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Title

Investigating the histopathologic correlates of ^{18}F-FDG PET heterogeneity in non small-cell lung cancer

Short title

Histopathologic correlates of ^{18}F-FDG PET in NSCLC

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Abstract

Purpose

Despite the growing use of 18-fluoro-2-deoxyglucose positron emission tomography (^{18}F-FDG PET) texture analysis to measure intratumoural heterogeneity in cancer research, the biologic basis of ^{18}F-FDG PET-derived texture variables (TV) is poorly understood. We aimed to assess correlations between ^{18}F-FDG PET-derived TVs and whole-slide image (WSI)-derived metrics of tumour cellularity and spatial heterogeneity.

Methods

Twenty-two patients with non-small cell lung cancer (NSCLC) prospectively underwent ^{18}F-FDG PET imaging before tumour resection. We tested 9 ^{18}F-FDG PET parameters: metabolically active tumour volume (MATV), total lesion glycolysis (TLG), mean standardised uptake value (SUVmean), first-order entropy, energy, skewness, kurtosis, grey-level co-occurrence matrix entropy, and lacunarity (SUV-lacunarity). From the haematoxylin and eosin-stained WSIs, we derived mean tumour-cell density (MCD) and lacunarity (Path-lacunarity). Spearman's correlation analysis and agglomerative hierarchical clustering were performed to assess variable associations.

Results

Tumour volumes ranged from 2.2-74 cm^3 (median 17.9 cm^3). MCD correlated positively with TLG (rs: 0.46, p-value: 0.007) and SUVmean (rs: 0.55; p-value: 0.008) and negatively with skewness and kurtosis (rs: -0.47 for both; p-value: 0.028 and 0.026, respectively). SUV-lacunarity and Path-lacunarity were positively correlated (rs: 0.5; p-value: 0.018). On cluster analysis, larger tumours trended towards higher SUV_{mean} and entropy with a predominance of tightly concentrated high SUV-voxels (negative skewness and low kurtosis on histogram); on WSI-analysis such larger tumours also displayed generally higher MCD and low SUV- and Path-lacunarity.

Conclusions

Our data suggest that histopathological MCD and lacunarity are associated with several commonly used ^{18}F-FDG PET-derived indices including SUV-lacunarity, MATV, SUV_{mean}, entropy, skewness, and kurtosis, and thus may explain biological basis of ^{18}F-FDG PET-uptake heterogeneity in NSCLC.

Key words:

Texture analysis

Whole-slide imaging

Tumour cellularity

^{18}F-FDG PET

Non-small cell lung cancer

Introduction

18-fluoro-2-deoxyglucose positron emission tomography (^{18}F-FDG PET) derived texture variables (TVs) are emerging as potentially useful biomarkers in cancer research. They quantify metabolic heterogeneity in different scales of space and direction and there is growing evidence supporting the role of TVs in making non-invasive inferences in varied oncologic applications, including tumour phenotyping and genotyping, response to treatment, and survival prognostication [1–4]. However, beyond the over-expression of glucose membrane receptors (e.g. GLUT-1) and upregulation of hexokinase activity in cancer cells, our understanding of the biological basis that can be inferred from histopathology that influences the spatial and intensity distribution of ^{18}F-FDG PET images is lacking and largely conjectural[5].

Tumour cell density and spatial heterogeneity on haematoxylin and eosin (H&E) microscopy are logical targets for studies seeking associations with ^{18}F-FDG PET imaging, since glycolysis is a cellular function and proliferating tumour cells are typically driven by anaerobic metabolism [6]. The clinical and experimental observation of low uptake of ^{18}F-FDG (measured by standardised uptake values, SUVs) in areas of low tumour cellularity supports this hypothesis [7,8]. To explore associations between tumour cell density and spatial variation with ^{18}F-FDG PET metrics, suitable corresponding whole-slide image-derived (WSI) metrics are required. Tumour cell density is difficult to estimate from WSIs because manual counting is impractical whereas subjective estimates are prone to error [9–11]. Secondly, a voxel-to-pixel match for TVs, derived from ^{18}F-FDG PET images with TVs derived from WSIs, may be limited in its ability to uncover true correlations due to the difference in scale between the two modalities and the presence of other influencing factors such as differences in glucose transporter (GLUT) expression [12]. Hence, simple, intuitive, and objective metrics of tissue heterogeneity need to be developed for comparison with ^{18}F-FDG PET-derived TVs.

Automated tumour cell counting, if done with acceptable error, is a practical alternative to subjective estimation, that could be applied to large samples of WSIs. Similarly, lacunarity - an intuitive metric of gaps in a geometric structure - can be applied to entire WSIs or ^{18}F-FDG PET volumes by simply converting them into binary maps representing areas of high versus low cellularity (or metabolic activity in case of ^{18}F-FDG PET images) [13,14].

We hypothesised that tumour cell density and lacunarity measured from WSIs are correlated with ^{18}F-FDG PET lacunarity and possibly other commonly reported TVs. The objectives of this proof-of-concept study were to develop algorithms to quantify mean tumour-cell density (MCD) and lacunarity from WSIs, to enable comparison with ^{18}F-FDG PET-derived lacunarity and other TVs.

Materials and Methods

Patients

Informed written consent was obtained from all patients in this institutional review board approved prospective study. The study population comprised 22 patients with NSCLC. Inclusion criteria were: a) Histopathologic diagnosis of NSCLC, b) ^{18}F-FDG PET/CT scans performed at our institution within 6 weeks before surgery c) Upfront surgery of the primary lung tumour. Patients were excluded if they had tumours smaller than 3 cm, since it has been shown ^{18}F-FDG PET/CT texture parameters are strongly influence by tumour volume (rather than tumour heterogeneity) in smaller lesions [3]. Twenty-two patients (mean age: 65.1 years; 8 men, 14 women) were thus included. Nineteen patients had adenocarcinoma (ADCA) and 3 had squamous cell carcinoma (SCCA). Clinical stages were: stage IA (n=5), stage IB (n=7), stage IIA (n=1), stage IIB (n=2), stage IIIA (n=6), and stage IIIB (n=1).

Image acquisition

Patients underwent ^{18}F-FDG PET/CT scans at a median 7 days before surgery (range 1 to 42 days). ^{18}F-FDG PET/CT scans were acquired on a standardised protocol on a Discovery 710 scanner (GE Healthcare, Chicago, US). Following a 6-hour fasting period, patients were injected with 350-400 MBq ^{18}F-FDG intravenously. As per department protocol, ^{18}F-FDG PET images were acquired from the base of the skull to the upper thighs 90 minutes after tracer injection. Volumetric image reconstruction was performed using the ordered subset expectation maximisation algorithm (2 iterations, 24 subsets, with post-construction smoothing filter of 4mm), slice thickness of 3.27 mm, and pixel size of 4.7mm. All corrections for scatter, randoms, dead time, and decay were applied as standard on the scanner. Attenuation correction was obtained with low dose un-enhanced CT (140 kVp and 65 mAs).

^{18}F-FDG PET-derived heterogeneity parameters

Reconstructed ^{18}F-FDG PET Digital Imaging and Communications in Medicine (DICOM) volumes were imported into in-house image texture-analysis software developed in MATLAB (Release 2013b, The MathWorks, Inc., Natick, Massachusetts, United States). A semi-automated tumour volume of interest (VOI) delineation workflow was adopted, employing an in-house implementation of the three-class fuzzy locally adaptive Bayesian (FLAB) segmentation algorithm [15]. First, a VOI was drawn manually by U.B (radiologist with 9 years' experience) around the tumour taking care to exclude metabolically active adjacent structures (e.g., myocardium, hilar lymph nodes) and to include at least 2-3mm rim of background. This VOI was then processed with FLAB to classify all voxels into 3 classes, i.e., tumour, background, and region of partial volume averaging. Voxels belonging to background class were discarded. A 64-bin quantisation scheme was used since it has been shown adequate to characterise typically encountered SUV ranges [16]. We tested

nine parameters: five first-order texture parameters (SUV_{mean}, first-order entropy, energy, skewness, and kurtosis), two tumour size-related parameters (metabolically active tumour volume [MATV] and total lesion glycolysis [TLG]), one second-order parameter, i.e., grey-level co-occurrence matrix [GLCM] entropy, and one model-based texture parameter, i.e., fractal dimension lacunarity (SUV-lacunarity). The justification to include these parameters in this study is as follows: SUV_{mean}, MATV, and TLG are related to tumour metabolism and volume, and are used widely in the clinic and research. Compared with SUV_{max}, whose value depends only on a single voxel, or SUV_{peak} (or the lean body mass corrected SUL_{peak}), whose value depends on a small $1cm^3$ volume, SUV_{mean} provides a more global estimate of tumour metabolism and is considered less prone to noise [17]. The first-order texture parameters characterise the global intensity distribution through histogram shape. Broadly speaking, the mean localises the position of the histogram peak, whereas skewness and kurtosis describe histogram symmetry and relative proportions of extreme values, respectively [16,18]. Compared with most regional and local parameters, first-order parameters have been shown to be highly reproducible in inter-observer and moderately-to-highly reproducible in inter-scan settings [16,18]. Most importantly, all included features besides SUV-lacunarity have been shown in recent studies to be potential biomarkers of tumour histopathology, treatment response and patient survival [2,19–22]. Lacunarity is a parameter that can be used to quantify the presence of gaps in a structure; the larger the gaps the higher the lacunarity [23]. We used lacunarity on the premise that large cell-poor regions (i.e., high WSI-derived lacunarity [Path-lacunarity]) would appear as large low-uptake cold spots on ^{18}F-FDG PET images (i.e., high SUV-lacunarity), contributing to image heterogeneity and lowering SUV_{mean}. Relevant details of the texture parameters can be found in online supplementary resource 1.

Histopathology slide staining and post-processing

Haematoxylin & eosin (H&E) staining was performed on 3-micron thick tissue sections fixed in 10% buffered formalin for 24 hours and embedded in paraffin. For H&E staining, paraffin-embedded tissue sections were stained manually by a technician using Gill No.3 Haematoxylin Solution (Sigma-AldrichGHS316). All slides were scanned at 20X magnification using a Hamamatsu Nanozoomer digital slide scanner (Hamamatsu Photonics K.K., Shizuoka, Japan). Low magnification (0.4 – 0.7X) views covering the entire tumour in a single frame (1920 x 1080 pixels) were exported into ImageJ v1.51d (National Institutes of Health, Maryland, USA) as Tag Image File Format files [24]. The tumour regions were delineated freehand by O.F (year-4 pathology trainee) for further processing. The segmented tumors were colour deconvolved into haematoxylin-only images (H-images) to allow identification of tumour nuclei [25].

We analysed the low magnification H-images to quantify tumour heterogeneity on the basis of spatial variation in nuclear density. Using the k-mean clustering algorithm, we first generated a nuclear density map (N-map) from an entire WSI (one per case) at low magnification. The N-map was a three-tone image with pixels denoting tissue categorised as “cell-poor”, “tumour-cell rich”, and “immune-cell rich”, in order of increasing nuclear density (Fig. 1). From the N-map, the relative weight of each tissue class was computed by dividing the number of pixels occupied by the particular class with total number of pixels occupied by the whole tumour.

We derived Path-lacunarity by converting each N-map into a binary image of high versus low nuclear-density regions and then using the gliding-box algorithm implemented in MATLAB [14].

To compute MCD, we implemented a cell-counting workflow as follows: First, we exported a 1920 x 1080 pixel image of a random 20X HPF view into ImageJ. After colour-deconvolution, we used manual thresholding to subtract background. Next, we used the built-in watershed algorithm to separate any overlapping nuclei. Considering each particle

on the resulting image as a single cell nucleus, we used the built-in ImageJ particle counter to count all particles in the image. This procedure was repeated on 20 random sites per WSI and the resulting cell counts were multiplied by the tissue weights derived from corresponding N-maps to obtain MCD. The total sampled area per slide was thus approximately 10.36 mm^2 (2.76% of the total area covered by a 25x15mm slide; conversion factor: 0.5 micron per pixel at 20X magnification) [26]. We validated the cell-counting workflow using five independent 20X high-power field (HPF) views (total 16431 nuclei) annotated manually by a pathologist (OW). Its median error in nuclear count per HPF was 20.36% [range 6.9% - 35%].

Statistical analysis

Tumour size, volume, and maximum diameter were reported as medians with ranges. Based on N-maps, relative proportions of immune-cell predominant, tumour-cell predominant, and cell-poor predominant regions of the WSIs were reported as medians with ranges. Examination of variable histograms and scatterplots of variable interactions showed that the assumptions of Pearson correlation, i.e., normal distribution of variables, linear relationships, and homoscedasticity, were not met [27] . Hence, Spearman rank correlation coefficient (rs) was measured to study the relationships among all ^{18}F-FDG PET derived and histopathology-derived parameters. Statistical significance was set at a p-value of <0.05 in this exploratory analysis [28]. To identify groups of correlated variables, agglomerative hierarchical clustering was done using 1-rs as a dissimilarity metric. R version 3.3.2 was used for statistical analysis [29].

Results

Measured from ^{18}F-FDG PET images, tumour volumes ranged from 2.2-75 cm^3 (median 17.9 cm^3) and diameters ranged from 1.6 cm to 5.2 cm (median 3.4 cm). The immune-cell predominant proportion ranged from 0.9% to 16.6% (median: 5.4%), the tumour-cell predominant proportion ranged from 15.1% to 55.4% (median: 40.1%), and cell-poor tissue ranged from 33.9% to 84.8% (median: 55.1%).

On correlation analysis, MCD correlated positively with TLG (rs: 0.46, p-value: 0.007) and SUV_{mean} (rs: 0.55; p-value: 0.008) and negatively with skewness and kurtosis (rs: -0.47 for both; p-value: 0.028 and 0.026 respectively). SUV-lacunarity and Path-lacunarity were also positively correlated (rs: 0.5; p-value: 0.018). All correlations are summarised in Fig. 2.

Cluster analysis revealed two groupings of variables which we labelled Group A and Group B (Fig. 3).

Variables within each group were positively correlated among themselves and negatively with variables belonging to the other group. For example, tumors with large MATV (Group A variable) had higher values for other Group A variables and thus showed greater tumour cell-rich proportion (MATV/tumour-rich proportion rs: 0.53 ; p-value: 0.01), higher MCD (MATV/ rs: 0.26; p-value: 0.236), higher SUV_{mean} (MATV/SUV_{mean} rs: 0.18; p-value: 0.43), and higher entropy (MATV/GLCM-entropy rs: 0.17; p-value: 0.46). Simultaneously, larger tumors showed lower values of Group B variables, i.e., negative skewness and low kurtosis (MATV/SUV skewness rs:-0.49; p-value: 0.02, MATV/SUV kurtosis rs: -0.38; p-value: 0.083). Since Path-lacunarity and SUV-lacunarity were Group B variables, larger tumours also generally had low lacunarity (MATV/SUV-lacunarity rs: -0.77; p-value: <0.0001; MATV/Path-lacunarity rs: -0.15; p-value: 0.5). Interpreted intuitively, these findings suggest that larger tumours had a wider spread of ^{18}F-FDG PET voxel intensities as indicated by histogram-derived entropy, kurtosis, and skewness, and had higher tumour cell-rich proportions. Although the spread of voxel intensities was larger and hence there was greater voxel-to-voxel variability in metabolism, these variations were spread out and

did not coalesce into large gaps in metabolic activity (or tumour cellularity on WSI) and thus lacunarity remained low. An example of each of a large tumour with high Group A/low Group B variables and a small tumour with low Group A/high Group B variables is shown in Fig.4 and Fig. 5.

Discussion

We developed a reproducible workflow with freely available software to quantify nuclear density from H&E WSIs. Using H&E stain accumulation on WSI images, we grouped tumour regions into tumour-cell rich, immune-cell-rich, and cell-poor regions. This grouping allowed us to sample tumour pathological MCD from entire WSIs, and also to compute a novel two-dimensional representation of tissue-level heterogeneity as a WSI-derived analogue of ^{18}F-FDG PET lacunarity. In our proof-of-concept study, we thus showed that it is possible to compare ^{18}F-FDG PET TVs with WSI-derived metrics of tumour cell density and spatial heterogeneity. The group-wise correlations we have described should enable future studies using ^{18}F-FDG PET TVs to explain some of the results on a biological basis.

We found that MCD correlated positively with TLG (rs: 0.46, p-value: 0.007) and SUV_{mean} (rs: 0.55; p-value: 0.008) and negatively with skewness and kurtosis (rs: -0.47 for both; p-value: 0.028 and 0.026, respectively). SUV-lacunarity and Path-lacunarity were also correlated (rs: 0.5; p-value: 0.018). Considering further indirect associations among ^{18}F-FDG PET-derived and WSI-derived variables allowed us to conjecture two metabolic patterns in our dataset.

Comparing vastly different modalities such as ^{18}F-FDG PET and H&E microscopy, is challenging due to difference in scale (resolution of a ^{18}F-FDG PET image is 4-5 mm and that of a 20X magnified WSI is 0.5 micron) and cost of large data processing (a single WSI

can contain up to 1 GB of data)[26]. Furthermore, whereas ^{18}F-FDG PET images are conventionally quantised for texture analysis, quantising large WSI to allow conventional texture analysis would require considerable post-processing and, in our opinion, may lose variable-to-variable match. Hence we chose a simpler workflow to analyse heterogeneity in WSIs that did not involve quantisation of pixel values but binary assignment to zeros or ones for cell-poor regions and highly cellular regions respectively. A positive resulting correlation between SUV- and Path-lacunarity and between MCD and various ^{18}F-FDG PET-derived variables supports our proof-of-concept workflow.

There are very few previous studies comparing tumour cellularity with ^{18}F-FDG PET derived texture features, with comparatively more studies comparing tumour cellularity with global metabolism indices such as SUV_{mean} and SUV_{max}. Most reports support the logical relationship between tumour cellularity and ^{18}F-FDG PET metabolism, as found in our study, with a few exceptions that we mention here [8,30–35]. In a bid to identify biologic explanation of ^{18}F-FDG PET-derived texture variables, Orlhac et al. compared texture indices derived from 28 co-registered images obtained from ^{18}F-FDG PET scans, autoradiography scans, and histopathologic sections of 3 rats bearing mammary tumours [12]. The authors did not find significant correlations between texture indices derived from ^{18}F-FDG PET images and those derived from histopathologic images, and attributed the lack of correlation to differential expression of GLUTs in tissue regions of similar cell density. Since our workflow was not based on co-registration of WSIs and ^{18}F-FDG PET images but on global trends in TVs based on MCD derived from WSI-samples, we believe our workflow allows greater flexibility in local differences between ^{18}F-FDG PET images and WSIs. Higashi et al. compared SUV_{mean} with manual tumour cell-counts and GLUT-I staining, among other variables [36]. They found a positive correlation between tumour cellularity and ^{18}F-FDG PET conditional upon strong GLUT-I staining, concluding that GLUT-I expression was the main predictor of ^{18}F-FDG PET activity. While GLUT

expression is an important factor modulating tissue sensitivity to ^{18}F-FDG PET uptake, the commonly observed fall of ^{18}F-FDG PET as a function of decreasing cell density with chemotherapy strongly supports tumour cell density as the primary variable in this relationship. Nonetheless, multivariate modelling of ^{18}F-FDG PET metabolism with H&E and immunohistochemical parameters including GLUT-I would be a refinement of our proof-of-concept design.

We found two groupings of ^{18}F-FDG PET-histopathologic variables using cluster analysis. The variable inter-relationships can be summarised using MATV as the primary variable: large tumours had greater MCD, high SUV_{mean}, and a predominance of high-SUV voxel with fewer outliers (low skewness and kurtosis). Such tumours also had low SUV-lacunarity compared to smaller tumours, suggesting less clustering of low-SUV voxels on ^{18}F-FDG PET images and of cell-poor tissue on WSIs. To our knowledge, there are no in-vivo studies comparing ^{18}F-FDG PET tumour-volumes with quantitative cell densities. However, the positive correlation between MATV, $SUV_{mean,}$ and GLCM-entropy found in our study has been reported previously [21].

Our study has potential limitations: our cell-counting algorithm counted both benign and malignant cells. The presence of stromal cells in MCD estimation would have theoretically lowered its correlation with SUV_{mean} proportional to the amount of stromal cells present, since stromal cells are less metabolically active than proliferating tumour cells [6]. We also acknowledge that MCD is not the only factor influencing ^{18}F-FDG PET activity in a tissue; variables such as tumour differentiation and expression of GLUT proteins would also influence ^{18}F-FDG PET activity and could be added in future implementations. Finally, since we did not co-register ^{18}F-FDG PET and WSIs, we did not correlate derived parameters on a voxel-to-pixel basis. However, we believe that the large difference in resolution between in-vivo ^{18}F-FDG PET imaging and WSI may make such comparisons difficult or impractical.

Conclusion:

We observed an association between mean tumour cell density and several ^{18}F-FDG PET derived metabolic indices, particularly MATV, SUV_{mean}, skewness, and kurtosis. We also found lacunarity, a quantifier of gaps in geometry, to be correlated on ^{18}F-FDG PET imaging and WSIs; thus it may be a suitable variable for inter-modality comparison. Our results suggest the feasibility of comparing TVs derived from ^{18}F-FDG PET images with variables quantified from WSIs. Future studies could provide a more accurate understanding of TVs by including further tissue-variables, e.g., GLUT expression, and employing computationally intensive tumour-cell segmentation algorithms.

List of Supplemental Digital Content

Supplemental Digital Content 1.pdf

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Tables

Table 1

Variable	**Value***
Clinical variables	
Age	68.4 years (55.1-87 years)
Tumour sub-type	
Adenocarcinoma	19
Squamous cell carcinoma	3
Sex M:F	8:14
Tumour stage	
IA	5
IB	7
IIA	1
IIB	2
IIIA	6
IIIB	1
18F-FDG PET variables	
SUVmean	7.2
Tumour diameter	3.4cm (1.6cm - 5.2cm)
MATV	17.9 cm3 (2.2cm3-75cm3)
WSI variables	
MCD	3500 (640-5457)
Path-lacunarity	4.1 (1.6-7.2)

*For continuous variables, median values are provided, with ranges given in between parentheses. MCD=Mean cell density, WSI=Whole-slide imaging

Figure legends

Fig.1 Illustrating key steps in our MCD and Path-lacunarity computation workflow. The original H&E image (a) is colour-deconvolved to yield the haematoxylin-only H-image (b). On the H-image (b), regions with greater haematoxylin clustering indicate high cell-density regions. Representative pixels of various tissue-types, i.e., immune-cell predominant, cancer-cell predominant, and cell-poor are marked with symbols (cross, square, and circle respectively). N-Map (c) is derived after running a 3-class k-means clustering algorithm after selecting representative pixels of each tissue type. (d) Path-lacunarity is computed from the N-map by re-coding all the high cellularity regions (red and blue regions in (c)) as '1' and low cellularity regions (green regions in (c)) as '0'.

Fig. 2 (a) Correlation matrix of the measured variables: Positive correlations are shown in blue and negative in red, with darker shade implying stronger correlation, as shown in colour-key provided. The histogram displayed inside the colour-key shows frequencies of different rs values from -1 to +1. (b) Chart of p-values corresponding to the correlation matrix in (a). P-values <0.05 are highlighted in colour. *values labeled '0' are <0.0001.

Fig. 3 (a) Heatmap with each row representing an individual variable and each row, a patient. Variables (rows) are grouped and ordered by the strength of correlation among them using agglomerative hierarchical clustering. Highly correlated variables exhibit similar changes in colour from patient to patient (for example, GLCM entropy and SUV entropy) (b) A dendrogram based on cluster analysis shows two groupings of variables (blue and red). The lengths of branches between correlated clusters increase with increasing dissimilarity between clusters. All connected variables to the right of the vertical dashed line have statistically significant rs values. Clustering supporting our hypothesis includes the grouping of MCD with SUVmean and with MATV and PET-TLG, and grouping of SUV-lacunarity with Path-lacunarity

Fig. 4 A prototypical example of a large, highly metabolically active ADCA in an 80-year old man (Case 16 on heatmap shown in Fig. 3a). (a) ^{18}F-FDG PET/CT axial section through the lungs shows the large ^{18}F-FDG-avid tumour. Specific values of several relevant variables are shown in the inset. (b)H-image shows dense tumour/immune cell population corresponding to the high SUV_{mean} and negative skew of the ^{18}F-FDG PET image. (c) Binary image illustrates low Path-lacunarity (2.8) as smaller sized black regions of low cellularity between white regions of high cellularity.

Fig. 5 A prototypical example of a small tumour in a 64-year old woman with ADCA (Case 2 on heatmap shown in Fig. 3a). This tumour is at the opposite end of the spectrum to the previous example. (a)^{18}F-FDG PET/CT axial image through the lungs exhibits a very low activity tumour that has the majority of its voxels displaying SUV values nearer the minimum (hence the positive skew). (b) H-image and (c) binary image show large black regions of low cellularity separating the white regions of high cellularity. Quantitatively, these gaps are represented by the high Path-lacunarity (4.7).