APMIS
ACTA PATHOLOGICA, MICROBIOLOGICA ET IMMUNOLOGICA SCANDINAVICA

APMIS 122: 482–489

DOI 10.1111/apm.12195

Prostate-specific membrane antigen expression in tumor-associated vasculature of breast cancers

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Wernicke AG, Varma S, Greenwood EA, Christos PJ, Chao KSC, Liu HE, Bander NH, Shin SJ. Prostate-specific membrane antigen expression in tumor-associated vasculature of breast cancers. APMIS 2014; 122: 482–489.

Prostate-specific membrane antigen (PSMA) has been found to be expressed in the tumor-associated neovasculature of multiple solid tumor types including breast cancers. However, thus far, the number of cases studied from some tumor types has been limited. In this study, we set out to assess PSMA expression in the tumor-associated vasculature associated with invasive breast carcinomas in a sizable cohort of patients. One hundred and six patients with AJCC stage 0-IV breast cancer were identified. Ninety-two of these patients had primary breast cancer [invasive breast carcinoma with or without co-existing ductal carcinoma *in situ* (DCIS) (74) or DCIS alone (18)]. In addition, 14 patients with breast cancer metastases to the brain were identified. Immunohistochemical staining for PSMA and CD31 was performed on parallel representative tumor sections in each case. Tumor-associated vascular endothelial cell PSMA immunoreactivity was semi-quantitatively assessed based on two parameters: overall percent of endothelial positivity and staining intensity. PSMA expression for tumor-associated vascular endothelial cells was scored 0 if there was no detectable PSMA expression, 1 if PSMA staining was detectable in 5–50%, and 2 if PSMA expression was positive in >50% of microvessels. CD 31 staining was concurrently reviewed to confirm the presence of vasculature in each case. Tumor-associated vasculature was PSMA-positive in 68/92 (74%) of primary breast cancers and in 14/14 (100%) of breast cancers metastatic to brain. PSMA was not detected in normal breast tissue or carcinoma cells. All but 2 cases (98%) showed absence of PSMA expression in normal breast tissue-associated vasculature. The 10-year overall survival was 88.7% (95% CI = 80.0%, 93.8%) in patients without brain metastases. When overall survival (OS) was stratified based on PSMA score group, patients with PSMA scores of 0, 1, and 2 had 10-year OS of 95.8%, 96.0%, and 79.7%, respectively ($p = 0.12$). When PSMA scores of 0 and 1 were compared with 2, there was a statistically significant difference in OS (96.0% vs 79.7%, respectively, $p = 0.05$). Patients with a PSMA score of 2 had a significantly higher median tumor size compared with patients in the lower PSMA score groups ($p = 0.04$). Patients with higher nuclear grade were more likely to have a PSMA score of 2 compared with patients with lower nuclear grade ($p < 0.0001$). Patients with a PSMA score of 2 had a significantly higher median Ki-67 proliferation index compared with patients in the lower PSMA score groups ($p < 0.0001$). Patients with estrogen receptor (ER)-negative tumors were more likely to have a PSMA score of 2 compared with patients with ER-positive tumors ($p < 0.0001$). Patients with progesterone receptor (PR)-negative tumors were more likely to have a PSMA score of 2 compared with patients with PR-positive tumors ($p = 0.03$). No significant association was observed between PSMA score group status and lymph node involvement ($p = 0.95$). Too little variability was present in Human epidermal growth factor receptor-2 (Her2/neu) amplified tumors to correlate with PSMA score group status. To date, this is the first detailed assessment of PSMA expression in the tumor-associated vasculature of primary and metastatic breast carcinomas. Further studies are needed to evaluate whether PSMA has diagnostic and/or potential therapeutic value.

Key words: Prostate-specific membrane antigen; breast cancer; anti-angiogenesis.

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Received 8 February 2013. Accepted 30 July 2013

Selective inhibitory targeting of angiogenesis either by interfering with angiogenic growth factors or by directly targeting tumor-associated blood vessels is an important strategy in the treatment of cancers (1). Exploiting this mechanism implies that endothelial cells comprising the (neo)vasculature of tumors demonstrate altered protein expression patterns relative to those of normal endothelium (1–3). As such, proteins selectively expressed in tumor-associated endothelial cells represent candidate targets for tumor-specific anti-angiogenic cancer therapy.

Prostate-specific membrane antigen (PSMA, folate hydrolase 1, glutamate carboxypetidase II), a zinc-dependent exopeptidase, is predominantly expressed in the prostate gland (4) where low levels are present in the normal prostatic secretory epithelium and significantly higher levels have been detected in prostatic adenocarcinomas (5). In recent years, several approaches targeting PSMA have been developed for diagnostic and therapeutic purposes (6–12). In the setting of metastatic prostate cancer, two clinical trials using a radiolabeled monoclonal antibody against PSMA have supported the potential of targeting PSMA (10, 11).

The extracellular domain of PSMA not only serves as a promising therapeutic target (13) in prostate cancer, but additionally, PSMA has been found to be selectively expressed in the vasculature of other solid tumors, but not in normal tissue vasculature (12, 14–16). These findings suggest that PSMA may have broader potential as a tumor-associated (neo-) vascular target in addition to one that is tumor-specific (prostate). Successful targeting of metastatic tumor sites in two clinical trials in patients with various solid tumor malignancies was accomplished using a humanized anti-PSMA antibody (J591), thus, demonstrating proof-of-principle that PSMA allows targeting of the neovasculature in solid tumors in addition to prostatic adenocarcinoma (17, 18).

To date, with the exception of GI (16) and oral cavity cancers, PSMA expression in the neovasculature of solid tumors has been limited in both the range of tumor types studied as well as small cohort sizes (12–16). Microvessel density is a well-characterized surrogate marker for angiogenesis/neovascularization in tumors; and has been shown to negatively correlate with prognosis in many studies (19, 20). We set out to assess PSMA expression in the tumoral microvessels in a large cohort of patients with primary and metastatic breast carcinomas to evaluate the diagnostic and/or therapeutic potential of PSMA in these patients.

MATERIALS AND METHODS

Patients' tumors specimens

After Institutional Review Board approval, 92 patients who had undergone surgical resection of their breast cancers were identified. Representative formalin-fixed paraffin-embedded tumor blocks from these specimens were obtained from the Department of Pathology and Laboratory Medicine of Weill Cornell Medical Center/New York Presbyterian Hospital. In addition, formalin-fixed paraffin-embedded tissue from brain metastases of 14 patients with breast cancer were obtained. Clinical and pathologic characteristics of all patients are summarized in Table 1. Primary breast tumor grade was assigned by applying the 'Elston-Ellis modified Scarff-Bloom-Richardson grading system' used in routine clinical practice as recommended by the College of American Pathologists (CAP) and American Joint Committee on Cancer (AJCC) (21). The proliferation index using Ki-67 immunostaining was scored as <10%, 10–14%, and >14% based on the percentage of tumor cells showing nuclear reactivity for Ki-67. These cut-off points have been shown to be prognostically significant in large landmark studies and are used in routine clinical practice to categorize the proliferation index (22). The 7th edition of AJCC staging manual was used to assign the pathological tumor stage (23).

Immunohistochemistry

Four-μm-thick tissue sections from representative tumor blocks were mounted on glass slides, deparaffinized by Histo-Clear (National Diagnostics, Atlanta, GA, USA), and rehydrated. Parallel slides from each case were stained with anti-PSMA and anti-CD31 as follows. For CD31, antigen retrieval was accomplished by pressure-cooking the slides for 1 min in 10 mmol/L citrate buffer (pH 6.0); for PSMA, slides were incubated in Target Retrieval Solution (pH 9.0; DAKO, Carpinteria, CA, USA) at 95 °C for 30 min. Endogenous peroxidase activity was blocked with Peroxidase Block (Envision System kit; DAKO) for 5 min. Slides were then incubated for 60 min with either anti-CD31 (clone 1A10; Novocastra/Vision BioSystems, Inc, Norwell, MA, USA) or anti-PSMA antibody (clone 3E6; DAKO) diluted 1:25 and 1:20, respectively, in Antibody Diluent (DAKO). Slides were then further treated with anti-mouse HRP polymer-labeled secondary antibodies for 60 min followed by 8 min in diaminobenzidine (DAB) solution (Envision System HRP; DAKO). Lastly, slides were counterstained with Harries Modified Hematoxylin (Fisher Scientific, Pittsburgh, PA; USA). In all assays, negative control slides were incubated with isotype-matched immunoglobulin. Positive controls consisted of prostatic adenocarcinoma specimens and tonsillar tissue for PSMA and CD31 staining, respectively.

Scoring

CD 31-stained slides were used in each case to confirm the presence of tumor-associated and non-tumor vasculature. Vascular endothelial cell immunoreactivity for PSMA was semi-quantitatively scored for both the intensity of staining and the proportion of tumor vessels that were positive.

Table 1. A summary of patient and tumor characteristics

	Number	Percentage
Total number	106	100
Primary	92	87
Metastases	14	13
Median age, at diagnosis years (range)	57 (32–92)	
Median size of invasive tumor (cm)	1.2 (0.1–7.0)	
T stage (total number)	92	100
pTis	18	20
pT1mic	2	2
pT1a	11	12
pT1b	12	13
pT1c	35	38
pT2	13	14
pT3	1	1
N stage (total number)	92	100
pNx	18	20
pN0	52	57
pNmic	3	3
pN1	14	15
pN2	3	3
pN3	2	2
M stage (total number)	106	100
M0	92	87
M1	14	13
Histology of primary breast cancer	92	100
DCIS	18	20
IDC	64	70
ILC	10	10
Receptor status	106	100
ER+	94	89
ER−	12	11
PR+	81	76
PR−	25	24
Her2neu+	27	25
Her2neu−	79	75
Ki-67 Index (%)	106	100
≤14	65	61
>14	41	39
Lymphovascular invasion	106	100
Present	27	25
Absent	79[1]	75
Nuclear grade	106	100
1	30	29
2	47	44
3	29	27

DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma.
[1]Excludes DCIS cases.

Positive PSMA expression was defined as membranous with or without cytoplasmic staining of endothelial cells lining microvessels. The method by which sampling, identification and selection of vessels was performed was similar to that used in other studies (19). After scanning the entire H&E stained tumor section at low power (100×), 'hot-spots' with highest microvessel density were identified. PSMA staining in microvessesls was then evaluated at high magnification (200×) in at least three high power fields. The larger vessels were excluded. Small clusters of

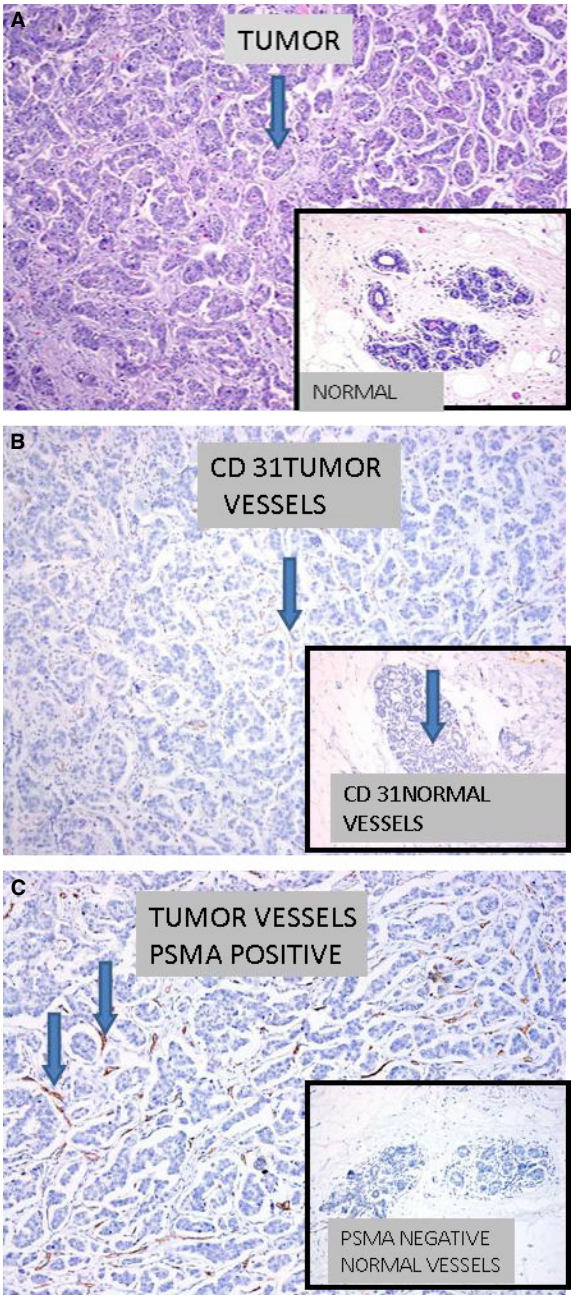

Fig. 1. PSMA expression in tumor-associated endothelium of primary invasive breast carcinoma. (A) H&E staining of the invasive breast cancer in a lumpectomy specimen, with the insert demonstrates normal breast tissue within the resected clear margin (an arrow points toward the vessel within the tumor). (B) CD31 staining demonstrates staining of the vasculature of the tumor and the normal endothelium in the breast tissue (arrows point to the CD31 staining of the vessels in both). (C) PSMA expression is demonstrated in the vasculature of the tumor only with the PSMA staining >50% (score 2) (arrows), but lack PSMA expression (score 0) is observed in normal breast tissue or its endothelial cells.

endothelial cells (even without a discernible lumen) were considered as a single microvessel. In the majority of cases, staining intensity directly correlated with extent of staining in tumor-associated vessels and as such, the score of each case was simplified as follows: tumors with no detectable endothelial PSMA expression were scored as '0'; tumors with PSMA staining in 5–50% of vessels were scored as '1'; and tumors that showed PSMA-positive staining in >50% of tumor-associated vessels were scored as '2'.

Statistical analysis

Statistical considerations – Descriptive statistics (including mean, standard deviation, median, frequency, percent) were calculated to characterize the study cohort. The ANOVA test was used to compare mean age among the three PSMA expression score groups (0, 1, and 2). The Kruskal–Wallis test was used to compare median tumor size and median Ki-67 proliferation index among the three PSMA expression score groups. The chi-square test was used to assess the association between PSMA expression score group and (i) histologic type, (ii) lymphovascular invasion, (iii) lymph node positivity, (iv) nuclear grade, (v) ER status, (vi) progesterone receptor [PR] status, and (vii) Human epidermal growth factor receptor-2 (Her2neu) status. Kaplan–Meier survival analysis was performed to calculate overall survival for the study cohort and the log-rank test was employed to compare overall survival among the three PSMA expression score groups. All p-values are two-sided with statistical significance evaluated at the 0.05 alpha level. Ninety-five percent confidence intervals (95% CI) for 10-year overall survival estimates were calculated to assess the precision of the obtained estimates. All analyses were performed in STATA Version 12.0 (StataCorp, College Station, TX, USA) and SPSS Version 20.0 (SPSS Inc., Chicago, IL, USA).

Table 2. Expression of PSMA in tumor-associated vasculature of breast cancer

	Total number	PSMA score (number per score group)
Primary breast cancer	92	0 = 24
		1 = 26
		2 = 42
Brain metastases from breast cancer	14	0 = 0
		1 = 0
		2 = 14

RESULTS

CD 31 positivity in vasculature was confirmed in all cases and was seen in both the tumor-associated and normal tissue-associated endothelial cells.

Primary breast carcinomas

Prostate-specific membrane antigen positivity was restricted to tumor-associated vasculature in 90 of 92 cases of invasive breast cancers (98%) (Fig. 1). In the remaining two cases, weak staining of a sub-

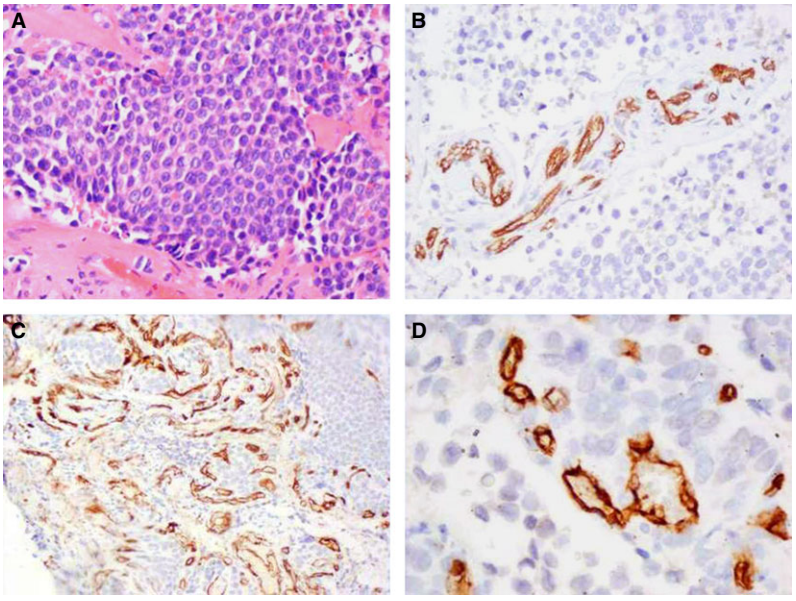

Fig. 2. PSMA expression in tumor-associated endothelium of metastatic breast cancer to the brain. (A) H&E staining of the resected brain metastasis. (B) CD31 staining demonstrates staining of the vasculature of the tumor. (C) PSMA expression in the vasculature of the tumor. (D) High power demonstration of the PSMA expression in the endothelial vessels of the brain metastasis.

set of normal tissue-associated vasculature by PSMA was also seen in addition to tumor-associated vasculature.

Normal glandular breast tissue and carcinoma cells were PSMA-negative in all studied cases. PSMA staining was not identified in any CD31-negative vessels within the tumor. The PSMA expression score ranged from 0 to 2 (Table 2). In 24/92 (26%) cases, no PSMA was detectable (score 0). The remaining 68/92 (74%) of tumors stained positive for PSMA to a varying degree (scores 1 and 2). No statistically significant association was observed between PSMA score group status and histologic type (invasive ductal carcinoma (IDC) vs invasive lobular carcinoma (ILC), p = 0.34). Similarly, patient age (p = 0.30) or presence of lymphovascular invasion (p = 0.31) was not associated with the increasing PSMA score group.

Brain metastases secondary to invasive breast carcinomas

All 14 breast cancers metastatic to brain demonstrated PSMA expression (Fig. 2). There were no PSMA scores 0 or 1, resulting in a PSMA score 2 in all 14 cases (Table 2). Of interest, 10 of the cases of metastases were compared with their respective primary breast carcinomas. In each of these cases, the metastatic lesions had the same PSMA expression score (i.e., 2) as their respective primary tumors.

The 10-year overall survival in this series of patients was 88.7% (95% CI = 80.0%, 93.8%) in patients without brain metastases (Fig. 3A). When the survival was stratified based on the PSMA score, patients with a score of 0 had a 10-year OS of 95.8% (95% CI = 73.9%, 99.4%), patients with a score of 1 had a 10-year OS of 96.0% (95% CI = 74.8%, 99.4%), and patients with a score of 2 had a 10-year OS of 79.7% (95% CI = 63.4%, 89.3%) (P = 0.12 by log-rank test) (Fig. 3B). When the OS was analyzed based on less than 50% staining vs more, patients with PSMA scores of 0 and 1 (50 patients, 6 deaths) had a 10-year OS of 96.0% (95% CI = 84.8%, 99.0%) as compared with the patients with a PSMA score of 2 (42 patients, 11 deaths) whose 10-year OS was 79.7% (95% CI = 63.4%, 89.3%) (Fig. 2C) (p = 0.05 by log-rank test).

PSMA expression was examined in relation to clinicopathological parameters in invasive breast carcinomas **(Table** 3**)**. Patients with a PSMA score of 2 had a significantly higher median tumor size compared with patients in the lower PSMA score groups (p = 0.04). Patients with higher nuclear grade were more likely to have a PSMA score of 2 compared with patients with lower nuclear grade

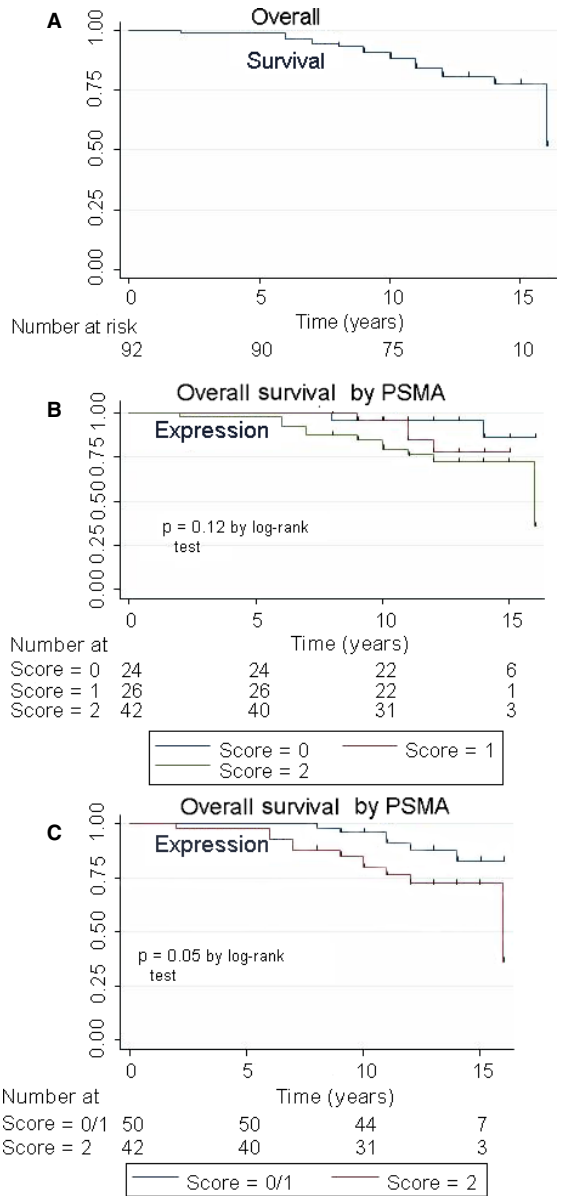

Fig. 3. Kaplan–Meier Survival Curves (A) Kaplan–Meier survival curve showing cumulative overall survival for patients with primary breast cancer (excluding brain metastases). (B) Kaplan–Meier survival curve showing overall survival for patients with based on a PSMA score. **(C)** Kaplan–Meier survival curve showing overall survival for patients with based on a PSMA score, when grouped by scores 0 and 1 (PSMA staining <50%) vs 2 (PSMA staining >50%).

(p < 0.0001). Patients with a PSMA score of 2 had a significantly higher median Ki-67 proliferation index (20%) compared with patients in the lower PSMA score groups (10% for scores of 0 and 1 each), (p < 0.0001). Patients with ER-negative

Table 3. Relationship of PSMA expression to clinicopathological parameters in invasive breast carcinomas

Tumor Characteristics	PSMA score 0 (N) 24	PSMA score 1 (N) 26	PSMA score 2 (N) 42	p-value
Median tumor size (cm)	1.00	0.95	1.4	0.04
Nuclear grade (% of total cases)	Grade 1 = 15 (63%) Grade 2 = 8 (33%) Grade 3 = 1 (4%)	Grade 1 = 13 (50%) Grade 2 = 10 (39%) Grade 3 = 3 (11%)	Grade 1 = 2 (5%) Grade 2 = 21 (50%) Grade 3 = 19 (45%)	< 0.0001
Lymph node Involvement (% of total cases)	3 (14%)	10 (45%)	9 (41%)	0.95
ER -negative tumors (% of total cases)	2 (8%)	0 (0%)	21 (50%)	< 0.0001
PR-negative tumors (% of total cases)	6 (25%)	9 (35%)	23 (55%)	0.03
Her2neu-expressing tumors (% of total cases)	0 = 3 (13%) 1+ = 2 (8%) 2+ = 18 (75%) 3+ = 1(4%)	0 = 0 (0%) 1+ = 1 (4%) 2+ = 23 (89%) 3+ = 2 (7%)	0 = 2 (5%) 1+ = 6 (14%) 2+ = 30 (71%) 3+ = 4 (10%)	0.35
Ki 67 index by increasing PSMA score (% of total cases)				< 0.0001
Ki67 score <10%	4 (16%)	9 (35%)	4 (10%)	
Ki67 score 10–14%	17 (71%)	12 (46%)	12 (29%)	
Ki67 score >14%	3 (13%) Median – 10%	5 (19%) Median – 10%	25 (61%) Median – 20%	

tumors were more likely to have a PSMA score of 2 compared with patients with ER-positive tumors ($p < 0.0001$). Patients with PR-negative tumors were more likely to have a PSMA score of 2 compared with patients with PR-positive tumors ($p = 0.03$). No significant association was observed between PSMA score group status and lymph node involvement ($p = 0.95$). Too little variability was present in Her2neu-amplified tumors to correlate with PSMA score group status.

DISCUSSION

Treatment of solid tumors with anti-angiogenic agents has become an established paradigm in cancer therapy (1, 24–26). The search for novel molecular targets has led to the discovery of PSMA as a neovascular target across a broad range of tumor types; however, studies of neovascular PSMA expression have been limited in tumor type and case number (14–16).

Neovascular PSMA expression levels appear to differ among different tumor types (27, 28). Our data indicate that PSMA expression in the neovasculature of primary breast cancers (74%) as well as (distant) metastatic disease (100%) is largely restricted to endothelial cells within the pathologically defined tumor area. Consistent with prior experience in gastric, colorectal (16) and head and neck cancer, there was no PSMA immunoreactivity in either normal breast tissue or carcinoma cells and only rarely in normal tissue-associated vasculature, albeit weak and focal. Furthermore, invasive breast carcinomas with unfavorable clinicopathologic features such as larger tumor size, higher nuclear grade, ER/PR negativity, and high Ki-67 index demonstrated significantly higher PSMA expression than cancers lacking these features. If this finding is validated by other studies, PSMA expression levels may provide helpful prognostic information in the future aiding the selection of patients for adjuvant systemic therapies.

We found high PSMA expression in brain metastases from primary breast carcinomas as well as in some primary invasive breast carcinomas (Table 2). The identical pattern of PSMA expression in 10 primary carcinomas and metastatic lesions from the same patient suggest that high PSMA expression may be conserved throughout cancer progression including distant metastases. Although assessed in a limited number of cases, if confirmed, the similarity of pattern of PSMA expression between the primary and metastatic sites should allow PSMA typing of the metastatic tumor vasculature by evaluating PSMA expression at the primary site. Such an approach may aid in selecting high PSMA-expressing tumors for PSMA-directed therapy. Haffner et al. demonstrated a similar correlation of patterns in the neovasculatures of gastrointestinal malignancies examined for PSMA expression (16).

Recently, an *in vitro* model of PSMA expression by primary human umbilical vein endothelial cells (HUVECs) cultured in Matrigel and induced by tumor-conditioned medium (TCM) derived from human breast cancer cells (MDA-MB-231) was described (29). In contrast to vascular endothelial growth factor (VEGF)-containing endothelial cell medium, TCM induced higher expression of PSMA in HUVECs. This *in vitro* model may enable studies

aimed at understanding the mechanism of PSMA expression in breast and other cancers (29).

We saw no significant difference between the PSMA score and overall survival in our study (Fig. 3B). However, when we examined OS based on ≤50% or >50% PSMA expression, we did see a statistically significant difference (Fig. 3C). Because there were only 17 deaths in this series, that probably precluded an adequate multivariate analysis.

A variety of methodologies have been developed to target PSMA - monoclonal antibodies (13, 30), peptides (6), RNA aptamers (8), vaccines (9), and small molecules (7, 31). Using these approaches for imaging and therapy of solid tumors would open new avenues in anti-angiogenic therapy. This molecule has already been studied widely as a target in metastatic prostate cancer (13, 30, 32). Two recent studies using a 111. In-labeled monoclonal antibody J591 against PSMA in patients with multiple solid tumor types demonstrated targeting of tumor sites and provided clinical evidence that an antibody-based approach against PSMA can be exploited for vascular targeting therapies (17, 18). Selection of patients most likely to benefit, that is, those with high PSMA expression, could be accomplished by immunohistochemical typing of a tumor specimen or by a PSMA imaging study.

CONCLUSION

This study provides evidence that PSMA is expressed in the tumor-associated vasculature of primary breast cancers and distant (brain) metastases. The use of PSMA targeting approaches in primary and metastatic breast cancers may therefore represent an alternative or adjunct to currently available treatment strategies.

CONFLICTS OF INTEREST

None.

This project was supported by the David H. Koch Foundation and the Robert Dow Philanthropic Fund. Dr. Paul Christos was partially supported by the following grant: Clinical Translational Science Center (CTSC) (2UL1TR000457-06).

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