas. Their location was in the same or adjacent point on the whole image. In one patient, distribution of FLT and Ki-67 staining level was the same as both are low. While in the left 7 patients, 2 presented the obvious opposite results. In patient No. 7, its SUV_{max} was 1.96, the lowest in 12 patients, and high uptake area cannot be contoured on it, but the whole Ki-67 staining slice was highly stained. While in patient no. 8, the high uptake area was contoured on the top right with a high SUV_{max} of 5.02, but there are no high proliferating tumor

cells. The other 5 didn't observe a distribution correlation.

Discussion

We validated the spatial co-localization between ¹⁸F-FLT PET uptake and immunohistochemical staining of the endogenous proliferation marker Ki-67 in primary non-small cell lung cancer.

Most previous studies correlated the biological characteristics of a random tumor sample with the uptake of FLT of the total tumor [14-16, 18], few focus on the correlation between intra-tumor heterogeneity of FLT uptake and histology. Our study complemented those of previous studies in which ¹⁸F-FLT was investigated as a tracer for PET imaging of cell proliferation without exploring the spatial correspondence between the patterns of FLT uptake and cellular proliferation. It was expected that high FLT uptake areas are spatial concordant with tumor areas of high proliferation.

Some of the experimental details need further discussion. The most serious problem in our study is the co-registration. According to the very few studies investigating spatial correlation between molecular imaging and pathologic information, resected tumor samples was made to undergo PET/CT after being sliced to insure the accurate registration [19]. However, for various reasons, it is hard to make patients undergo PET/CT scanning just before surgery. So a time interval of several days is inevitable between PET scan and surgery, and accurate registration is inaccessible, on this basis, we just analyses the general distribution of high uptake and high labelling areas to see whether they are in the same or near part of the whole image rather make a precision validation. Although both the removal of the tumor and the slicing might influence tumor architecture and account for errors in image registration, the PET scan of the surgical specimen and ki-67 microscopy images could be approximately matched.

There is an abnormal uptake of ¹⁸F-FLT in all patients with a mean SUV_{max} of 3.6. And take the H-E staining microscopy as the standard, the best cut-off % SUV for each slice was $49\% \pm 27.56\%$ (range, 5%-90%), showing further explanation of the abnormal uptake of FLT.

And we compared quantitative analysis of ^{18}F -FLT uptake with standard methods for quantification of proliferation by immunohistochemical markers. However unexpectedly, we did not find a significant positive correlation between the Ki-67 LI and SUV_{max} . This finding runs counter to several previous studies indicating significant positive correlations between SUV_{max} for ^{18}F -FLT PET and Ki-67 LI. There are several factors that potentially can account for the lack of correlation between Ki-67 LI and SUV_{max} in our study. (1) The small number of patients. (2) For all patients, only one slice was available for Ki-67 staining, the Ki-67 LI may not represent the proliferating level for the whole tumor, but the SUV_{max} is of the whole tumor. (3) There was a time interval between PET and surgery. Although the median interval of 4 days was relatively short compared with other studies reported in the literature.

On the other hand, we studied the global co-localization of areas of high proliferation and high uptake of ^{18}F -FLT. In total, only 5 in 12 patients can show a co-localization between FLT uptake and Ki-67 expression, with one patient the FLT uptake and Ki-67 LI were both in low level, while the other 4 showed that the areas of FLT high-uptake were spatially concordant with the areas of active proliferation. 2 other patients showed a completely opposite results: a low SUV_{max} of FLT with a high Ki-67 expression and a high FLT uptake with a low Ki-67 expression. We can only got a highly consistent distribution in less than half of the patients. However, Esther found a weak correlation between ^{18}F -FLT uptake and Ki-67 staining intensity in oral cavity tumors [18], and Marian Axente demonstrated that higher ^{18}F -FLT uptake was co-localized with the areas of active proliferation in FaDu tumor models [20]. Besides, many previous studies have demonstrated that ^{18}F -FLT standard uptake value had a significant correlation with the tumor cell proliferation.

One limitation of this study is the small number of cases, so that subgroup analyses were difficult. Therefore, we are continuing our work to enroll more patients, both as a confirmation for this study and for future subgroup analysis.

On the basis of the current results, the distribution of ^{18}F -FLT uptake was not well co-localized with proliferating tumor cells indicated by Ki-67

staining. Therefore, ^{18}F -FLT may not represent in vivo proliferating tumor cells accurately, and the value of ^{18}F -FLT as a measure of validating underlying biological background in non-small cell lung cancer seems limited. However, this does not necessarily imply that there is no spatial concordance between FLT uptake and cell proliferation. Further research and more precise protocol are needed until definite conclusions can be drawn about its significance as a biomarker for tumor cell proliferation.

Conclusion

Noninvasive ^{18}F -FLT PET assessing the proliferative status may be a valuable aid to guide dose painting and BIMRT in NSCLC, but it needs to be validated before introduction in the clinic. We demonstrated the feasibility of an in-vitro method to correlate molecular imaging with histology in human NSCLC. This validation study didn't demonstrated a highly consistent distribution between ^{18}F -FLT uptake and Ki-67 positive staining tumor cells. The application of ^{18}F -FLT PET/CT into radiation planning should be carefully considered and further studied.

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Disclosure of conflict of interest

None.

Address correspondence to: Xue Meng, Department of Radiation Oncology, Shandong Cancer Hospital and Institute, Shandong Academy of Medical Sciences, Jiyuan Road 440, Jinan 250117, Shandong Province, China. Tel: 86-0531-67626142; Fax: 86-0531-67626141; E-mail: mengxue5409@126.com

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