J of Nuclear Medicine Technology, first published online May 24, 2022 as doi:10.2967/jnmt.122.264141

Title: Gallium-68-Pentixafor PET/CT demonstrating in vivo CXCR4 receptors' overexpression in rare lung malignancies: Correlation with the histological and histochemical findings.

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Running Title: [68Ga]-Pentixafor PET for CXCR4 imaging
Manuscript category: Brief Communication

Abstract:

Objectives: Gallium-68 [^{68}Ga]-Pentixafor PET/CT imaging allows non-invasive assessment of CXCR4 expression in various malignancies, but its use in rare lung cancer variants is not reported. **Methods:** [^{68}Ga]-Pentixafor PET/CT imaging was performed in 6 patients (3M:3F; mean age=57.0±16.80 years) with suspected lung masses. Whole-body PET/CT images were acquired at 1-h after the i.v. injection of 148.0-185.0 MBq of the tracer. PET/CT images were reconstructed and analysed. The image findings were correlated with histopathological and quantitative (CXCR4-receptors) FACS analysis. **Results:** Histopathological diagnosis of haemangioendothelioma, sarcomatoid carcinoma and hemangiopericytoma was confirmed in 1-patient each. Lung metastasis was diagnosed in the remaining 3/6 patients with primary sarcoma (n=1), RCC (n=1) and unknown primary (n=1). Increased tracer uptake in the primary lung mass with SUV_{max} values of 3.0, 6.3 and 13.0 were noted in hemangiopericytoma, sarcomatoid carcinoma and haemangioendothelioma cases respectively. The mean values of SUV_{max}, MFI and % stained cells were highest in haemangioendothelioma. Among 3 patients with lung metastases, the highest SUV_{max} value of 9.5 was observed in primary sarcoma patient. **Conclusion:** [^{68}Ga] Pentixafor selectively targets the *in vivo* whole-body disease burden of CXCR4 receptors. This approach thus holds good promise for developing suitable radio-theranostics in lung cancers expressing these targets.
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Key words: [^{68}Ga]Pentixafor; PET/CT imaging; CXCR4 receptors; lung cancer; rare variants; metastasis.

Introduction:

Despite ever evolving research and advances in the diagnostic & treatment strategies, the lung carcinoma (LC) remains the leading cancer killer worldwide [1]. The diagnostic work-up in suspected lung tumours involves tissue diagnosis including histopathology and immuno-histochemistry analysis and imaging. A presumptive differentiation between small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) can be made on the basis of clinical presentation and radiological findings [2]. Functional tumor imaging using 2-deoxy-2[18F]fluoro-D-glucose ([18F]F-FDG) positron emission tomography (PET)/computed tomography (CT) offers complementary information by assessing tumor burden and helps in staging [3]. However, non-invasive PET/CT imaging of receptors' expression and heterogeneity of specific receptors can provide complementary information [4].

There is evidence that SCLC and NSCLC display CXCR4 receptors' over-expression and it is associated with high tumor aggressiveness, metastasis and recurrence [5]. CXCR4 expression is analysed using immuno-histochemistry (IHC) and fluorescence activated cell sorting (FACS) analysis on biopsy samples [6]. Non-invasive imaging using high throughput PET probes targeting CXCR4 receptors may provide important diagnostic or prognostic information in such patients [7]. Few studies have described the feasibility of radiolabeling a cyclic pentapeptide (Pentixafor) with [^{68}Ga] and the recent use of [^{68}Ga]-Pentixafor have yielded encouraging pre-clinical and clinical results for *in vivo* imaging of CXCR4 expression in solid tumors as well as in hematological malignancies [8, 9, 10]. The use of [^{68}Ga] Pentixafor PET/CT imaging for selective targeting of CXCR4 receptors has made much progress in haematological malignancies, whereas, its role in solid tumors has been scarcely reported [11,12,13].

Since, there are more than 30 human malignancies which are known to over-express CXCR4 receptors, therefore the imaging applications of [^{68}Ga]-Pentixafor PET/CT imaging for targeting these receptors are fast expanding in other malignancies. It has recently been reported that [^{68}Ga]-Pentixafor PET/CT imaging in patients demonstrate high tumor uptake in patients of lung cancer (SCLC, NSCLC), glioblastoma multiforme (GBM) and multiple myeloma (MM). This tracer exhibited strong affinity/specificity for the *in vivo* localization/imaging of CXCR4 receptors in these malignancies [11, 14,15,16].

In this study, we present the diagnostic utility of [^{68}Ga]-Pentixafor PET/CT imaging in few cases of rare lung cancer variants and lung metastatic cases with distant primaries which has not been reported previously.

Materials and methods

Six patients (3M:3F; mean age 57.00±16.80 years; range 33-73 years) with clinical and radiological suspicion of lung cancer were recruited prospectively in the study. A written and informed consent was taken from all the study subjects who participated in the study. The study protocol was cleared by the Institute Ethics Committee (IEC) as Doctoral thesis of the first author. All the patients underwent [^{68}Ga]-Pentixafor PET/CT, bronchoscopic or PET/CT -guided lung biopsy, routine histopathology, immuo-histochemistry (IHC) and quantitative CXCR4 receptors' analysis by Fluorescence-activated cell sorting (FACS).

[^{68}Ga]- Pentixafor PET/CT Data acquisition and analysis

Briefly, 148.0 to 185.0 MBq radioactivity of [^{68}Ga]-Pentixafor was injected intravenously. Whole-body PET (GE Discovery, USA) and CECT (using standard CT acquisition parameters) images were acquired consecutively 1- h after the tracer administration. PET acquisition was done 3min/frame (7-8 frames) from the base of skull to proximal thighs. Attenuation corrected PET images were reconstructed iteratively using OSEM algorithm. The reconstructed images projected in three planes (cross-sectional; coronal and sagittal) were used for visual and quantitative (SUV_{max}) analysis.

Fluorescence-activated cell sorting (FACS) Analysis

In FACS analysis, the freshly biopsied lung sample was processed and 5.0µl of fluorescein isothiocyanate (FITC) labelled CD184 (BD Pharmingen, USA) were used to further label the CXCR4 positive tumor cells in the tissue suspension. Flow cytometer (FACS Calibur, BD, USA) was used to analyse the stained/unstained cells' population and the results were expressed as MFI and the percent stained CXCR4 positive cells.

[^{68}Ga]-Pentixafor PET/CT image findings (SUV_{max}) were compared with histopathology and with the quantitative parameters of FACS assay i.e. the mean fluorescence intensity (MFI) and the percent stained cells.

Results

The results of histopathology, FACS and [^{68}Ga]- Pentixafor PET findings are presented in table-1. High tracer uptake was noted in all lung lesions. In 3 patients (#1,2,3), rare primary lung pathologies were identified. Highest SUV_{max} of 13.04 with the highest corresponding MFI value of 682.0 were noted in the patient (#2) with histopathological evidence of haemangioendothelioma (Figure-1). The SUV_{max} and MFI values in patients 1 & 3 (sacromatoid carcinoma & hemangiopericytoma) were noted to be 6.34, 110.5 and 3.0, 27.90; respectively. The corresponding SUV_{max} and MFI values in 3 cases of secondary lung metastasis from sarcoma (#4), RCC (#5) and unknown primary (#6) were found to be 9.5: 191.20; 6.0:62.0 & 7.5: 216.0 respectively. The results of [^{68}Ga]-Pentixafor PET/CT, FACS and histopathological analysis in patient (#4) are presented in Figure-2.

Pearson correlation analysis indicated a significant correlation between SUV_{max} and MFI (r=0.90), SUV_{max} and percent stained cells (r=0.79) and between MFI and percent stained cells (r=0.72).

Discussion

In the present study, [^{68}Ga]-Pentixafor PET/CT imaging demonstrated high uptake (SUV_{max}=13.0; MFI=682.0) in haemangioendothelioma. The SUV_{max} values in other two pathologies i.e. Sarcomatoid carcinoma and hemangiopericytoma varied as a function of MFI values. Interestingly, amongst the three cases of lung metastases, the highest SUV_{max} of 9.5 was seen (with MFI=191.0) in the lung metastatic lesion with sarcoma as the primary disease. These findings presented a positive correlation (r=0.90) between Ga-68 Pentixafor uptake and the CXCR4 receptors' expression/density which in turn indicated the high specificity of the tracer for these receptors. Likewise, the SUV_{max} values also correlated (r=0.79) with the percent stained cells' population.

We were the first to report normal biodistribution of [^{68}Ga]-Pentixafor in a healthy volunteer, the highest SUVmean & SUVmax values were seen in urinary bladder (146.0,239.0), spleen (6.80; 10.10) followed by kidneys (4.99; 20.55) respectively [17]. The variable physiological uptake of [^{68}Ga]-Pentixafor was seen in spleen in different imaging studies. The same was found to have association with stage of the disease and clinical outcomes as reported in a study on 145 solid tumor patients [18]. A positive correlation was found between [^{68}Ga]-Pentixafor splenic uptake and platelet and/or leukocyte counts in lung cancer and neuroendocrine tumors suggesting splenic uptake could possibly play a role in systemic immunity/inflammation [18].

We have previously reported that the uptake of [^{68}Ga]-Pentixafor in SCLC patients was higher than in NCSLC and other lung cancers variants and the uptake varied as a function of CXCR4 receptors' density [11, 14]. However, the pattern of tracer uptake and the *in vivo* evidence of CXCR4 expression in rare lung malignancies have not been studied before.

[^{68}Ga]-Pentixafor PET tracer had been shown to have excellent affinity for CXCR4 receptors in pre-clinical as well as clinical studies [19,20]. According to the available literature, non-invasive imaging of CXCR4 expression in SCLC is feasible and [^{68}Ga]-Pentixafor as a novel PET tracer might serve as a readout for confirming the CXCR4 expression [20]. Watts et al. reported that [^{68}Ga]-Pentixafor uptake denoting CXCR4 expression is higher in SCLC compared to NSCLC patients [11,14]. Evaluation of CXCR4 expression is a pre-requisite for potential CXCR4 directed radio-chemo therapies in lung cancer and especially in SCLC which has high CXCR4 expression amongst all other variants of lung cancer.

The reports on CXCR4 expression in rare lung tumors included in this study are not available. However, the role of over-expression of CXCR4 receptors in tumor growth and progression in sarcoma and RCC primaries and in metastasis to lungs have been demonstrated [21,22]. [^{68}Ga]-Pentixafor PET imaging may thus be expanded beyond SCLC and NSCLC to unravel the CXCR4 receptors' density and to understand the process of metastatic spread and the intra-/inter-individual heterogeneity of these tumours [23]. In a recent study, an urgent clinical need to develop novel therapeutics for devastating NSCLC disease targeting CXCR4/CXCL12 axis has been advocated and has further stressed that this is the time now to move forward and attempt to incorporate CXCR4 inhibitors in novel immune-based lung cancer therapeutic protocols [24].

Conclusion:

[^{68}Ga]-Pentixafor selectively targets and accurately maps the *in vivo* whole-body disease burden of CXCR4 receptors, which is not possible by tissue sampling methods. This technique thus holds a great promise for translating this approach by labelling the vector with α/β emitters to a therapeutic scenario in such aggressive lung cancer variants having limited treatment options.

Acknowledgements:

The author (BS) is thankful to the Department of Science & Technology (DST) for extramural research funding under DST-FIST program (grant number- SR/FST/LSI-548/2012) to the Department of Nuclear Medicine at my Institute for the purchase of Automated Chemistry Module and other chemicals and peptides.

Authors' contributions :

AW (first author): conceived and designed the study, conducted the imaging and experimental work, analysed and interpreted data, manuscript draft writing; **BS (Corresponding author)**: Conceived and designed the study, data analysis and final editing of the manuscript; **HS-** Image interpretation ; **HK**- data analysis and manuscript writing; **AB**- histopathological analysis **MV and SK Arora**- FACS CXCR4 receptors quantification; **DB**- Patients' recruitment and clinical analysis .

Conflict of Interest:

The authors declare that they have no conflict of interest.

Funding : The study was carried out under funding from DST-FIST (grant number- SR/FST/LSI-548/2012)

Ethical approval : All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

Key points:

Question: To demonstrate the in vivo expression of CXCR4 receptors in rare lung cancers by [^{68}Ga]-Pentixafor PET/CT non-invasively.

Pertinent Findings: [^{68}Ga]-Pentixafor PET/CT detected the presence of CXCR4 receptors in rare lung cancers and metastases. The tracer uptake varied as a function of the receptors' density showing high specificity for in vivo imaging of CXCR4 receptors' disease burden in lung cancers.

Implications for patients' care: This technique thus holds a great promise for translating this approach by labelling the vector with α/β emitters for therapeutic applications in such aggressive lung cancer variants having limited treatment options.

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Table-1 . [^{68}Ga]-Pentixafor PET/CT findings (SUV_{max}) values, MFI, percent stained cells (FACS analysis) and histopathology findings in 6 patients with rare lung tumors and metastatic lung disease.

Patient No.	**Age**	**Sex**	**Histopathology**	**[^{68}Ga]-Pentixafor PET Findings**	**FACS Analysis**	
				SUVmax value	MFI	Percentage stained CXCR+ tumor cells (%)
1.	73	F	Sarcomatoid carcinoma (Primary tumour)	6.34	110.50	2.70
2.	33	F	Haemangioendothelioma (Primary tumour)	13.0	682.0	73.60
3.	68	M	Hemangiopericytoma (Primary tumour)	3.0	27.90	2.50
4.	70	F	Lung metastasis (Primary Sarcoma)	9.5	191.20	45.2
5.	40	M	Lung metastasis (Primary RCC)	6.0	62.0	47.0
6.	58	M	Lung metastasis (unknown primary)	7.5	216.6	59.2

Figure-1. [^{68}Ga]-Pentixafor PET/CT MIP image (A), cross sectional PET/CT fused image (B) showing increased tracer uptake(SUV_{max}=13.0) with the corresponding CT image (D). FACS analysis using CD184-PE showing the stained CXCR4 positive tumour cells'in the scatter plot and histogram (C), with histopathological disease evidence (E) showing epithelioid tumour cells (H & E 40x) and IHC stain with CD31 showing diffuse membranous positivity (F) in a 73 years old female patient (#2) having primary lung haemangioendothelioma.

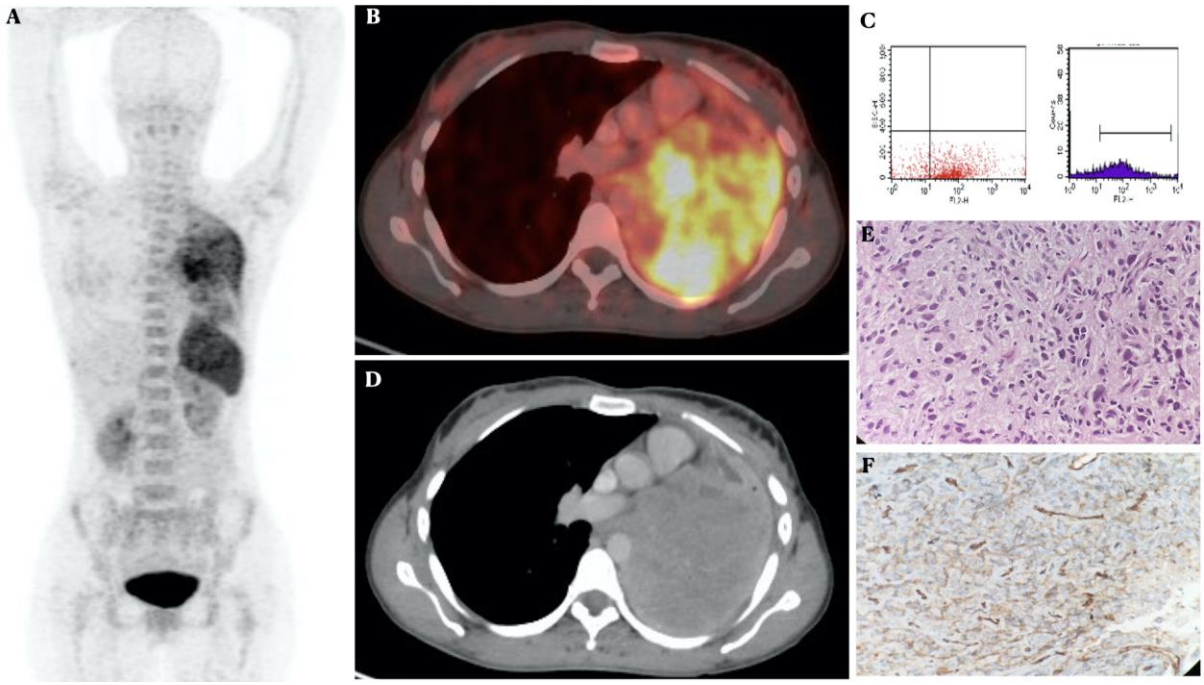
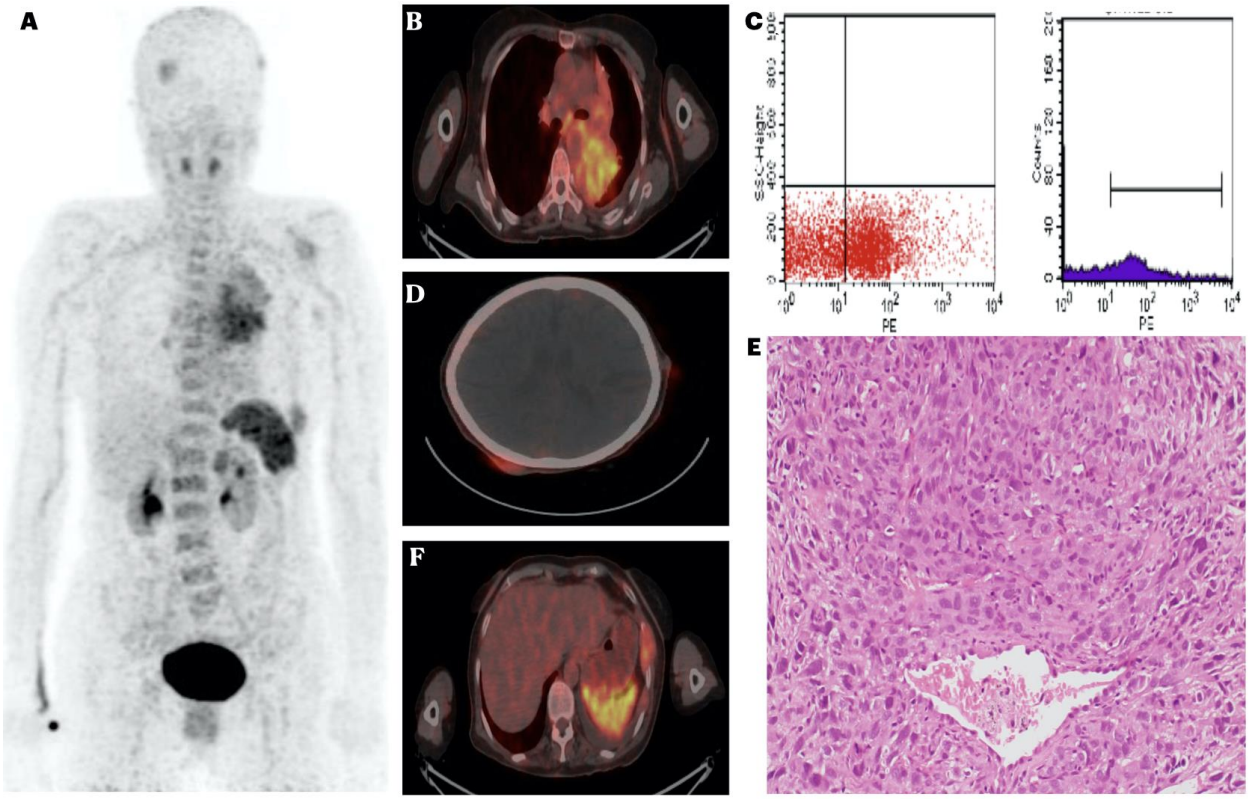


Figure-2. [^{68}Ga]-Pentixafor PET/CT MIP image (A) showing increased tracer uptake in lung and in multiple sarcomatic lesions, the cross-sectional PET/CT fused image (B) showing increased (SUV_{max}=9.5) tracer uptake in the metastatic lung lesion, subcutaneous lesions posterior and left lateral aspect (D) of the scalp (SUV_{max}=4.6) , lytic expansile lesion with soft tissue component involving lateral aspect (F) of the 5th rib (SUV_{max}=5.4) , FACS analysis using CD184-PE showing the stained CXCR4 positive tumour cells'in the scatter plot (C) and histogram with histopathological disease evidence (E) in a 70 years old female patient (#4) having secondary lung cancer disease.



Graphical Abstract