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ORIGINAL ARTICLE

The role of extracellular matrix metalloproteinase inducer protein in prostate cancer progression

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Abstract Extracellular matrix metalloproteinase inducer (EMMPRIN/CD147) is a multifunctional membrane glycoprotein overexpressed in many solid tumors, and involved in tumor invasion and angiogenesis. We investigated EMMPRIN expression in human prostate cancer (CaP) tissues and cells, and evaluated whether EMMPRIN expression is related to tumor progression and matrix metalloproteinase (MMPs) expression in human CaP. An immunohistochemical study using tissue microarrays of 120 primary CaPs of different grades and 20 matched lymph node metastases from untreated patients was performed. The association of EMMPRIN expression with clinicopathological parameters was evaluated. Co-immunolocalization for EMMPRIN and MMP-1, MMP-2 or MMP-9 in primary tumors was examined using confocal microscopy. Flow cytometry and immunoblotting were used to examine EMMPRIN expression in 11 metastatic CaP cell lines. Heterogeneous expression of EMMPRIN was found in 78/120 (65%) CaPs, correlated significantly with progression parameters including pretreatment PSA level ($P < 0.05$) and increased with progression of CaP (Gleason score, $P < 0.05$; pathological stage, $P < 0.01$; nodal involvement, $P < 0.05$ and surgical margin, $P < 0.05$). Heterogeneous cytoplasmic MMP-1, MMP-2 and MMP-9 associated with EMMPRIN immunolabeling was observed, particularly in tumors with Gleason scores >3 + 4. Metastatic CaP cell lines, except DuCaP, expressed abundant EMMPRIN protein, indicating highly (~45 to ~65 kDa) and less (~30 kDa) glycosylated forms, although with no relationship to cells being either androgen responsive or nonresponsive. Our results suggest that EMMPRIN may regulate MMPs and be involved in CaP progression, and as such, could provide a target for treating metastatic CaP disease.

Keywords CD147/EMMPRIN · Matrix metalloproteinase · Prostate cancer · Metastasis · Tumor-associated antigen

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Introduction

Prostate cancer (CaP) is a major health problem for Western men. Despite current therapies, many patients develop metastases. Progression of CaP from androgen-dependent to hormone-refractory disease is often accompanied by lymph or blood-borne dissemination of malignant cancer cells and micrometastases that spread to the bone.

Degradation of extracellular matrix (ECM) surrounding primary tumors and metastases is critical for invasion and metastasis of epithelial tumor cells [1]. The zinc-dependent endopeptidases, matrix metalloproteinases (MMPs) can degrade ECM, and are involved in cancer invasion, metastasis and angiogenesis [2]. Some MMPs may also protect against tumor growth and metastases (reviewed [3]). MMP expression by stromal [4] and endothelial cells [5] can be regulated by various soluble or cell-bound factors including extracellular MMP inducer protein (EMMPRIN, CD147, M6, Basigin), a transmembrane glycoprotein, and member of the immunoglobulin (Ig) superfamily [6, 7]. EMMPRIN can stimulate MMP expression by tumour cells, stromal fibroblasts and endothelial cells [8, 9] through cell-to-cell contact or by shedding of EMMPRIN vesicles [6, 7]. MMPs can also solubilize cell-associated EMMPRIN, enhancing its biological activity by circumventing cell contact requirements [10]. EMMPRIN stimulates the expression of MMP-1 (collagenase), MMP-2 (gelatinase A) and MMP-3 (stromelysin) in endothelial cells [11, 12], and induces their expression, together with MT1-MMP (membrane type-MMP), in fibroblasts [13]. EMMPRIN may thus induce tumor invasion/metastasis by activating MMP production and modulating cell-substrate and adhesion processes [14].

Heterogeneous EMMPRIN expression has been described in tissue microarrays (TMAs) of human normal prostate and CaPs, [44.7% + untreated CaP ($n = 30$) and 66.7% + hormone-refractory prostate cancers (HRPC) ($n = 38$)] [15], however there is no report of its distribution in benign prostate tissues, metastatic lesions, CaP cell lines, and different cancer grades, or of the relationship between EMMPRIN and MMP expression in CaP.

We examined EMMPRIN expression in specimens from patients with primary untreated CaP of different grades and matched lymph node (LN) metastases using TMAs and whole sections. Metastatic CaP cell lines were studied for EMMPRIN protein expression using flow cytometry and Western blotting; highly glycosylated forms of EMMPRIN have been associated with MMP activation [16]. Our results suggest that EMMPRIN is important in CaP progression and dissemination, and may have potential as a therapeutic target.

Materials and methods

Tissues/clinical data

As described previously [17], 120 CaP tissues were obtained with informed consent from patients with localized CaP undergoing radical resection of the prostate (RRP) or trans-urethral resection of the prostate (TURP) at Urology Sydney, St George Private Hospital, from 2000 to 2005. Controls ($n = 40$) were from normal biopsies or from morphologically normal areas of CaP tissue. Ethical approval was obtained from the South East Area Health Human Research Ethics Committee South Section. Specimens were grouped as: Group I, normal prostate glands from men (age <40 years, range 26–38, $n = 10$, and age >50 years, range = 55–83 years, $n = 10$), normal area of prostate glands from CaP patients (median age 67 years, range 62–84, $n = 20$) or benign prostate hyperplasia (BPH) (median age 61 years, range 53–72, $n = 15$); Group II, 120 CaP specimens (72 RRP, 48 TURP), containing prostatic intraepithelial neoplasia (PIN) ($n = 10$), Gleason <7 ($n = 30$), Gleason = 7 (3 + 4) ($n = 15$), Gleason = 7 (4 + 3) ($n = 20$), Gleason >7 ($n = 45$) median age 61 years (range 46–76) and Group III, CaP LN metastases from above primary RRP patients (median age 64 years, range 57–76, $n = 20$).

Formalin-fixed tissues were routinely processed, paraffin-embedded and H&E sections were reviewed. Tumor foci were identified, circled in ink, and graded (Gleason system). Pathological stage (RRP) was determined using the TNM system. Clinical data in RRP patients ($n = 72$): average age at surgery, 63 years (range 49–72); medium follow-up, 18 (2–50) months. A detectable level of PSA (>0.2 ng/mL) following surgery was defined as biochemical recurrence [18]. Pertinent clinical information (pretreatment PSA level, Gleason score, clinical stage, surgical margin status, assessment by clinic visit, phone, or e-mail contact to determine overall, cancer-specific, and recurrence-free survival) was recorded. All patients were advised to undergo a serum PSA test twice/year.

TMAs

TMAs were constructed (see [17]), with three tissue cores (diameter 1.0 mm)/donor block within the marked areas being arrayed into a recipient paraffin block (35 × 20 mm) of the semi-automated Beecher Instruments (Silver Springs, MD USA). Sections (5-μm) were cut, collected on Superfrost Plus slides (Lomb Scientific, Australia) and H&E staining performed.

Antibodies

Rabbit anti-EMMPRIN (N-terminus) polyclonal antibody (PAb) (2 μg/ml, Zymed, San Francisco, USA), and mouse anti-EMMPRIN MAb (extrcellular portion) (4 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for immunhistochemistry, flow cytometry and Western blotting, respectively. Both EMMPRIN antibodies reacted specifically with human EMMPRIN, and reactivity had been further confirmed with human breast cancer BT-20

and colon cancer SW480 cell lines as specified by the manufacturers.

Other antibodies used were as follows: mouse anti-MMP-2 MAb (4 μg/ml; NeoMarkers, San Francisco, CA, USA); mouse anti-MMP-1 and MMP-9 MAbs (4 μg/mL, Oncogene Research Products, San Diego, CA, USA); mouse anti-GAPDH MAb (0.5 μg/ml Ambion Inc., Applied Biosystems, Foster City, CA, USA); Alexa Fluor-488 goat anti-mouse IgG and AlexaFluor-594 goat anti-rabbit IgG (Molecular Probes, Eugene, Oregon, USA); biotinylated donkey anti-rabbit Ig (GE Healthcare Pty Ltd., Australia); goat anti-mouse IgG and goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Upstate, Millipore Billerica, MA, USA); anti-mouse IgG_1 isotype control (Dako Glostrup, Denmark); rabbit immunoglobulins (Ig) and ExtrAvidin peroxidase conjugate (Sigma Aldrich Pty Ltd, St Louis, MO, USA).

Immunohistochemistry

For immunolabeling, paraffin-embedded TMAs or whole tumor sections were deparaffinized in xylene, followed by a graded series of alcohols (100, 95, 75 and 50%) and re-hydrated in phosphate buffered saline (PBS) (pH 7.4). Slides were immersed in 0.01 M ethylenediamine tetraacetic acid (EDTA) (pH 8.0) at 80°C for 15 min to enhance antigen retrieval, then rinsed in PBS.

For EMMPRIN immunostaining visualised using peroxidase and VectorNovaRed (Vector Labs. Pty Ltd, Australia), TMAs were initially treated with 5% hydrogen peroxide and rinsed in PBS. After blocking in 10% normal donkey serum (NDS) in PBS for 30 min, sections were incubated overnight at 4°C in rabbit anti-EMMPRIN PAb (2 μg/mL), washed with PBS, then incubated in biotinylated donkey anti-rabbit IgG (1:100) for 1 h at room temperature (RT), rinsed in PBS and then incubated in ExtrAvidin peroxidase (1:200). After rinsing in PBS, immunoreactivity was visualized using Vector NovaRED (red-brown positive cells). Sections were counterstained with hematoxylin. For a negative control, nonspecific rabbit Ig (2 μg/mL) was used.

For co-immunolabeling with EMMPRIN and MMP-1, -2 or -9 antibodies, sections were blocked with 10% normal goat serum (NGS) in PBS for 30 min at RT, then incubated overnight at 4°C in rabbit anti-EMMPRIN PAb and mouse anti-MMP-1, -2 or -9 MAb, respectively. After PBS washing, sections were incubated in goat anti-rabbit Alexa 594 (for EMMPRIN) and goat anti-mouse Alexa 488 (for MMPs) for 2 h at RT, and rinsed in PBS. Negative controls were treated identically, using nonspecific immunoglobulins (IgG_1 or rabbit Ig). Sections were examined using a Zeiss LSM 5 Pascal laser scanning confocal microscope and LSM 5 Pascal Image software. Multichannel excitation bleedthrough was minimized using fluorochromes with a large difference in peak excitation (488 and 594 nm, respectively). Emission bleedthrough was minimized by Multi-tracking, where signal crosstalk between neighboring channels was corrected by performing a sequential image capture routine. This process combined images acquired with a single excitation beam/single detection channel acquisition process. This methodology corrects for the effects of emission crosstalk.

Assessment of TMA immunostaining

TMA immunostaining was assessed for staining intensity (grades 0–3) using light microscopy (Leica microscope, Nussloch, Germany) and a 40× objective, and three cores/case were scored. The criteria used for assessment were as previously reported [18], where: 0 (negative, <25%); 1+ (weak, 25–50%); 2+ (moderate, 50–70%); 3+ (strong, >75%) of the tumor cells stained. This analysis is comparable with whole section analysis [19]. All cores (3/3) constituted positive staining for each case. Many tissues showed heterogeneity between cores; an average score was, determined from specimens scored blindly by two observers (MCM and YL), taken. Where discordant, differences were resolved by joint review after consulting with a third observer (WD).

Cell lines/culture

Androgen nonresponsive (PC-3, PC-3M, PC-3MM2, DU145, LNCaP-C4-2, LNCaP-C4-2B) and androgen responsive (DuCaP, LNCaP, LNCaP-C4, LNCaP-LN3, LNCaP-FGC) CaP cell lines from different sources were studied (Table 1) using tissue culture reagents supplemented with 10% (v/v) heated-inactivated fetal bovine serum (FBS) (Invitrogen Australia Pty Ltd, Melbourne, VIC, Australia), unless otherwise stated. PC-3, PC-3M, PC-3MM2, DU145 and LNCaP-FGC cells were cultured in RPMI-1640; LNCaP-LN3 cells in 1:1 RPMI-1640:F12-K; LNCaP, LNCaP-C4, LNCaP-C4-2 and LNCaP-C4-2B cells in T-medium [20]; and DuCaP cells in DMEM. All were grown in a humidified incubator at 37°C/5% CO_2. After 48 h of culture, sub-confluent cells were raised twice with Dulbecco's phosphate-buffer saline (DPBS) (pH 7.2), detached with 0.25% trypsin/0.05% EDTA in PBS at 37°C, collected and resuspended in buffer (see below).

Flow cytometry

Cell-surface EMMPRIN expression was detected by indirect immunofluorescence staining on ice using cold DPBS with 5% FBS as diluent and for washing. Cells ($0.5–1.0 \times 10^6$) were washed twice (200 g, 5 min), incubated

Table 1 Characterisitics of CaP cell lines

Cell line	Site of origin	Source	Androgen response
PC-3	Bone (human)	ATCC-CRL-1435	N
PC-3M	PC-3 subline (mouse liver metastases following intrasplenic implantation of PC-3)	MD Anderson Hospital, Austin, TX, USA	N
PC-3MM2	PC-3 subline (mouse bone metastases following intracardiac injection of a PC-3M subline)	MD Anderson Hospital, Austin, TX, USA	N
DU145	Brain (human)	ATCC	N
DuCaP	Dura mater (human)	Hallym University, Seoul, Korea	R
LNCaP	Lymph node (human)	Leland Chung, Emory University, GE, USA	R
LNCaP-C4	Co-inoculation in mice of LNCaP and fibroblasts from a human osteosarcoma	UroCor, Inc.	R
LNCaP-C4-2	Growth in castrated mice of C4 cells	UroCor, Inc.	N
LNCaP-C4-2B	Bone metastasis in castrated mice following orthotopic implantation of C4-2	Leland Chung, Emory University, GE, USA	N
LNCaP-FGC	LNCaP subline	ATCC	R
LNCaP-LN3	Lymph node (mouse)	MD Anderson Hospital, Austin TX, USA	R

N nonresponsive to androgen, *R* responsive to androgen

with mouse anti-EMMPRIN MAb (4 μg/mL) or mouse IgG_1 isotype control (4 μg/mL) for 45 min on ice, then washed, resuspended and incubated in Alexa Fluor 488 goat anti-mouse (1:1,000) for 45 min in the dark, washed again, resuspended in 1 mL cold DPBS with 5% FBS and filtered through a 63 μm mesh before flow cytometry. Cells were analyzed on a Becton-Dickenson FACsCalibur (San Jose, CA, USA) flow cytometer collecting 10,000 events/sample; autofluorescence was subtracted. Data were analyzed using CELLQuest software (Becton-Dickinson).

Western blotting

After two washes in ice-cold PBS, CaP cells were lysed in mammalian cell lysis/extraction reagent (M-PER, Pierce) and lysates were cleared by centrifugation (5 min, at 14,000*g*, 4°C). Total protein was determined using a DC Protein Assay (Bio-Rad, Hercules, CA, USA) and samples were stored at −80°C. Equal loaded proteins (15 μg) were separated under reducing conditions on SDS-acrylamide gels (120 V, 3 h). Resolved proteins were transferred to Invitrolon 0.45 μm PVDF membrane (Invitrogen) in Tris-glycine/methanol buffer at 250 mA for 3 h, blocked with 5% skim milk powder in 0.05% Tween 20/Tris Buffered Saline (TTBS) at 4°C overnight, rinsed in TTBS, before incubating in rabbit anti-EMMPRIN PAb (Zymed, 2 μg/ml, N-terminus) or mouse anti-EMMPRIN MAb (Santa Cruz, 4 μg/ml, extracellular portion) at 4°C overnight, followed by HRP-conjugated secondary antibody (1:25,000 in 1%BSA/TTBS) for 2 h. After washing, visualization was with ECL (SuperSignal West Dura Extended Duration Substrate, Pierce) followed by exposure to X-OMAT film (Kodak) and photographic development. To confirm equal loading of protein lysates, immunoblots were stripped (Restore Western Blot Stripping Buffer, Pierce), re-probed using mouse anti-GAPDH MAb (0.5 μg/mL) then processed as above. Immunoblots were scanned and processed in Adobe Photoshop.

Statistical analysis

The Chi-squared test was used to assess for associations between EMMPRIN expression (staining score 0–3) and clinicopathological data for different groups of patients and between staining intensity for EMMPRIN in different prostate tissue types. All P values were 2-sided; $P < 0.05$ was considered significant. Analyses were performed using the GraphPad Prism 4.00 package (GraphPad, San Diego CA).

Results

EMMPRIN expression in prostate tissues

The TMAs contained 120 CaP tissues, 40 (33%) with a Gleason score ≤6 and 80 (67%) with a Gleason score ≥7. Scattered areas of weak (≤grade 1) heterogeneous epithelial cell membrane EMMPRIN immunostaining were observed in 5/20 (25%) normal prostates and 2/15 (13%) BPH specimens (Fig. 1a, b, low power; Fig. 2a, b, high

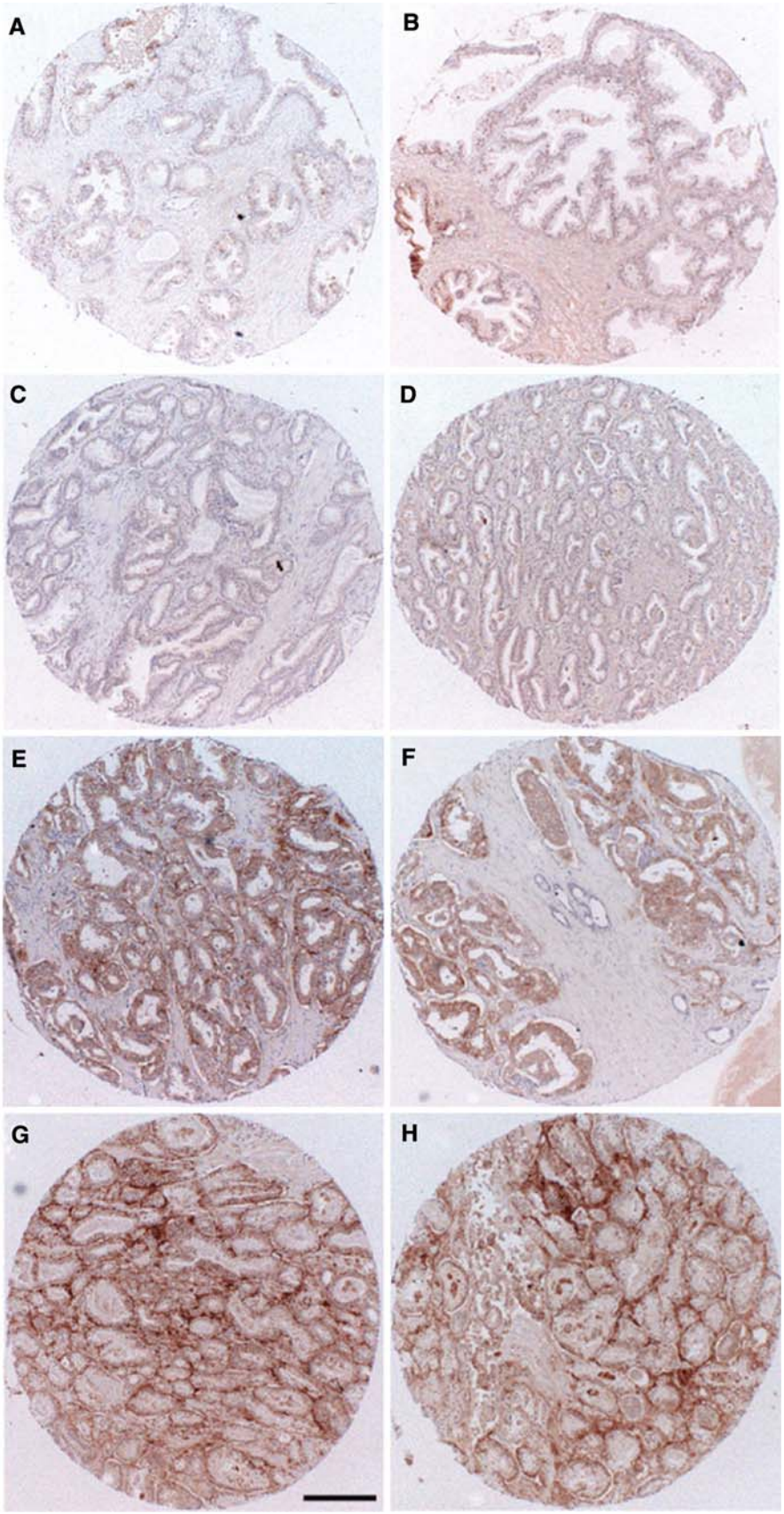

Fig. 1 Representative images from TMAs containing normal control prostate, BPH and CaP specimens showing different grades of EMMPRIN expression. **a**, **b** <Grade1 EMMPRIN with few areas of EMMPRIN-positive epithelial membrane staining (heterogeneous expression) in normal prostate and BPH specimens, respectively. **c**, **d** Weak EMMPRIN expression (grade 1) in CaP specimens with low Gleason scores (3 + 3). **e**, **f** Medium-level cell membrane EMMPRIN (grade 2) is clearly seen in CaP in regions of tumor with Gleason scores 3 + 4 and 3 + 3, respectively. Stromal regions do not appear EMMPRIN positive. **g**, **h** High-level EMMPRIN expression (grade 3) in CaP tissues with Gleason scores of 4 + 3 and 4 + 5, respectively. Tumor cells across the whole core are strongly EMMPRIN positive, with some evidence of stromal EMMPRIN expression. EMMPRIN-immunoreactive areas are *red/brown* in color. **a**–**h**, *Bar* 200 μm

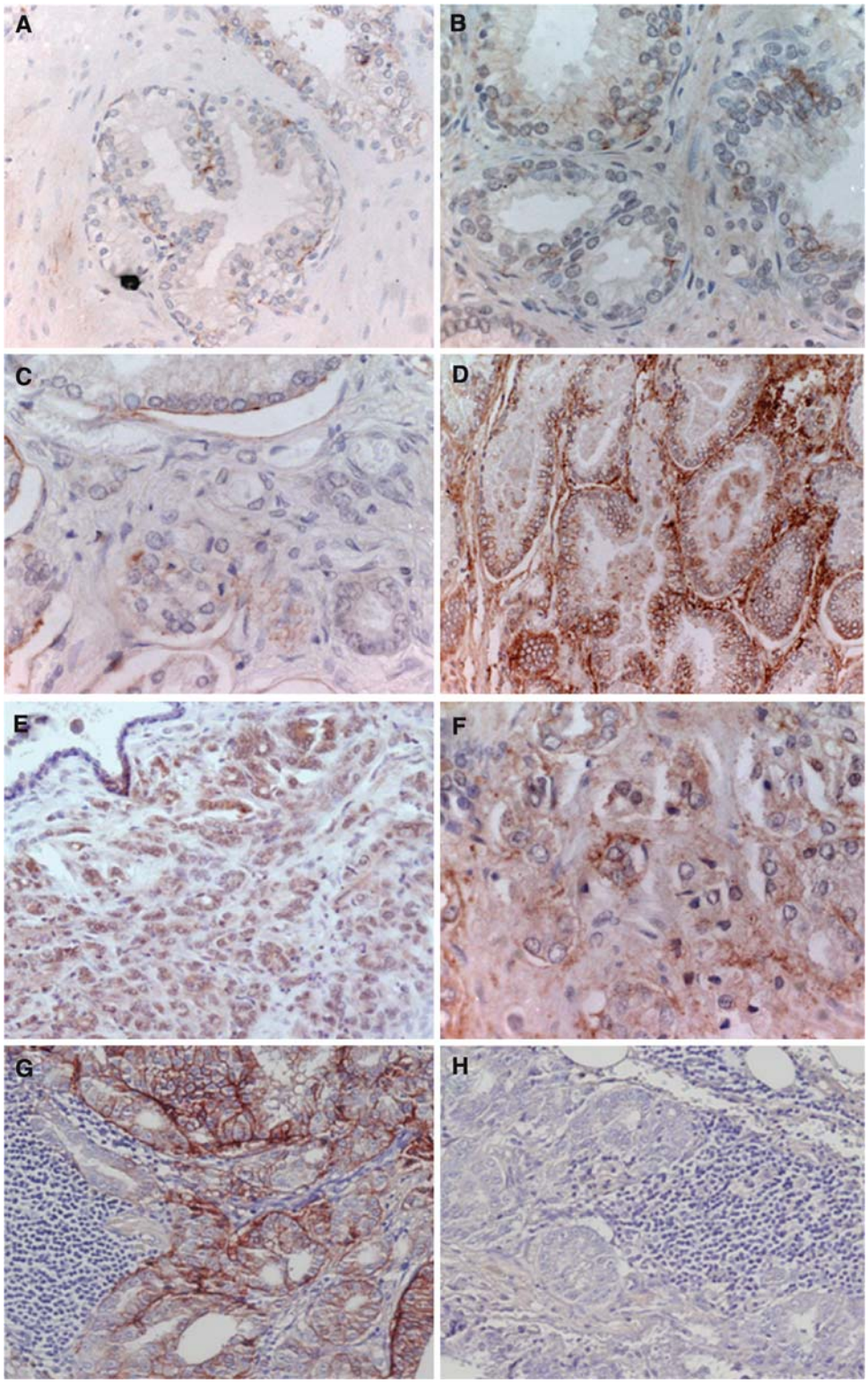

Fig. 2 Representative high magnification images of prostate TMAs showing EMMPRIN expression in control, BPH, CaP specimens and lymph node metastases. **a**, **b** Low-level EMMPRIN staining (<grade 1) in normal and BPH specimens, respectively, with few areas of EMMPRIN epithelial membrane immunostaining. Examples of grade 1–3 EMMPRIN immunostaining in CaP are shown in **c**–**f**. **c** Weak staining (grade1) in CaP tissues with Gleason score 3 + 3. **d** Strong cell membrane localization of EMMPRIN (grade 3) in CaP tissues with Gleason score 3 + 4. **e**, **f** Moderate staining of tumor cells in CaP tissues with Gleason scores 4 + 5 and 4 + 3, respectively. Stromal regions in **f** also show weak EMMPRIN immunostaining. **g** EMMPRIN expression in lymph node metastases with Gleason score 4 + 4; an area of lymphocytes is seen on the left of the figure. **h** A section from the same lymph node as (**g**) incubated in rabbit IgG shows no immunolabeling. (EMMPRIN-immunoreactive areas are *red/brown* in color)

power) whilst 78/120 (65%) CaP tissues were positive for EMMPRIN (1+ − 3+), of which 66/78 (85%) were high-grade tumors. For EMMPRIN positive primary CaP sections, heterogeneous weak (1+), moderate staining (2+) and strong staining (3+) were found in 40/120 (33%), 32/120 (27%) and 6/120 (5%), respectively; increased staining correlated with increasing grade of CaP ($P < 0.05$) (Figs. 1c–h, 2c–f). All PIN sections were negative for EMMPRIN (not shown). EMMPRIN expression was found in 4/20 (20%) LN metastases (Fig. 2g). Primary tumors from patients with EMMPRIN positive LN metastases, also expressed EMMPRIN.

Positive staining of EMMPRIN in higher grade CaPs was statistically increased over normal prostate tissues and low-grade tumors ($P < 0.01$), and was mainly cell membrane-associated, although cytoplasmic staining was observed. Tumor stroma stained positive for EMMPRIN in 17/80 (21%) CaP with Gleason score ≥7 but not in low Gleason score CaPs. No immunolabeling was seen in CaP sections incubated in rabbit Ig (Fig. 2h).

Correlation with clinical parameters

Of the 72 RRP patients, only 5/72(7%) relapsed with biochemical progression (PSA >0.4), and none died of CaP during the follow-up period. Median time to relapse was 38 (14–50) months. In this group, 29% of tumors were with Gleason score <7; 71% of tumors were with Gleason score ≥7. Of the tumors, 33% were small (pT1), 31% organ-confined (stage pT2) and 36% had extracapsular extension (stage pT3) (Table 2). Although individual CaPs from all stages or Gleason scores stained positive for EMMPRIN, marked differences between subgroups were observed. For statistical analysis, tumors were considered positive if any staining was detected. Overall, 68 (49/72) were EMMPRIN positive (Table 2). EMMPRIN staining in primary tumors was significantly correlated with clinicopathological parameters (Table 2) including pre-treatment PSA level ($P < 0.05$), and significantly increased with progression of CaP (Gleason score, $P < 0.05$; pathologic stage, $P < 0.01$; nodal involvement, $P < 0.05$; surgical margin, $P < 0.05$). There was no correlation between the expression of EMMPRIN and PSA-defined recurrence ($P > 0.05$).

Table 2 Clinicopathological characteristics and EMMPRIN expression in RRP patients ($n = 72$)

Variable %	EMMPRIN Positive (49/72) 68(%)	EMMPRIN Negative (23/72) 32(%)	*P* value
Pre-treatment			
PSA level (ng/ml)			
<10	21/40 (53)	19/40 (47)	**0.0001***
≥10	30/32 (94)	2/32 (6)	
Gleason Score			
<7	10/21 (48)	11/21 (52)	**0.0170***
≥7	39/51 (76)	12/51 (24)	
Pathologic stage			
pT1	10/24 (42)	14/24 (58)	**0.0311***
pT2	18/22 (82)	4/22 (18)	
pT3	21/26 (81)	5/26 (19)	
Nodal involvement			
No	39/52 (75)	13/52 (25)	**0.0422***
Yes	10/20 (50)	10/20 (50)	
Surgical margin			
Negative	29/49 (59)	20/49 (41)	**0.0184***
Positive	20/23 (87)	3/23 (13)	
PSA-defined recurrence			
No	45/67 (67)	22/67 (33)	0.5526
Yes	4/5 (80)	1/5 (20)	

Seventy-two radical resection of the prostate (RRP) patients were evaluated

PSA prostate-specific antigen, *PSA-defined recurrence* biochemical treatment failure after prostatectomy for clinically localized cancer defined at a PSA level >0.2 ng/ml

* $P < 0.05$ significant. Bold values indicate significant correlation

Co-immunolabeling of primary tumors with EMMPRIN and MMP-1, MMP-2 or MMP-9

Heterogeneous areas of EMMPRIN and MMP immunolabeling were found in normal, BPH, CaPs and LN specimens as shown in representative images (Fig. 3). EMMPRIN imunofluroescence labeling displayed a similar distribution to the immunoperoxidase staining seen in TMAs (cf. Figs. 1, 2), and was generally localized to epithelial cell membranes, although at lower levels and less frequently in normal (not shown) and BPH specimens (Fig. 3a). More obvious EMMPRIN immunolabeling of epithelial cell membranes was observed in CaP specimens (Fig. 3b–e). Pockets of distinct cytoplasmic MMP-2 immunostaining were seen in tumor cells and adjacent stromal cells in CaP (Fig. 3c) with vascular-associated cells in some regions; this was more obvious in CaPs with Gleason scores >7 (not shown). EMMPRIN and MMP-2 immunolabeling did not co-localize, but rather tumor and stromal cells were observed to express either protein. In some regions, adjacent tumor cells also expressed either EMMPRIN or MMP-2 (Fig. 3a–c).

MMP-1 and MMP-9 immunolabeling was generally seen in the cytoplasm of epithelial cells, being more widespread than MMP-2. Co-localization of EMMPRIN immunolabelling with MMP-1 and MMP-9 expression was observed in epithelial cells in some tumor regions (Fig. 3d, e), together with stromal MMP-9 expression (Fig. 3e).

EMMPRIN expression in metastatic CaP cell lines

Using flow cytometry, androgen nonresponsive (PC-3, PC-3M, PC-3MM2, DU145, LNCaP-C4-2, LNCaP-C4-2B) and androgen responsive (LNCaP, LNCaP-C4, LNCaP-LN3, LNCaP-FGC) CaP cell lines, except DuCaP, expressed high levels of EMMPRIN compared to the IgG isotype control (Fig. 4a) confirming observations of cell surface EMMPRIN/CD147 immunolabeling in primary human CaP.

Western blot analysis demonstrated abundant forms of EMMPRIN that were highly- (HG, ~45–65 kDa) and less-glycosylated (LG, ~30 kDa) in all cell lines except DuCaP (representative example, Fig. 4b).

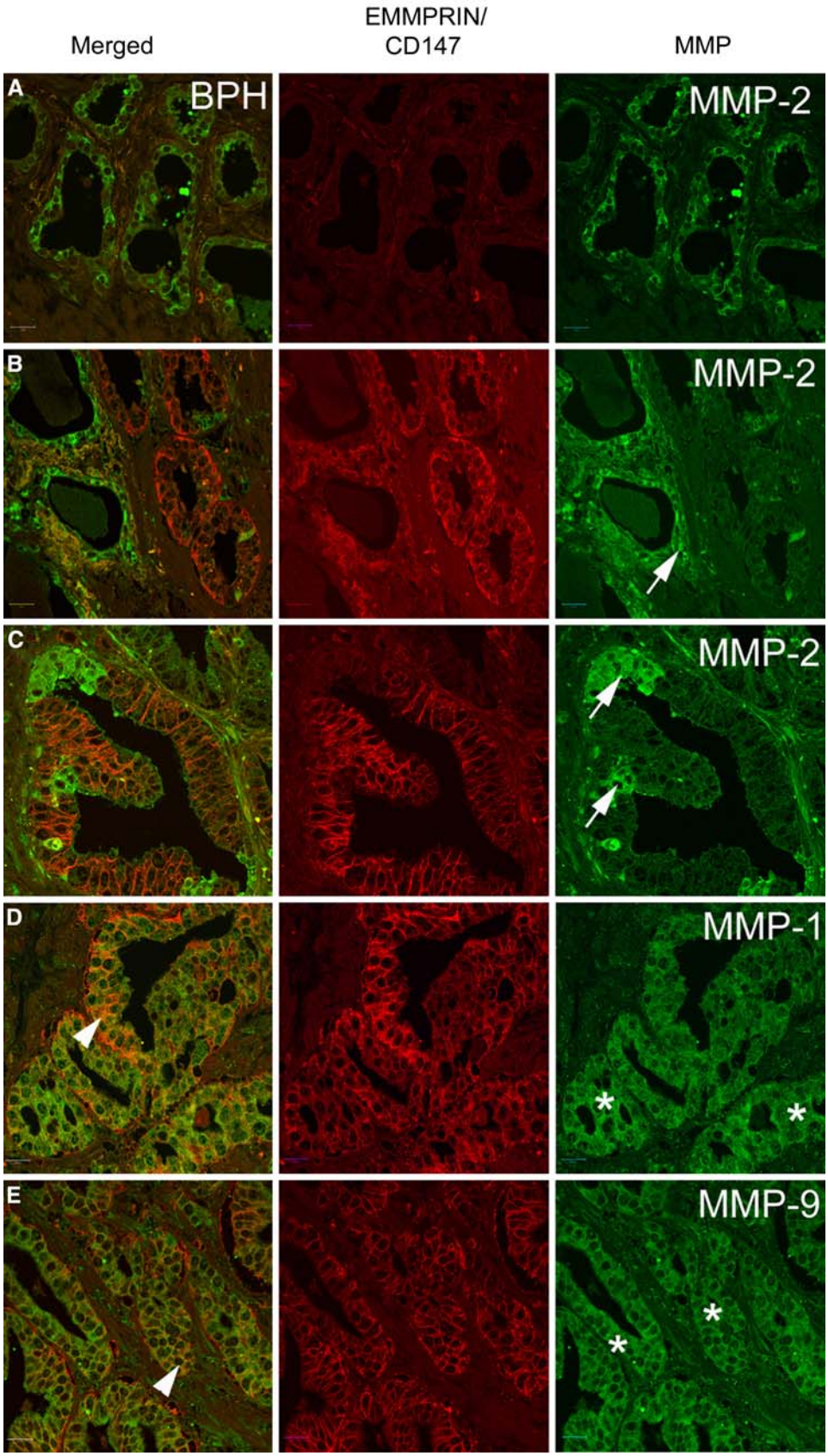

Fig. 3 Representative confocal images of EMMPRIN (*red*; Alexa-594) and MMP (*green*; Alexa-488) immunolabeling of paraffin-embedded sections of BPH and CaP specimens of varying Gleason scores. *Merged images*, *red* and *green* channels are shown separately. Heterogeneous areas of EMMPRIN and MMP immunolabeling are observed for normal, BPH, CaPs and lymph node specimens. **a** EMMPRIN immunolabeling is generally seen on epithelial cell membranes, at much lower levels and less frequently for BPH specimens (*middle panel*). BPH specimens also immunolabel for MMP-2 (*right panel*) in some areas. **b**, **c** EMMPRIN immunolabeling of epithelial cell membranes is clearly seen (*middle panel*), with MMP-2 immnostaining of tumor cells (*arrows*) and adjacent stromal cells (*right panels*). EMMPRIN and MMP-2 do not appear to be co-expressed (*left panel*, *merged*), but are rather seen in the adjacent areas of either tumor cells or stromal cells in CaP tissues with Gleason score 3 + 3 (**b**), or more closely apposed in CaP tissues with Gleason score 4 + 3 (**c**). **d**, **e** Tumor cells in CaP tissues with Gleason score 4 + 3 show more widespread cytoplasmic MMP-1 (*) (**d**) and MMP-9 (*) (**e**) immunolabeling, than for MMP-2. EMMPRIN immunostained tumor cells show evidence of co-immunolabeling with both MMP-1 and MMP-9 in some regions (*left panel*, *merged*, *arrowheads*). Some regions also show stromal expression of MMP-9 (**e**, *right panel*)

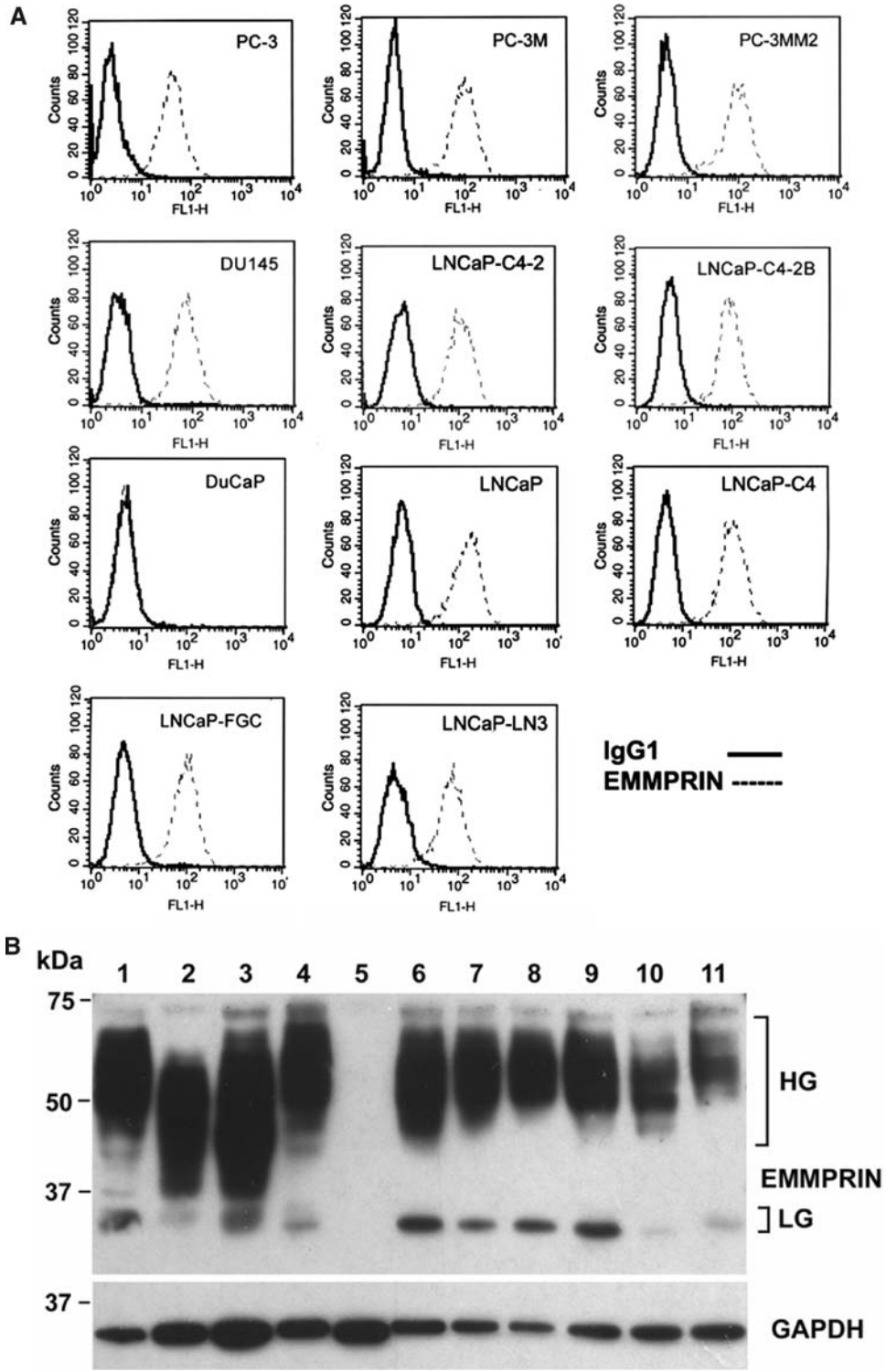

Fig. 4 EMMPRIN expression in 11 CaP cell lines detected by flow cytometry and Western blot. **a** Representative flow cytometry histograms showing high levels of EMMPRIN expression for all CaP cell lines, except DuCaP, which is similar to the IgG_1 isotype control. Data are presented as histograms, using mouse IgG_1 negative control to determine background fluorescence (*solid line*). The *dotted line* indicates EMMPRIN expression (*FL1-H* log fluorescence intensity). **b** Representative Western blot showing EMMPRIN expression in all CaP cell lines, except DuCaP (*lane 5*; *upper panel*). The positions of highly glycosylated (HG; ~45–65 kDa) and less glycosylated (LG; 30 kDa) EMMPRIN are indicated. Loading is demonstrated in the *lower panel*, with the membrane stripped and reprobed with a monoclonal antibody to GAPDH. Molecular mass markers (kDa) are shown on the *left*. (*Lanes*: *1* PC-3, *2* PC-3M, *3* PC-3MM2, *4* DU145, *5* DuCaP, *6* LNCaP, *7* LNCaP-C4, *8* LNCaP-C4-2, *9* LNCaP-C4-2B, *10* LNCaP-FGC, *11* LNCaP-LN3)

Discussion

CaP is the most commonly occurring cancer in men and advanced metastatic CaP is currently incurable. Identifying the factors that drive metastasis may offer insights into how to limit or prevent CaP progression. While several studies have shown that the presence and modulation of EMMPRIN may play important roles in normal physiological processes [21] and cancer progression [6, 7, 22], its expression and biological role in primary CaPs and in the metastatic microenvironment has not been fully investigated.

Using TMAs, we found that overexpression of EMMPRIN occurs in advanced CaP specimens but not in PIN, benign tissues, and normal prostates. Some LN metastases

and matched primary cancer tissues also expressed EMMPRIN. To our knowledge, this is the first report of EMMPRIN expression in different grades of CaP, and LN metastases, in a large set of human CaP samples. A previous report described strong EMMPRIN expression in normal columnar secretory prostate cells [15]. We observed low-level heterogeneous expression in normal prostate tissues compared to positive EMMPRIN expression in 49/72 (68%) of untreated CaPs. This difference in expression patterns may be related to the complexity of prostate tissues and differences in methodology including antibody specificity, selection and processing of specimens.

Elevated EMMPRIN expression has been shown to correlate with the progression of various malignancies including ovarian, esophageal squamous, breast, hepatocellular, cervical, and colorectal carcinomas [23–28]. Our study demonstrated that EMMPRIN expression in CaP correlated significantly with the preoperative PSA, histologic grade, clinical stage, nodal involvement and tumor margin. No difference in biochemical-free survival was found between the groups because of the short follow-up period in this patient cohort, with only 7% of patients experiencing biochemical progression. While a significant correlation between EMMPRIN and CaP development and metastasis was found, the potential causative role of EMMPRIN in these processes remains to be established.

Transcriptome analysis and comparative genomic hybridization of individual tumor cells isolated from bone marrow found that EMMPRIN was the most frequently expressed protein in primary tumors and micrometastases of CaP patients [29] We have also demonstrated overexpression of EMMPRIN in primary PC-3 xenografts, in local regional LN metastases in a NOD-SCID mouse model (unpublished data). Some studies suggest that EMMPRIN expression is associated with invasive capacity [14], and it has been observed in micrometastatic bone marrow deposits and malignant pleural effusions in breast cancer patients [30]. In a separate study, we have also found evidence of heterogeneous EMMPRIN immunolabeling of tumor cells near the edge of bone lesions in PC-3 tibial xenografts in a NOD-SCID mouse model (unpublished data). In contrast, for our primary/LN matched patient series, only 20% of LNs immunostained for EMMPRIN, indicating that the processes regulating EMMPRIN expression are complex, and may depend on factors and cellular interactions within the tumor/host microenvironment. Down-regulation of EMMPRIN expression may occur after tumor cells have migrated from the primary tumor and subsequently formed metastatic deposits at other sites, as seen for example in lymph nodes from our CaP patients. A recent in vitro study found that expression of MMP-regulating genes including *EMMPRIN* was down regulated when the transcriptional suppressor *Snail* (involved in cell movement and epithelial to mesenchymal transition important for cancer progression [31]) was suppressed in malignant melanoma cells [32]. We also detected high levels of cell-surface EMMPRIN expression by flow cytometry on >90% of cells in 11/12 metastatic CaP cell lines derived from different sites in CaP patients or as sublines from animal models, consistent with metastatic cancer cell clones retaining the potential to express EMMPRIN antigen. As discussed above, this may be down regulated in vivo at metastatic sites and requires further investigation.

Growth and invasion of CaP cells involves breakdown of ECM by proteinases including MMPs, which are involved in tumor progression, growth, invasion, angiogenesis and metastasis [33, 34]. Previous studies have demonstrated that EMMPRIN promotes tumor cell invasion by stimulating stromal cells to produce elevated levels of MMPs, including MMP-1, MMP-2, MMP-9 and MT1-MMP [2]. In the present study we observed for the first time, co-immunolocalization of EMMPRIN with MMP-1 and MMP-9 in primary CaP tissues at different stages, but not in benign prostate tissues and normal prostates. We hypothesize that tumor cells expressing EMMPRIN may stimulate stromal and tumor cell MMP production, promoting CaP growth and invasion. This may occur by heterotypic cell-to-cell interactions, for example, between tumor cells and fibroblasts [20], through an as yet unidentified receptor, or by homophilic interactions, with paracrine activity between tumor cells through a signaling mechanism requiring tyrosine kinase activity [35]. EMMPRIN can also be released from the surface of cancer cells via shedding of constitutive microvesicles [36, 37] that rapidly break down to release to full length, soluble, bioactive EMMPRIN [36]. Soluble EMMPRIN can act in a paracrine fashion on stromal cells adjacent to and at sites distant from the tumor, further stimulating the production of MMPs and additional EMMPRIN.

MMPs may also participate in late events during cancer metastatic spread, when cancer cells enter, survive and exit blood vessels or lymphatics. We have shown that elevated EMMPRIN expression in CaP is associated with expression of MMP-1, MMP-2 and MMP-9. Supporting these observations, Pulukuri et al. [38] have reported that increased levels of secreted MMPs are detectable only in androgen-nonresponsive CaP cell lines (PC-3 and DU 145), but not in less aggressive LNCaP cells. Tumor cell invasion is significantly inhibited by treating PC-3 cell culture medium with antibodies to MMP-1, but not to MMP-3 or MMP-7, and slightly inhibited when MMP-2 and MMP-9 are simultaneously depleted [37]. These results suggest that up regulated expression of MMP-1, MMP-2 and MMP-9 may be critical to CaP progression. Metastasis in CaP patients is also correlated with high levels of plasma/urine MMP-2 and MMP-9 [39, 40]. Increased MMP-2 expression in

malignant prostate epithelia is associated with a decreased disease-free survival in CaP patients and is an independent predictor of increased tumor aggressiveness [41]. Inhibition of MMP-9 by antisense oligonucleotides, ribozyme technology or synthetic MMP inhibitors is found to attenuate angiogenesis, human CaP cell invasion and tumorigenicity [42] as well as metastatic potential in a mouse xenograft study [43]. Downregulation of EMMPRIN by siRNA in PC-3 cells has consistently been shown to decrease invasiveness and secretion of MMP-2 and MMP-9 [44]. Our preliminary gelatin zymography results indicate that metastatic PC-3, PC-3M and PC-3MM2 cells (strongly EMMPRIN positive) express latent MMP-9.

EMMPRIN is a multifunctional cell surface protein whose functional diversity may relate to distinct post-translational modifications. Differential modification through glycosylation may be cell-type specific or associated with malignancy. Highly glycosylated EMMPRIN induces MMP activation via signaling pathways that are not well defined [7]. We observed variable glycosylation of fully processed EMMPRIN proteins, including HG forms (65 kDa and ~45 kDa), in 11 CaP cell lines. HG forms of EMMPRIN self-aggregate [35] to induce MMP production [16]. However, LG forms of EMMPRIN interact with the tumor suppressor protein, caveolin-1 that regulates caveolae-dependent signaling, to inhibit HG forms and cell-surface clustering of EMMPRIN, leading to impairment of MMP induction [16, 45]. How EMMPRIN glycosylation is regulated and its possible association with caveolin-1 in CaP remains to be elucidated.

A recent study found that EMMPRIN expression was increased in multidrug resistant (MDR) breast cancer cell lines and that anti-EMMPRIN antibody treatment of these cells could inhibit the activity of MMP-1, MMP-2 and MMP-9, and in vitro invasion [46]. Furthermore, treatment of MDR breast cancers with P-glycoprotein substrates was found to adversely affect therapeutic outcomes by modulating the production of EMMPRIN, MMP-2 and -9, and EGFR, suggesting that these effects may be initiated by the transporter function of P-glycoprotein [47]. The role of MDR proteins in CaP progression is unclear, and future studies will investigate the functional interactions between EMMPRIN, these proteins and MMP expression.

CaP disseminates from the prostate via lymph and blood causing metastases. Many of the factors and tumor-associated antigens involved could potentially provide targets for CaP therapy. Although MMPs may play a key role in tumor cell progression and metastasis, the use of synthetic MMP inhibitors, whilst promising in preclinical studies has not proven effective against advanced cancers in clinical trials [48, 49]. This may reflect the protective role of some tumor and stroma-derived MMPs in certain tumor types, as seen for example in mouse models of squamous cell and breast carcinomas [3, 50, 51]. MMP-12 derived from host tissue macrophages has also been reported to inhibit angiogenesis and thus tumor growth in a mouse model of pulmonary metastases [52]. The early involvement of MMPs in cancer such that the metastatic cascade is already established before use of MMP inhibitors, may also have limited the effectiveness of these agents in human clinical trials where inhibitors were generally administered at late stages of disease [53, 54]. The possibility of targeting MMP-inducing proteins such as EMMPRIN may also be considered in CaP, as suggested by a recent study which found that using CD147 antibodies could inhibit MMP production and tumor growth of hepatocellular carcinoma cells in an orthotopic nude mouse model [55].

In summary, we have demonstrated for the first time that more frequent, higher levels of heterogeneous EMMPRIN expression occur in high grades of CaP compared with benign or normal tissues, and that positive EMMPRIN staining correlates significantly with various progression parameters. The heterogeneous immunolabeling pattern of EMMPRIN, MMP-1, MMP-2 and MMP-9 and localized expression in vasculature and stromal cells highlight the importance of the tumor microenvironment in the pathogenesis of CaP. The abundant forms of EMMPRIN found in most metastatic CaP cell lines further indicate that variable glycosylation of EMMPRIN is present, with implications for MMP activation. Taken together, these results suggest that EMMPRIN is involved in CaP growth and dissemination, and may be a useful therapeutic target for treating CaP.

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