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Conflict of Interest Statement

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Abstract

Background

Prostate-specific membrane antigen (PSMA) is a prostatic epithelial protein that is used as a radiotracer (68Ga-PSMA-11) for prostate cancer staging. PSMA-PET/CT performed for prostate cancer has been observed to detect melanoma metastases. The aim of this study was to investigate the performance of PSMA immunohistochemistry on resected melanoma metastases to explore its use as a diagnostic imaging biomarker for melanoma.

Methods

41 stage III/IV melanoma specimens were stained with PSMA immunohistochemistry. All specimens required both disease and control regions. Two pathologists scored the specimens and a receiver operating characteristic (ROC) curve was plotted. Western blot and multiplex immunofluorescence were also performed.

Results

The area under the ROC curve was 0.82, suggesting PSMA has excellent discriminatory power in melanoma metastases. Sensitivity is 82.9% and specificity 73.2%. Immunohistochemistry and Western blot reveal that PSMA staining in melanoma consistently and most intensely occurs in tumor neovasculature. Multiplex immunofluorescence shows that melanocytes may also weakly express PSMA.

Conclusion

Performance of PSMA immunohistochemistry in melanoma metastases rivals that reported in prostate cancer studies. This study indicates that PSMA shows promise for use as a novel biomarker in melanoma and justifies further research in the clinical setting with potential as a PET/CT radiotracer and intraoperative fluorescence marker for melanoma.

Keywords

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Introduction

Prostate-specific membrane antigen (PSMA) or glutamate carboxypeptidase II is a type-II transmembrane protein produced and expressed by prostatic epithelium[1]. It is found in both normal and neoplastic prostatic tissue[1]. In 2012, ^{68}Ga-PSMA-11, a urea-based small molecule inhibitor of PSMA was developed in Germany and has since become the most widely used of all the PSMA ligands in the assessment of prostate cancer[2]. PSMA-PET/CT has demonstrated superiority to conventional CT and bone scan imaging in the staging of prostate cancer, being particularly useful in biochemically recurrent prostate cancer[3-8] and its use may change management in up to 50% of patients[9, 10]. Recently, it has become apparent that PSMA may have potential as a diagnostic imaging biomarker in melanoma, specifically as a PET radiotracer. A recently published report has described an in-transit melanoma metastasis detected by PSMA-PET/CT, performed for staging of prostate cancer[11].

However, PSMA is not "specific" for prostatic tissue, as it has now been described as being expressed in multiple other tissue types. PSMA-PET uptake has been found in normal organs (weakly expressed in kidney, duodenum, parotid, submandibular gland, spleen, lacrimal gland, liver)[9], benign conditions (sarcoidosis[12], Paget's disease[13]), benign (thyroid adenoma[14], schwannoma[15], meningioma[16]) and malignant neoplasms (colorectal[17], pancreatic neuroendocrine tumor[18], RCC[19], cholangiocarcinoma[20], HCC[21], bladder[22], breast[23], GIST[24], multiple myeloma[25], ovarian and endometrial[26]). In contrast to the prostate, where PSMA is located on prostatic epithelial cells, PSMA expression in these other neoplastic pathologies is located on endothelial cells of capillary vessels in peritumoral and endotumoral areas. This suggests PSMA expression may be related to angiogenesis of tumor neovasculature[1], however a precise explanation to explain this phenomenon remains elusive[27]. There is *in vitro* evidence that anti-PSMA antibodies react with malignant melanoma neovasculature[28] and, combined with the recent

report showing PSMA-PET/CT avidity, suggests a possible role for PSMA as a novel diagnostic imaging biomarker.

Currently, there are limitations with both CT and FDG-PET/CT in diagnosing melanoma metastases, particularly nodal metastases[29, 30]. Identification of a melanoma imaging biomarker offers the possibility of improved accuracy in staging and surveillance. In addition, with the development of fluorescence-guided surgery, a diagnostic biomarker for melanoma has the potential to help with intra-operative identification of tumor extent and to guide resection margins[31].

The development of imaging biomarkers requires that biomarker compounds undergo technical and biological validation prior to clinical validation[32]. Therefore, this study aims to establish the sensitivity and specificity of PSMA immunohistochemistry for melanoma metastases previously resected from patients. In addition, it aims to describe the pattern of PSMA expression in melanoma and to identify associations between PSMA expression and the following pathological variables: melanoma subtype, ulceration, thickness and site of metastasis.

Materials and Methods

Patients and Setting

At a tertiary referral centre for melanoma, a retrospective, observational study was performed using historical stored tissue specimens. Patients who had undergone surgery for stage III or IV melanoma were identified from a hospital database. Tissue samples stored since 2011 were available to be retrieved from storage and so the search was limited to patients undergoing surgery since then. Patient characteristics and clinicopathological data were retrieved from medical records.

This study was approved by the institutional ethics committee and was performed in accordance with the principles outlined by the Helsinki Declaration. Only patients who had appropriate, documented pre-operative consent for the use of tissue for research, and whose samples were stored in hospital tissue-banking facilities, were included.

Specimen preparation

Samples were assessed by a senior pathologist (SH) for adequacy. Specimen inclusion criteria required that the histology slides contained both a region of metastatic melanoma (disease region) and a region of normal tissue (control region). Exclusion criteria included samples containing tissue completely replaced with melanoma as they contained no internal control region, or disease or control regions that were considered to be too small for accurate analysis (there was no specific size for this but was judged on an individual basis). Sections from the corresponding formalin-fixed, paraffin-embedded blocks were then cut at a thickness of 3 μm and mounted onto charged slides. Immunohistochemical staining with PSMA was performed at UCL Advanced Diagnostics (London, UK) using liquid concentrated PSMA antibody clone 1D6 at 1:50 dilution (Leica Biosystems, Newcastle, UK).

The PSMA-stained slides were then analysed and scored by two senior histopathologists (SH, NF). Scores were assigned to both the disease region and the control region. Since PSMA has been shown to stain the tumor neovasculature in melanoma, scores were calculated by counting the number of blood vessels positively stained with PSMA averaged over 10 fields using a 10X eyepiece and 20X objective. A blood vessel was considered positive if the majority of endothelial cells stained with higher intensity than the surrounding tissue. These counts were then averaged between the two scoring pathologists to establish a final score. In addition to vessel counts, specimens were assessed subjectively for PSMA

staining intensity using both standard immunohistochemistry and multiplex immunofluorescence (below).

To further characterize the location of PSMA expression in melanoma metastases, a Western blot was performed on four human melanoma cell lines – Mel624, DO4, A375 and MeWo - alongside a lymph node metastasis of prostate cancer, known to contain PSMA. Cells were lysed by snap-freezing on dry ice and the lysate was then centrifuged and transferred to a polyvinylidene difluoride Hybond-P membrane (Amersham; Buckinghamshire, UK). Immunodetections were performed using anti-PSMA rabbit polyclonal antibody (Cell Signaling; Beverly, MA, USA), in conjunction with a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (GE-Healthcare, Buckinghamshire, UK). Equal loading was assessed using α-tubulin mouse monoclonal primary antibodies (Sigma-Aldrich; Gillingham, UK). The Super Signal chemiluminescent system (Pierce; Rockford, IL, USA) was used for detection.

In addition, multiplex immunofluorescence was performed. 4 µm sections of tissue were stained with 1:500 dilution of PSMA antibody (Abcam 133579; Cambridge, UK) and Opal™ reagents (Akoya Biosciences; Menlo Park, US). Stained slides were imaged using a Vectra® 3.0 microscope (Akoya Biosciences; Menlo Park, US) and inForm® image analysis software (Akoya Biosciences; Menlo Park, US).

Statistical analysis

Given that PSMA expression in melanoma has not previously been explored, there is no established definition for what constitutes positive staining. Therefore, a receiver operating characteristic (ROC) curve was plotted using various score points for PSMA staining scores to establish the optimal cut-off

point. This point and above was considered positive PSMA staining and below considered negative. Area under the curve (AUC) and 95% confidence intervals from the ROC were reported. Similarly, sensitivity, specificity, positive predictive and negative predictive values were then calculated at the optimal cut-off point.

In addition, descriptive analyses of the patient's diagnosis, disease stage, treatment received, pattern and intensity of PSMA staining were undertaken using frequencies and percentages in categories, and medians and ranges for continuous, non-normal data. PSMA scores from tumor and non-tumor samples were also summarized by disease sites, melanoma subtype and ulceration subgroups.

Results

From database searches, 65 patient specimens were screened for inclusion. 18 of these were deemed unsuitable by initial pathology review due to insufficient amounts of either tumor or non-tumor areas. A further 6 specimens were unable to be retrieved, leaving a total of 41 specimens included for analysis. The clinicopathological characteristics of these patients are shown in Table 1. The median Breslow thickness of primary melanoma was 2.95 mm (range 0.8-19 mm). Only two patients had been pre-treated with either targeted therapy (one), immunotherapy (zero) or radiotherapy (one).

Performance of PSMA staining

The receiver operating characteristic (ROC) curve for PSMA staining in melanoma metastases is shown in Figure 1. The area under the ROC curve (AUC) is 0.82 (95% confidence interval: 0.73-0.90). From the ROC curve, the optimal cut-off point could be identified. Using this cut-off (above which stains were considered positive; below which negative), the sensitivity of PSMA for melanoma metastases is 82.9% and the specificity is 73.2% (Table 2).

PSMA-staining scores, subdivided by site of metastasis, melanoma subtype and ulceration status can be seen in Table 3. A sub-group comparison, to investigate for any difference in PSMA expression depending on these factors, was deemed inappropriate due to the small numbers in the sub-group populations. However, as seen in Table 3, it is noteworthy that nodular melanomas displayed considerably higher median scores than any other subgroup.

Pattern of PSMA staining

Examples of PSMA staining patterns in melanoma metastases are shown in Fig 2 (A-H). PSMA was seen to stain the endothelial cells lining the tumor neovasculature in melanoma metastases, whilst the malignant melanocytes did not themselves stain with PSMA (Fig 2 G&H). When normal surrounding tissue (nodal or subcutaneous tissue) stained with PSMA, the cells showing the PSMA expression were also vascular endothelial cells. The pattern of staining of blood vessels within the tumor often occurred along fibrous septae that traversed the tumor deposit (Fig 2B).

Western blot analysis of human melanoma cell lines showed further absence of PSMA expression in the melanocytes (Fig 3). However, multiplex immunofluorescence revealed PSMA staining by tumor neovasculature but also uptake by tumor melanocytes (Fig 4). Staining was inconsistent, with some specimens showing widespread staining in tumor cells (Fig 4A) and others showing staining only in tumor vessels (Fig 4B). When staining was observed in tumor cells, the appearance was of cytoplasmic patches rather than membrane staining.

There were also some notable circumstances in which PSMA did not stain the tumor. Tumors mostly composed of spindled melanoma cells almost never stained with PSMA (Fig 2 C&D). Similarly,

necrotic areas within tumors did not stain (Fig 2 A&B). Furthermore, melanoma often contains melanin pigment-laden areas. This pigment masks the similar-coloured PSMA immunohistochemical stain, causing these tumors to be particularly difficult to detect pattern and intensity of staining.

Intensity of PSMA staining

The intensity of staining with PSMA in the melanoma metastasis was greater than the intensity of staining in the surrounding non-tumor tissue (Fig 2E). As described above, this staining was mostly observed in blood vessels both within the tumor and without; but staining in the tumor vessels was much more intense. This increased intensity was seen with standard immunohistochemistry (Fig 2E) and confirmed with multiplex immunofluorescence (Fig 4 A&B). In instances where tumor melanocytes stained with PSMA, this was also at much lower intensity (Fig 4D).

Discussion

This is the first study to investigate PSMA as a novel diagnostic imaging biomarker in melanoma. We have demonstrated that PSMA immunohistochemistry has a high sensitivity (82.9%) and specificity (73.2%) for detecting melanoma metastases that rivals its performance in prostate cancer (sensitivity 65.9%, specificity 82.9%)[33]. There is no previous research to guide the definition of positive PSMA staining in melanoma. Therefore, we used multiple different cut-off points for the number of vessels staining per high power field to create a ROC curve. This displayed an AUC of 0.82, which suggests this is a test with excellent discriminatory power.

The pattern and intensity of PSMA staining in melanoma has been seen most strongly and consistently to mark the tumor neovasculature within melanoma metastases in this series. PSMA does appear

occasionally to stain vessels in normal tissue as well as malignant tumor melanocytes, but in both these instances, it is inconsistent, and the intensity of staining is weak (Fig 2&4).

This pattern of PSMA staining in tumor vessels was seen microscopically with standard immunohistochemistry and was reinforced with Western blot analysis, where PSMA was not detected in any melanoma cells lines. However, with the addition of fluorescence multiplex immunohistochemistry, PSMA could be detected in the melanoma cells in some samples. Due to logistical constraints, it was not possible to perform multiplex immunofluorescence on all samples, so quantification was not possible. It is unclear why the melanoma cells were seen to stain with PSMA in the multiplex immunofluorescence but not with standard immunohistochemistry. Different PSMA antibodies were used for each technique (Leica and Abcam) and may have slightly different reactivities. Also, it may be that the multiplex technique is more sensitive to lower levels of expression. Whilst it has been previously shown that PSMA is only expressed in the melanoma neovasculature[11], our study suggests that, in fact, melanoma cells themselves may also express PSMA.

Unfortunately, numbers in this series were not large enough to draw meaningful conclusions regarding any differences in PSMA expression based on different melanoma subtypes or sites of metastasis. The latter is an important point. This cohort only included nodal or in-transit metastases. PSMA used as an imaging biomarker for melanoma would also need accurately to detect solid organ metastases, frequently seen in melanoma. This needs to be tested further. Furthermore, whilst numbers are small and conclusions must be drawn cautiously, nodular melanomas displayed very strong staining with PSMA compared to other subtypes and may represent a group of patients for whom PSMA-based investigations are particularly useful.

There was one histopathologic type which consistently showed a lack of PSMA staining: a spindled morphology. It is not clear whether this is due to spindled metastases having less intrinsic PSMA expression or whether it is because spindled metastases have less, or possibly even different, neovasculature from other melanoma subtypes. Neither spindle cell melanoma nor desmoplastic melanoma (a common variant of spindle cell melanoma) featured as the primary melanoma diagnosis in our cohort. In the specimens where spindled morphology featured and PSMA staining was minimal (four patients), the primary melanoma was superficial spreading melanoma in two and not recorded in the other two. Whilst a similar lack of PSMA expression was seen in necrotic metastases, necrosis usually exists in the centre of a viable tumor and this viable portion does seem to express PSMA (Fig 2B).

There were only 2 patients in this series who had been pre-treated for their melanoma, one with targeted therapy and one with radiotherapy. Combined with immunotherapy, these treatment modalities are used much more commonly today than they were during the period our samples were collected. The effect that these treatments will have on PSMA expression is unclear and warrants further investigation.

Current guidelines for staging in melanoma, stage IIIB and above, recommend PET/CT with FDG radiotracer[34]. However, organs such as the brain and liver have high background FDG uptake, limiting the utility of FDG-PET/CT for imaging these sites. As such, MRI brain is required and MRI liver, particularly in ocular melanoma with a high propensity for hepatic metastases, is frequently required also. Whilst PSMA has been seen to be expressed in normal liver, the intensity of the expression is relatively weak compared to the high intensity observed in melanoma metastases and it is expected that the different signal intensities would allow clear distinction between tumor and normal liver on a PSMA-PET. The implication of this study is that PET/CT using PSMA radiotracer may obviate the

need for these MRI scans and may possibly even provide greater diagnostic accuracy. Moreover, a specific radiotracer for melanoma may have utility in selecting patients who may benefit from nodal dissection and may guide the extent of surgery. This potential should inform future studies and justifies a clinical study evaluating the diagnostic performance of PSMA-PET in advanced melanoma.

In addition to its diagnostic utility, research continues into novel prostate cancer treatments based on PSMA; for example, near infrared photoimmunotherapy, using an anti-PSMA antibody, is showing promise in animal models for treating prostate cancer[35]. In other cancer types, fluorescence-guided surgery (using fluorescent probes attached to tumor-specific molecules that can be visualised with specific light filters, for example ultraviolet) continues to show promise as a technique to aid intra-operative tumor margin assessment and guide resection extent[31]. In melanoma specifically, fluorescence-guided surgery using indocyanine green (ICG) has become standard of care in many institutions for melanoma sentinel-node biopsy. Whilst ICG is not specific for melanoma, it demonstrates the potential of fluorescence-guided surgery to aid in the identification of tumor-bearing nodes using tumor-specific markers.

In conclusion, we believe that PSMA shows promise for use as a novel imaging and intraoperative biomarker in melanoma, and this study provides justification for the exploration of clinical diagnostic and therapeutic techniques which target PSMA in patients with melanoma.

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Table 1. Clinical and pathological characteristics

Characteristic	n	%
Specimen type		
- Lymph node	35	85.4
- In-transit/subcutaneous	6	14.6
Stage		
- IIIB	9	22.0
- IIIC	28	68.3
- IIID	1	2.4
- IV	3	7.3
Subtype		
- Superficial spreading	15	36.6
- Nodular	6	14.6
- Acral	2	4.9
- Mucosal	1	2.4
- Unknown primary	9	22.0
- Not recorded	8	19.5
Anatomic site of primary		
- Trunk	10	24.4
- Upper limb	3	7.3
- Lower limb	17	41.5
- Head & neck	1	2.4
- Perineum/genitalia	1	2.4
- Unknown	9	22.0

Thickness (Breslow, mm)		
- 0-1	5	12.2
- 1.01-2	5	12.2
- 2.01-4	10	24.4
- >4	10	24.4
- Unknown	11	26.8
Ulceration		
- Present	10	24.4
- Absent	31	75.6
Prior immunotherapy		
- Yes	0	0
- No	41	100
Prior targeted therapy		
- Yes	1	2.4
- No	40	97.6
Prior radiotherapy		
- Yes	1	2.4
- No	40	97.6

Table 2. Performance of PSMA staining in stage III-IV melanoma. Binary table for PSMA-stained vessel counts at optimal cut-off point (0.35).

	Disease region	Control region	Total
≥ 0.35	34	11	45
< 0.35	7	30	37
Total	41	41	82

Sensitivity: 82.9% (95% CI: 67.9 – 92.8)

Specificity: 73.2% (95% CI: 57.1 – 85.8)

Positive predictive value: 75.6% (95% CI: 60.5 – 87.1)

Negative predictive value: 81.1% (95% CI: 64.8 – 92.0)

Table 3. PSMA immunohistochemistry staining scores (number of vessels per high-power field) by site, melanoma subtype and ulceration status. (All values listed are: median (range)).

	Disease region	**Control region**
Site		
In-transit/subcutaneous (n=6)	5.35 (0.35–28.30)	0 (0–0)
Lymph node (n=35)	5.75 (0–27.55)	0 (0–25.05)
Melanoma subtype		
Acral (n=2)	1.10 (0–2.20)	0 (0–0)
Mucosal (n=1)	1.4 (N/A)	0 (N/A)
Nodular (n=6)	13.03 (5.35–27.55)	0 (0–8.95)
Superficial spreading (n=15)	2.35 (0–28.30)	0 (0–25.05)
Unknown Primary (n=9)	6.00 (0–22.50)	0 (0–4.50)
Not recorded (n=8)	4.6 (0-14.9)	0.35 (0-1.55)
Ulceration		
No (n=31)	4.30 (0–28.30)	0 (0–25.05)
Yes (n=10)	7.73 (1.40–27.55)	0 (0–5)

Figure Legends:

Fig 1. Receiver Operating Characteristic (ROC) curve for PSMA in melanoma metastases

Fig 2. H&E (A, C, E, G) and PSMA (B, D, F, H) stained micrographs of melanoma lymph node metastases. A&B (20X magnification) show a metastasis with central necrosis (arrow) and PSMA highlighting the endothelium of vessels lining a fibrous septum (*) running through the node. C&D (100X magnification) reveals the lack of PSMA staining seen in a metastasis with spindled morphology. At low magnification (E&F, 20X magnification), metastases (solid arrows) can be seen to stain with PSMA at much greater intensity than the surrounding lymph node (open arrows). At higher magnification (G&H, 100X magnification) within a metastatic deposit, PSMA can be seen to stain the individual vessels traversing the metastasis.

Fig 3. Western blot of four human melanoma cell lines (left 4 columns) and a prostate cancer lymph node lysate (right column), with loading control (bottom row). This suggests PSMA expression is in the stroma of the melanoma metastases rather than the malignant cells.

Fig 4 – Multiplex immunofluorescence (20X magnification) of melanoma metastases in disease regions (A&B) and control regions (C&D). Anti-PSMA antibody (red stain) can be seen highlighting the blood vessels (solid arrows, A&B) in tumor regions. There is variable staining in the melanocytes, with some staining strongly (A) and others not staining (B). The control region shows staining in the blood vessels (open arrows, C&D) but at a much weaker intensity than in the disease region.