Radiomics Biomarkers correlate with CD8 expression and Predict Immune Signatures in Melanoma Patients

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Running Title: Radiomics Biomarkers predict melanoma immune signatures

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Abstract

Treatment for metastatic melanoma includes targeted and/or immuno-therapy. Although many patients respond, only a subset have complete response. As late stage patients often have multiple tumours in difficult access sites, non-invasive techniques are necessary for the development of predictive/prognostic biomarkers.

PET/CT scans from 52 stage III/IV melanoma patients were assessed and CT image parameters were evaluated as prognostic biomarkers.

Analysis indicated patients with high standard deviation or high mean of positive pixels (MPP) had worse progression-free survival (p=0.00047 and p=0.0014, respectively) and worse overall survival (p=0.0223 and p=0.0465, respectively). Whole-exome sequencing showed high MPP was associated with BRAF mutation status (p=0.0389). RNA-sequencing indicated patients with immune ‘cold’ signatures had worse survival, which was associated with CT biomarker, MPP4 (p=0.0284). Multiplex immunofluorescence confirmed a correlation between CD8 expression and image biomarkers (p=0.0028).

Implications: CT parameters have the potential to be cost-effective biomarkers of survival in melanoma, and reflect the tumour immune-microenvironment.
INTRODUCTION

The worldwide incidence of cutaneous melanoma has steadily increased and represents a significant health problem (1). For patients with resected stage III/IV disease, standard treatment includes systemic therapies. However, only a proportion of patients experience long-term survival (2). Not all patients have equal risk of relapse and biomarkers are needed to more precisely deliver care.

An effective immune response is necessary for long-term survival in melanoma (3). Tumours with favourable immune responses have CD8+ cell infiltrates (4) and distinct gene expression signatures consistent with an immune “hot” microenvironment (5). Patients with immune hot tumours have longer overall survival (OS) with immunotherapies (6).

For patients with metastatic melanoma, PET/CT (Positron emission tomography–computed tomography) imaging is routinely performed as standard-of-care (7). This provides key clinical information and enables radiomics analysis, a non-invasive tumour assessment using statistical parameters derived from medical imaging.

Emerging data suggests that radiomics might provide prognostic biomarkers across several cancer types. In colorectal cancer, multi-parametric PET/CT analysis identified KRAS mutant tumours with hypoxic or proliferative phenotypes (8). Similarly in non-small cell lung cancer, quantitative CT analysis was significantly associated with KRAS mutations (8). A pan-cancer study developed a machine-learning process based on CT images to assess CD8 expression and PD-L1 immunotherapy response to predict tumour immune phenotype and survival (9).

In melanoma, a study of 42 patients, undergoing anti-VEGF therapy, showed CT image characteristics were highly accurate in predicting OS (10).
The primary aim of this study was to identify PET/CT image characteristics as prognostic biomarkers of survival in stage III/IV melanoma patients (n=52). To understand the underlying molecular mechanisms, we combined whole-exome sequencing data (WES), RNA sequencing (RNAseq) analysis and multiplex immunofluorescence.
MATERIALS AND METHODS

Study group

We performed a single centre study comprising 52 stage III/IV melanoma patients. Tumour tissue and blood were collected, at surgery. Pre-treatment PET/CT scans were available. Patients were recruited through the Cancer Evolution Biobank (HREC/10/PAH/153, UQ/2011001286) between July 2014 and July 2018. All participants provided written informed consent. Project approval was granted by the Metro South Human Research Ethics Committee (HREC/16/QPAH/671) and by the University of Queensland (UQ/2017000149). The study was conducted in accordance with the Declaration of Helsinki.

Patients were treated at the Princess Alexandra Hospital melanoma unit, Queensland, Australia. Treatment included BRAF inhibitor and/or immunotherapy, detailed in Supplementary Table S1. Clinical follow up data (HREC/18/QMS/48596) was used to determine OS and progression-free survival (PFS) (Supplementary Table S2). OS was the time from surgery until death from disease. PFS was the time from surgery until the first recurrence of disease confirmed through biopsy or conclusive radiological evidence. BRAF p.V600 mutation status was obtained through clinical pathology records. No patients were lost to follow up.

Tissue was stored in RNAlater. DNA/RNA were extracted using the Qiagen AllPrep DNA/RNA mini kit according to the manufacturer’s protocol (Qiagen, Germany). Tumours were also formalin-fixed paraffin-embedded (FFPE). Hematoxylin and eosin (H&E) slides were reviewed by an anatomical pathologist (GS) for tumour content.
**Positron Emission Tomography and Computed Tomography**

PET/CT scans were assessed retrospectively to extract imaging parameters for each tumour. Computed tomography texture analysis was performed using a commercially available filtration-histogram method (Feedback plc, Cambridge, UK) (11). A region of interest (ROI) was drawn around the tumour. It was then measured using several filters (fine to course) described as spatial scaling factors (SSF) ranging between 2 and 6mm (Visual Overview, step 1). Lesions comprising at least 20 pixels (CT pixel size 1.0mm) were analysed to ensure sufficient spatial variation in image intensity for extraction of meaningful texture features. For each tumour, a quantitative data set was compiled, consisting of: mean (M), mean of positive pixels (MPP), standard deviation (SD), entropy (E), kurtosis (K), skewness (S). All parameters were measured using each SSF.

PET parameters: metabolic tumour volume (MTV), maximum standard uptake volume (SUVmax), total lesion glycolysis (TLG) and number of pixels were also included in the analysis.

**Statistical Analysis**

Survival analysis was performed using a tree-structured model to stratify patients into low or high subgroups based on a single PET or CT parameter. Recursive binary partitioning determined the association between OS/PFS and covariates (R Foundation for Statistical Computing) and identified the optimal cut-off to stratify patients into survival groups (Supplementary Table S3). Log-rank Kaplan-Meier analysis assessed the predictive/prognostic value of each covariate using the cut-off values (GraphPad Prism 8.3.1). The Benjamini-Hochberg (BH) procedure corrected for multiple testing. The significance level for all analysis was 0.05.
Whole-exome sequencing

WES on matching tumour/buffy coat samples was available from a previous study for 33 patients (12). Data is available through the European Genome-phenome Archive, study ID EGAS00001004619 (data set ID: EGAD00001006375) (Supplementary Table S2). For 32 patients, the matched tumour deposit was also assessed by PET/CT.

WES was performed on the Illumina Hiseq4000 to a depth of 500X in the tumour and 100X in the buffy coat (n=29). Tumour/buffy coat pairs from an additional 4 patients were sequenced on the Illumina NextSeq. Tumour mutation burden (TMB) was reported as the number of mutations per megabase (Mut/Mb) in the coding region. Detailed methods for mutation detection and calling are described in Aoude et al (12). QIMR Berghofer Human Research Ethics Committee granted approval for genomic analysis (QIMR Berghofer HREC P3577).

SNP Array

For samples sequenced on the HiSeq4000, SNP arrays (2.5M Illumina) and the qpure bioinformatics tool were used to assess the tumour content (13). For samples sequenced on the NextSeq500, cellularity was assessed using the mean allele fraction. All samples contained >20% tumour content.

RNA sequencing

RNAseq was performed on tumour RNA with a RIN score >5 and tumour cellularity >20% (n=21) (Supplementary Table S2). Libraries were generated using the TruSeq Stranded mRNA kit and sequenced with 100bp paired end reads. Reads were aligned to GRCh37 using STAR (version 2.5.2a) (14) and Cutadapt (version 1.11). Quality control metrics were computed using RNA-SeQC (version 1.1.8)(15) and gene expression estimated using RSEM
Gene read counts were normalised to transcripts-per-million (TPM). RNAseq data is available through the European Genome-phenome Archive study ID EGAS00001004619 (data set ID: EGAD00001006375).

Published immune modulator genes were assessed to identify patients with immune hot/cold signatures (17). Unsupervised clustering was performed using the log2 TPM expression values using Euclidian distance and complete-linkage clustering. The heatmap show the row-wise centred and standardised z-scores.

**Multiplex immunofluorescence and histomorphometry**

Multiplex immunofluorescence analysis was performed on 31 FFPE tumours (Supplementary Table S2) to assess expression of immune markers: CD4, CD8, PD-L1 and PD-L2. Tumours selected had > 50% tumour content.

After deparaffinisation, hydration and antigen retrieval (Dako pH9, 100°C, 20 min), sections were treated with peroxidase and blocked. Slides were stained using the Ventana BenchMark Special Stains platform (Roche Diagnostics).

Nuclei were DAPI stained. The antibodies used were: CD4 (#M7310, Dako, 1:800, mouse secondary-Opal 650); CD8 (#M7103, Dako, 1:4000, mouse secondary-Opal 690); PD-L1 (#51296S; Cell Signalling Technology; 1:400, rabbit secondary-Opal 570); PD-L2 (#MAB1224-100, R&D Systems, 1:400, mouse secondary-Opal 520).

Slides were scanned (20X) on a Zeiss AxioScan Z1 and images analysed and size reduced using Zen3.1 (Zeiss). The Visiopharm Image Analytical System (Version 2017.2.4.3387) quantified the staining. DAPI nuclei were automatically segmented defining the areas. All markers were quantified using the imbedded Visiopharm system workflow. The marker staining levels were reported as a ratio of the marker intensity to the assessed area.
RESULTS

Clinico-Pathologic Analysis

We examined a cohort of 52 melanoma patients (Supplementary Table S1) to determine whether PET/CT image characteristics were associated with survival. In stage III patients (n=47) we assessed the largest positive lymph node. In resected stage IV patients (n=5), we analysed the largest metastatic deposit. A subset of patients, 19/52 (37%) harboured a BRAF p.V600E mutation. Two patients had an alternative BRAF variant (p.L601E or p.T599I) and were considered wild-type for this study. The median PFS was 9.1 months (range, 1-46 months). The median OS was 27.6 months (range, 3-50 months). Median follow up for survivors was 32.3 months (range, 12-50 months).

PET/CT variables and Survival Outcomes

We assessed individual PET (MTV, SUVmax, TLG, pixels) and CT parameters (M, MPP, SD, E, K, S). Each parameter was measured using a range of filters described as spatial scaling factors (SSF) ranging from 2-6mm (Visual Overview). As an example SD2 denotes SD measured at SSF2 (2mm).

We performed univariable survival analyses using each PET/CT parameter as a continuous variable, and included clinico-pathologic features (BRAF status, ulceration of primary lesion, stage and TMB). SD was significantly associated with PFS and OS (Fig. 1a, cox regression) regardless of the SSF used (SD2-6). MPP was also associated with PFS using all filters, but not OS. Both the SD and MPP parameters were found to have greater prognostic significance than clinico-pathologic features in this cohort and passed correction for multiple testing (BH<0.05). From the filter set, MPP at spatial scaling factor 4 (MPP4) and SD
at spatial scaling factor 3 (SD3) were most significantly associated with survival.

Downstream analysis focused on these markers.

None of the PET parameters were associated with survival (Fig. 1a).

The patient cohort was divided into low/high groups using the optimal cut-offs for survival (Supplementary Table S3). High MPP4 (>34.3) was significantly associated with poor PFS (Hazard Ratio (95% CI) = 3.050 (1.572-5.918), p=0.0014, log-rank (Mantel-Cox) test) (Fig. 1b). MPP was significantly associated with PFS using all filters (MPP2-MPP6) indicating that this was a robust measurement (Supplementary Table S3). A high MPP4 score was also significantly associated with poor OS (Hazard Ratio (95% CI) = 3.319 (1.201-9.173, p=0.0465, log-rank (Mantel-Cox) test) (Fig. 1b).

A high SD3 (>55) was significantly associated with poor PFS (Hazard Ratio (95% CI) = 2.876 (1.363-6.070, p=0.00047, log-rank (Mantel-Cox) test) (Fig. 1b). Importantly, SD remained statistically significant when measured across a range of filters (Supplementary Table S3). Additionally, high SD3 was significantly associated with poor OS (p=0.0223; Hazard Ratio (95% CI) = 3.086 (1.049-9.075), log rank) (Fig. 1b). The survival analysis for MPP4 and SD3 remained significant when only stage III patients were included (Supplementary Fig. S1), indicating this result was not driven by stage.

Multivariate cox-regression analysis assessed clinico-pathologic variables and compared them to MPP4 low/high and SD3 low/high as prognostic factors (Fig. 1c). SD3 and MPP4 were significantly associated with PFS, p=0.007 and p=0.012 respectively. Additionally, MPP4 was associated with OS, p=0.035. Though a clear trend was seen with stage, the clinico-pathologic variables were not statistically significant in this small cohort.

**BRAF status is associated with PET/CT markers**
WES analysis determined whether tumour genomic features were associated with PET/CT parameters (n=33 patients). *BRAF* was the most frequently mutated gene (48%) followed by *NF1* (39%) and *H/K/NRAS* (33%) (Fig. 2a) in alignment with other studies (18).

Both MPP4 high (p=0.0389, Fishers exact) and SD3 high (p=0.0437, Fishers exact) (Fig. 2b) were associated with the presence of a BRAF p.V600 mutation. TMB was not related to either MPP4 or SD3 (Supplementary Fig. S2).

**Immune hot/cold signatures are associated with MPP4**

RNAseq analysis assessed whether the expression of immune modulator genes was related to MPP or SD (n=21 patients). Unsupervised clustering resulted in an immune cold and an immune hot cluster (Fig. 2c) (17). The immune hot signature correlated with low MPP4, while the immune cold signature related to high MPP4 (p=0.0284, unpaired t-test) (Fig. 2d). SD3 showed no association (Fig. 2d).

**CD8+ cells correlate with MPP4 and patient survival**

To define the association between the immune hot phenotype and MPP4, we performed multiplex immunofluorescence on four immune markers, CD4, CD8, PD-L1 and PDL-2. CD4 is found on the surface of T-helper cells and macrophages. CD8 is expressed by cytotoxic cells (T-cells, NK cells, cortical thymocytes, dendritic cells). Based on the RNAseq data, CD4 and CD8 were more highly expressed in the immune hot group (CD4 fold-change = 2.9 and CD8 fold-change = 8.9).

PD-L1 (encoded by *CD274*), expressed by activated T-cells, is a target of current immunotherapies. PD-L2 (encoded by *PDCD1LG2*) is linked to PD-L1, though its involvement is yet to be fully ascertained. RNAseq data showed *CD274* and *PDCD1LG2* were more highly
expressed in the immune hot group (CD274 fold-change = 4.8 and PDCD1LG2 fold-change = 8.0).

FFPE sections (n=31) were stained with the immune markers, and intensity was quantified (Supplementary Fig. S3). The number of tumour infiltrated CD8+ cells was 2.3 times higher in the MPP4 low patients (p=0.0166, Fig. 3a). PD-L1, PD-L2 and CD4 were not differentially expressed. None of the immune markers were associated with SD3 (Fig. 3b).

We assessed whether CD8 protein expression was correlated with MPP4 and SD3 by comparing patients with high CD8 expression (>5%) to those with low expression. The cut-off was the median level of CD8 expression across the sample group. Low CD8 expression was associated with a high MPP4/SD3 measurement (p=0.0028, chi-square = 11.742, Fig. 3c). These findings confirmed the RNAseq results. Fig. 3d shows staining for MPP4 low (MelR062) and MPP4 high (MelR166) tumours.
DISCUSSION

Melanoma patients undergo routine PET/CT scans as part of their clinical management. Using a non-invasive tumour assessment, widely available in radiology practices, we found a prognostic signature derived from PET/CT imaging that represented a ‘hot’ tumour immune microenvironment.

We showed that CT parameters, MPP4 and SD3, were associated with patient survival. These biomarkers appeared to have better prognostic value than primary tumour ulceration status, which forms part of the staging system. This should be investigated in a larger cohort and expanded to include different disease stages as sample size is a limitation of this study.

RNAseq analysis indicated that patients with immune hot tumours had low MPP4 values \( (p=0.0284) \). Furthermore, MPP4 low tumours had more CD8+ cells \( (p=0.0166) \). High CD8+ infiltration correlated with better PFS \( (p=0.0005) \). These results are concordant with other pan-cancer analysis using radiomics to quantify CD8+ cells in the tumour \( (9, 10, 19) \). Our findings build on previous studies showing that CT parameters correlate with the immune signatures and patient survival.

Identifying a correlation between the radiomics parameters and tumour biology (BRAF status, immune signatures and CD8 expression) demonstrates the prognostic performance of this technique. However, this needs to be confirmed in validation cohorts.

An important outcome of this study would be to determine whether these biomarkers can be applied to unresectable stage IV patients where tumour tissue is unavailable. Moreover, stage IV patients may have multiple tumour sites with inter- and intra-tumour heterogeneity further complicating treatment outcomes \( (20, 21) \). This technique could allow patients with unresected disease to be treated with a precision medicine approach as radiomics
parameters are derived from routine images. Furthermore, if risk of recurrence could be determined at stage III, a subset of patients with low risk of relapse, could avoid the potential toxicities of systemic therapy, which can be irreversible (22, 23).

Imaging is an established clinical tool. The quantitative imaging techniques in this study are simple and readily applied to existing protocols. This highlights the utility of radiomics in the clinical decision making process. Prognostic or predictive biomarkers would be of significance for melanoma research and clinical practice. Inclusion of novel imaging biomarkers in clinical trials can enable more accurate stratification of patients and identification of sub-groups enriched for response.

The potential of this study is to leverage routine, readily accessible, non-invasive imaging technology to derive insight into the underlying immune landscape of melanoma. Providing a personalised approach to reduce treatment failure, morbidity and costs associated with the treatment of metastatic melanoma.
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AUTHORS’ CONTRIBUTIONS

LGA, VFB, KM and APB contributed to the study design, methodology and data analysis. APB, BMS, VA, GB and SY recruited the patients. BW, PL and KM performed the PET/CT image analysis. GS performed the pathology review. KP performed laboratory experiments related to tissue and blood samples. SB, HO, LTK, MMN, JVP and NW contributed to the acquisition and analysis of whole-exome and RNAseq data. SBW, VFB, JJB and CJB performed multiplex immunofluorescence analysis. LGA and VFB wrote the manuscript. All authors edited, read and approved the final manuscript.
REFERENCES


FIGURES

Figure 1. Survival in metastatic melanoma patients stratified using CT texture analysis.

a. Cox regression analysis integrating survival with continuous variables from PET/CT imaging. PET parameters were: metabolic tumour volume (MTV), maximum standard uptake volume (SUVmax), total lesion glycolysis (TLG) and number of pixels. CT parameters were: mean (M), mean of positive pixels (MPP), standard deviation (SD), entropy (E), kurtosis (K), skewness (S). Each CT parameter was measured using spatial scaling factor (SSF 2-6 mm) where SSF denotes the size of the feature highlighted in the scan. Additional biomarkers were analysed: BRAF status, ulceration of primary tumour, stage and tumour mutation burden (TMB).

b. Kaplan-Meier (log-rank) survival analysis showing MPP4 low/high groups and progression-free survival (PFS), **p=0.0014; MPP4 and overall survival (OS), *p=0.0465; SD3 and PFS, ***p=0.00047; SD3 and OS, *p=0.0223.

c. Hazard ratio combining clinic-pathologic information and radiomics markers. Clinico-pathologic variables were compared to CT parameters using multivariate cox-regression analysis. Assessment was performed for both PFS and OS.

Figure 2. Genomic analysis. Whole-exome Sequence analysis was performed. a. Alterations (missense, nonsense and splice site mutations) in the most frequently mutated driver genes are represented. Frequencies of each mutation are indicated. Mutations in NRAS, KRAS and HRAS are combined under N/K/HRAS. Samples are arranged according to their BRAF p.V600E mutation status which is associated with b. MPP4 (p=0.0389, Fisher’s exact) and SD3 (p=0.0437, Fisher’s exact). c. RNAseq Immune hot/cold signatures. Unsupervised clustering of immune modulator gene panel showing MPP4 high is associated with ‘cold’
immune signature and MPP4 low is associated with a ‘hot’ immune signature. Red indicates immune hot. Blue indicates immune cold. d. Unpaired t-test showing MPP4 values in immune hot/cold groups, *p=0.0284 and SD3 values in immune hot/cold groups, not significant.

**Figure 3. Validation of immune biomarkers by multiplex immunofluorescence.** Tissue sections were stained for CD8 (Cy7), PD-L1 (Cy3), CD4 (Cy5) and PD-L2 (FITC). Positive cells were quantified using the intensity of the tumour area selected. a. Two-way ANOVA test comparing intensity/area ratios in MPP4 low/high patients. MPP4 low patients had significantly more CD8+ cells, *p=0.0166. PD-L1, PD-L2 and CD4 levels were not significantly different between MPP4 low/high patients. b. Two-way ANOVA test showing no association between immune biomarkers and SD3. c. Chi-square analysis showing high CD8 expression was associated with low MPP4 and low SD3. Conversely, low CD8 expression was associated with high MPP4 and high SD3, **p=0.0028, chi-square = 11.742. The median level of CD8 expression, 5%, was used as the cut-off. d. Multiplex immunofluorescence images representing MPP4 low (MelR062) and MPP4 high (MelR166) phenotypes.

**Visual Overview. Experimental overview and clinical significance.** From left to right, Step 1 shows CT texture analysis highlighting the metastatic deposit in the lymph node in blue. Histogram analysis of the region of interest (ROI) has been undertaken using a fine filter at special scaling factor 2 (SSF 2, blue) and course filter at special scaling factor 6 (SSF 6, pink). Step 2 indicates the genomic and transcriptomic analysis including whole-exome sequencing (WES), RNA sequencing (RNAseq) and immune signatures. Step 3 shows validation of immune markers using multiplex immunofluorescence staining of patient tumour tissue. Step 4 correlates immune markers with patient survival outcomes.
**Figure 3**

**Marker expression and MPP4**

![Bar chart showing MPP4 expression](chart1)

**Marker expression and SD3**

![Bar chart showing SD3 expression](chart2)

**CD8 expression and MPP4/SD3**

![Bar chart showing CD8 expression and MPP4/SD3](chart3)

**Images**

*Panel d* shows immunofluorescence images of DAPI, CD8/Cy7, PD-L1/Cy3, CD4/Cy5, PD-L2/FITC, and a merge for cells labeled with MelR062 and MelR166.
Visual Overview

1. PET/CT imaging
   I. Tumour ROI
   II. Histogram Analysis

2. Genomics and Transcriptomics
   I. WES
   II. RNAseq
   III. Immune Hot/Cold Signature

3. Validation
   Multiplex Immunofluorescence

4. Clinical utility
   Patient Survival Outcomes
Molecular Cancer Research

Radiomics Biomarkers correlate with CD8 expression and Predict Immune Signatures in Melanoma Patients


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