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EMMPRIN Regulates Cytoskeleton Reorganization and Cell Adhesion in Prostate Cancer

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Abstract

Background—Proteins on cell surface play important roles during cancer progression and metastasis via their ability to mediate cell-to-cell interactions and navigate the communication between cells and the microenvironment.

Methods—In this study a targeted proteomic analysis was conducted to identify the differential expression of cell surface proteins in human benign (BPH-1) vs. malignant (LNCaP and PC-3) prostate epithelial cells. We identified EMMPRIN (extracellular matrix metalloproteinase inducer) as a key candidate and shRNA functional approaches were subsequently applied to determine the role of EMMPRIN in prostate cancer cell adhesion, migration, invasion as well as cytoskeleton organization.

Results—EMMPRIN was found to be highly expressed on the surface of prostate cancer cells compared to BPH-1 cells, consistent with a correlation between elevated EMMPRIN and metastasis found in other tumors. No significant changes in cell proliferation, cell cycle progression or apoptosis were detected in EMMPRIN knockdown cells compared to the scramble controls. Furthermore, EMMPRIN silencing markedly decreased the ability of PC-3 cells to form filopodia, a critical feature of invasive behavior, while it increased expression of cell-cell adhesion and gap junction proteins.

Conclusions—Our results suggest that EMMPRIN regulates cell adhesion, invasion and cytoskeleton reorganization in prostate cancer cells. This study identifies a new function for EMMPRIN as a contributor to prostate cancer cell-cell communication and cytoskeleton changes towards metastatic spread, and suggests its potential value as a marker of prostate cancer progression to metastasis.

Keywords

Prostate Cancer; EMMPRIN; Cytoskeleton; shRNA; Filopodia

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INTRODUCTION

Metastatic prostate cancer is a major contributor to cancer related mortality in men. Normal prostate epithelial cell homeostasis is maintained by a dynamic balance between cell proliferation and apoptosis. Normal cells undergo anoikis (a unique mode of apoptosis) upon detachment from extracellular matrix (ECM). Cancer cells however develop mechanisms to evade anoikis and acquire the ability to detach and migrate into new sites that provide a nurturing microenvironment for continued growth (1). During the metastatic spread of primary tumor cells, proteins on cell surface are critical in mediating cell to cell and cell to environment communication.

EMMPRIN is a cell surface glycoprotein of IgG superfamily encoded by a gene localized to 19p13.3 (2, 3). EMMPRIN is an integral membrane protein, but may be released as a soluble protein by vesicle shedding (4, 5). It initiates the function through homophilic interactions between EMMPRIN molecules on neighboring cells (4, 5). EMMPRIN is expressed in numerous normal and malignant cells and mediates diverse processes such as angiogenesis, neuronal signaling, cell differentiation, wound healing, and embryo implantation (6). Mice lacking EMMPRIN demonstrate various defects, including low embryonic survival, infertility, deficiencies in learning and memory, abnormality in odor reception, retinal dysfunction, and mixed lymphocyte reaction (6–10). Elevated expression of EMMPRIN is found in several human cancers and correlates with the metastatic potential of tumor cells, specifically in breast and ovarian cancer epithelial cells during progression to metastasis (11–14). In the context of the tumor microenvironment, EMMPRIN induces matrix metalloproteinase (MMP) production in stromal fibroblasts and endothelial cells as well as in tumor cells (11–13, 15–17). Elevated MMPs result in ECM degradation and subsequent detachment and metastasis of cancer cells. In addition, EMMPRIN can promote tumor cell invasion via activation of urokinase-type plasminogen activator (18), stimulate tumor angiogenesis by elevating vascular endothelial cell growth factor (VEGF) through Akt signaling (19), and causes multi-drug resistance in tumor cells via hyaluronan-mediated up-regulation and ErbB2 signaling activation (20). EMMPRIN is implicated in metastasis via its ability to confer resistance of breast cancer cells to anoikis by inhibiting BIM (21), and its association with lipid raft or caveolae via interactions with key membrane proteins, including caveolin-1, monocarboxylate transporters, annexin II, (22) and integrins $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$ (23), all critical in the spatial distribution and activity of EMMPRIN.

Previous studies suggested that EMMPRIN expression is associated with prostate cancer progression (24, 25), and loss of EMMPRIN reduces the invasion potential of human prostate cancer cells (26). This evidence however has been correlative and little is known about the mechanistic significance of EMMPRIN in prostate cancer progression and metastasis beyond its ability to induce MMPs. In this study we profiled the EMMPRIN expression pattern in human prostate cell lines of benign and metastatic origin and characterized the function of EMMPRIN in tumor cell aggressive behavior. EMMPRIN suppression led to a significant decrease in prostate cancer cell attachment to the ECM, migration and invasion, as well as filopodia formation while it enhanced cell-cell interactions. The results provide a new insight into the ability of EMMPRIN to regulate prostate cancer cell adhesion, invasion and cytoskeleton organization.

MATERIALS AND METHODS

Cell Lines

The HEK293 and the human prostate cancer cell lines PC-3, DU-145 and NCaP, were obtained from the American Type Culture Collection (Manassas, VA). The nontumorigenic benign human prostatic epithelial cells BPH-1, (derived from human prostate epithelium of

benign pathology) was generously provided by Dr. Simon W. Hayward (Department of Urological Surgery, Vanderbilt University Medical Center). Cells are maintained in RPMI-1640 medium (Gibco™, Grand Island, NY), supplemented with 10% fetal calf serum (CSS), 100U penicillin and 100-mg/ml streptomycin, at 5% CO₂ incubator at 37 °C.

Western Blot Analysis

Confluent cell cultures (80%) were washed with PBS, scraped, and cell pellets were harvested. Cells were disrupted with RIPA buffer (50 mM Tris-HCl, pH7.4, 1% NP40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mg/mL each of aprotinin, leupeptin, pepstatin, and 1 mM Na₃VO₄). Cell lysates were centrifuged at 5,000×g (15mins), resolved by SDS-PAGE, and transferred to Immobilon-P membranes (Millipore, Bedford, Mass.). Upon incubation with the primary and secondary antibodies, immunoreactive bands were detected using a chemiluminescent approach with the ECL kit (Pierce, Rockford, IL). Membrane fractions were prepared using the protein isolation kit (Pierce). Monoclonal antibodies against EMMPRIN, ZO-2, actin and tubulin were purchased from Santa Cruz Biotech (Santa Cruz, CA). Monoclonal antibodies against ZO-1 and AF6 were obtained from Invitrogen Zymed (San Francisco, CA) and BD Transduction (Lexington, KY), respectively.

RT-PCR analysis

Total RNA was extracted from cells using an RNAeasy kit (Qiagen, Valencia, CA). RNA samples (0.25µg) were subjected to reverse transcription (RT) PCR reaction in a 20-µl volume with poly-oligoT primer. The resulting cDNA was subjected to PCR using EMMPRIN specific primers. The first set of primers started with exon 1 and ended at exon 11: EF2 (5'-ATG GCG GCT GCG CTG TTC GTG-3') and ER11 (5'-GGA GCA GGG AGC GTC CTC GGG-3'). The second set of primers started with exon 2 and ended at exon 11: EF1 (5'-ATG AAG CAG TCG GAC GCG TCT C-3') and ER11. GAPDH primers (5'-CAG CAA TGC ATC CTG CAC-3' and 5'-GAG TTG CTG TTG AAG TCA CAG G-3') were used as control in the same PCR reactions. Thirty cycles of PCR reactions were performed and each cycle included 45 sec, 94°C; 45sec, 55°C; and 45sec, 72°C. The PCR products were analyzed on a 1.2% agarose gel. Amplicons are purified, cloned and sequenced by IDT (Coralville, IA).

shRNA Plasmid Construction and Transfection

Short hairpin RNA (shRNA) interference oligos, were designed using OligoEngine software (Seattle, WA) to specifically target EMMPRIN (NM_198589). Three oligos that target EMMPRIN (variant 2 mRNA) at nucleotides 98–116 (TGGCTCCAAGATACTCCTC), 277–295 (CCATGGGCACGGCCAACAT) and 776–794 (AGGCAAGAACGTCCGCCAG), are named as 98i, 277i and 776i, respectively. A scramble shRNA (TTCTCCGAACGTGTCACGT) was used as control. The oligos are cloned to pSUPER (neo + GFP) plasmid from OligoEngine according to the manufacturer's instruction. Plasmids were amplified in DH5α cell and confirmed by sequencing.

Subconfluent cell populations were used for transfection using the FuGENE system (Roche, Indianapolis, IN). Briefly, the plasmid and Fugene reagent were combined and incubated for 20–30mins at room temperature. After transfection (36hrs), cells were subjected to cell sorting based on GFP expression and GFP positive cells were subsequently subjected to Western blotting. Stable transfectants were cloned under Geneticin selection (Invitrogen) (300µg/ml), the generated clones were maintained in RPMI 1640 medium (150µg/ml Geneticin).

Cell Viability Assay

The MTT assay (based on the ability of viable mitochondria to convert MTT, a soluble tetrazolium salt, into an insoluble formazan precipitate) was used to assess cell viability. Cells were seeded into 96-well plates (2,500 cells/well) and incubated in growth medium (18–24hrs). After incubation with the MTT solution for 4 hrs, absorbance was read at A₅₇₀ and the colorimetric reaction product was quantitated spectrophotometrically (BioTek, PowerWave XS, Winooski, VT).

Evaluation of Cell Cycle and Apoptosis

BrdU/PI (Bromodeoxy uridine and propidium iodide) method was used for the analysis of cell cycle progression and apoptosis. Cells (1×10^6 /ml) were incubated with BrdU (20mM) (60min at 37°C), suspended in PBS, and fixed with ice-cold 95% (v/v) ethanol. Fixed cells were subsequently permeabilized using pepsin (0.04% w/v, 0.4mg/mL in 0.1N HCl). BrdU was probed with FITC labeled anti-BrdU (BD, San Jose, CA). Apoptosis among the different cell populations was evaluated using the terminal UTP end-labeling (TUNEL) technique. (Leica, Germany).

Cell Adhesion Assay

The ability of cells to attach to key ECM components, (fibronectin and laminin) was tested using fibronectin or laminin-coated 6-well multiwell plates (BD Bioscience). Prostate cancer epithelial cells were plated (10^5 /well), and incubated at 37°C for 30mins, prior to fixing with methanol, and washed with PBS. Cells were counted from three random fields/well.

Evaluation of Cell Migration and Invasion

Confluent monolayer cells were wounded by scraping. Cultures were washed twice with medium, and then incubated at 37 °C for 16hrs to allow migration toward the gap. The number of migrating cells was determined under the microscope. The invasion potential of prostate cancer cells was assessed using Biocoat Matrigel invasion chambers (Becton Dickinson). Briefly, cells (5×10^4) resuspended in RPMI1640-based medium were added (250μl) into the invasion chambers and chambers were subsequently inserted into 24-well plates. Stained cells were photographed and counted.

Confocal Microscopy

Cells were plated on fibronectin-coated glass coverslips and fixed with 4% paraformaldehyde. Cells were permeabilized in 0.1% (v/v) Triton-X 100 and were subsequently stained with rhodamine-phalloidin (Jackson ImmunoResearch, West Grove, PA). After rinsing with PBS (3×), slides were mounted with Vectorshield (Vector Lab, Burlingame, CA). Slides were examined under a laser-scanning confocal microscope (Leica Lasertechnik, Heidelberg, Germany).

Cell Aggregation Assay

Cells aggregation assay was performed as previously described (27). Briefly, cells were suspended into single cells and dissociated cells were allowed to associate in medium (1hr) in 5% CO₂ at 37°C, with gentle rotation of the plates. The number of cell aggregates in the parental control PC-3 and EMMPRIN shRNA transfectant cells was counted.

Statistical Analysis

Data are expressed as Mean ± SD. Mann-Whitney and Student's t tests were used to comparatively analyze the differences between groups in the various experiments.

RESULTS

EMMPRIN Expression in Human Prostate Cancer Cell Lines

Targeted proteomic analysis comparing the cell surface proteomes of BPH-1 (immortalized benign prostate hyperplasia cell line) and LNCaP and PC-3 (human prostate cancer cell lines derived from metastatic lesions) revealed the differential expression of EMMPRIN.

EMMPRIN was found to be highly expressed on the cell surface of prostate cancer epithelial cells but not the benign prostate cells. Western blot analysis was subsequently conducted to validate the proteomics screening data and the results are shown in Figure 1. Using total cell lysates (Fig. 1, panel A), EMMPRIN showed a broad range molecular shift corresponding to different degrees of glycosylation as previously shown in breast cancer cells (11–13).

Malignant prostate cells, PC-3 and LNCaP appeared to have more highly glycosylated EMMPRIN than BPH-1 while the total protein levels were similar in all three cell lines.

Glycosylation of EMMPRIN contributes to its membrane localization. Thus plasma membrane fractions were isolated from all prostate cell lines and subjected to Western blotting. As shown in Figure 1B, EMMPRIN levels in the plasma membrane fractions of LNCaP and PC-3 cells were significantly higher than in BPH-1 cells. These results are consistent with the cell surface proteome studies, implicating higher EMMPRIN translocation to the plasma membrane in prostate cancer cells than in benign cells. The molecular mechanism of membrane targeting and translocation is beyond the scope of this article and is currently being pursued in a parallel study.

The alternative splicing isoforms of EMMPRIN in the prostate cell lines were also determined. Four splicing isoforms of EMMPRIN have been deposited in the NCBI database and most studies focus on variant 2 that harbor two Ig domains. Two sets of primers were designed for RT-PCR: one starting at exon 1 and ending at exon 11, and the other one starting at exon 2 and ending at exon 11. The RT-PCR products were analyzed by agarose electrophoresis and the results are shown in Figure 1 (Panel C). The RT-PCR products were cloned and subjected to DNA sequencing. The sequencing results demonstrated that there are three different splicing variants existed in human prostate cells: variant 2 (band 3, 828bp), variant 4 (band 1, 634bp) and variant 3 (band 2, 793bp). Other bands indicated by filled triangles were non-specific RT-PCR products. Variant 2 appeared to be the major transcript in human prostate cells and there were no evident differences in the splicing isoforms among the different cell lines

EMMPRIN Silencing in PC-3 Prostate Cancer Cells

The functional significance of EMMPRIN in prostate cancer progression remains unknown. Thus we examined whether high levels of EMMPRIN in PC-3 cells, functionally contribute to the aggressive behavior of metastatic prostate cancer cells. Since PC-3 exhibit high endogenous EMMPRIN expression, we used the RNA interference approach to silence EMMPRIN in these cells. Three pairs of oligos targeting to EMMPRIN exon 5, 6 and 11 were designed and successfully cloned into pSUPER plasmid (containing GFP marker). Due to the low transfection efficiency in PC-3 cells (about 30% using FuGENE), cells with the GFP marker were sorted at 36hrs after transfection and were subjected to Western blot analysis. The results shown in Figure 2A indicate that EMMPRIN protein levels are significantly reduced by all three shRNA species. Stable clones in which EMMPRIN was silenced under G418 selection, had lower EMMPRIN levels compared to scramble controls (Fig. 2, panels A and B). The shRNA 277 clone, in which the middle region of EMMPRIN gene was targeted, had less of an effect in reducing EMMPRIN expression.

Effect of EMMPRIN Loss on Prostate Cancer Cell Proliferation and Apoptosis

To determine the role of EMMPRIN on prostate cancer cell growth, we initially examined the consequences of EMMPRIN silencing on prostate cancer cell proliferation, cell cycle and apoptosis. Interestingly, down-regulation of EMMPRIN resulted only in a modest inhibitory effect on prostate cancer cell growth (Supplementary Figure S1, panel A). Cell cycle analysis demonstrated no significant effect on cell cycle progression in shRNA EMMPRIN PC-3 transfectants (Fig. S1, panel B). Evaluation of apoptosis based on the TUNNEL assay revealed that loss of EMMPRIN had no significant consequences on the rate of cell death among these cell populations (Fig. S1, panel C). Thus EMMPRIN is not involved in the control of prostate cancer cell growth or apoptosis.

EMMPRIIN Loss Decreases Prostate Cancer Cell Adhesion, Migration and Invasion

Many cell surface proteins are involved in cell adhesion and EMMPRIN can be a potential partner with such adhesion molecules. To determine the functional contribution of EMMPRIN to prostate cancer cell adhesion to the ECM, we examined attachment ability of EMMPRIN silenced PC-3 transfectants to key components of the ECM, fibronectin and laminin. As shown on Figure 3 (Panel A), there was a 40% decrease in the number of cells attached to fibronectin for the EMMPRIN knockdown cells compared to the scramble control cells. A similar magnitude of suppression of cell adhesion to laminin was observed in the EMMPRIN shRNA stable clones compared to scramble control cells (Fig. 3, panel B) or PC-3 parental cells (approximately 30–50% suppression). We subsequently examined the consequences of EMMPRIN loss on prostate cancer cell migration. EMMPRIN silencing yielded a significant reduction in cell migration ability in all three shRNA prostate cancer cell lines (Fig. 3, panel B), with the s277i clone exhibiting the most significant suppression. In addition, we examined the impact of EMMPRIN loss on the invasion ability of PC-3 cells. Figure 3 (Panel C), shows a significant decrease in cell invasion observed in EMMPRIN shRNA transfected cells compared to control cells. Thus loss of EMMPRIN significantly decreased the adhesion, migration and invasion abilities of metastatic prostate cancer cells.

EMMPRIIN Enhanced Filopodia Formation in Prostate Cancer Cells

To determine whether EMMPRIN promotes cell migration by facilitating cytoskeleton reorganization, we examined the ability of EMMPRIN shRNA PC-3 cells, to form filopodia. Cells attached to fibronectin-coated cover-slips were subjected to immunofluorescence analysis for vinculin and F-actin presence and localization. The image on Figure 4 (Panel A), indicates that EMMPRIN silencing inhibited prostate cancer cell spreading on fibronectin, while a stronger F-actin staining was detected forming a stress fiber but without typical focal adhesion complex (Fig.4, panel A). Confocal microscopy revealed a significant suppression of filopodia formation as a consequence of EMMPRIN loss. An approximate 50% reduction in the number of filopodia is detected in EMMPRIN knockout cells compared to control cells (Fig. 4, panel B). In addition, EMMPRIN silencing also led to a decrease in the strength of the filopodia. Immunofluorescence analysis (Fig. 4, panel A) revealed considerably larger filopodia in control cells compared to limited and small filopodia observed among EMMPRIN knockdown PC-3 prostate cancer cells.

Effect of EMMPRIN Knockdown on Cell Aggregation and Tight Junction Proteins

We subsequently examined the effect of EMMPRIN on the dissociation/detachment of cancer cells. A cell aggregation assay was conducted in the PC-3 control and EMMPRIN shRNA PC-3 prostate cancer cells. As shown in Figure 5 (Panel A), there was increased cell aggregation in EMMPRIN silenced PC-3 cells. Subsequent experiments determined the effect of EMMPRIN silencing on the expression of tight junction proteins. The levels of

plasma membrane proteins JAM-A and JAM-B were unchanged in the EMMPRIN knockdown clones (Fig. 5, panel B). A significant increase however in the levels of tight junction associated proteins ZO-1, ZO-2, AF6 and β -catenin was detected consequential to EMMPRIN loss. These data imply that EMMPRIN may impair cell-cell interactions by facilitating the dissociation/detachment of tumor epithelial cells from each other.

DISCUSSION

To determine the cell surface protein differences between malignant and benign prostate cells and their significance in prostate cancer metastasis, we performed mass spectrometry analysis to profile the expression of cell surface proteins in human prostate cancer cells derived from metastatic lesions and benign prostate epithelial cells. One of the proteins highly expressed on the cell surface of metastatic prostate cancer cells, but not benign cells, was identified to be extracellular matrix metalloproteinase inducer (EMMPRIN, also known as basigin, CD147, OX47 or 5A11). EMMPRIN has been previously shown to be involved in cancer development via its ability to stimulate MMP production and consequently control extracellular matrix remodeling and anchor independent growth (28). In addition, EMMPRIN has been shown to regulate angiogenesis by engaging the AKT-PIK3 pathway (19), and to up-regulate urokinase-type plasminogen activator (18). EMMPRIN can also interact with key adhesion proteins such as integrins (23), implicating its role in cancer cell migration and invasion. The present study provides the first evidence on the functional consequences of EMMPRIN loss on prostate cancer cell growth, proliferation, apoptosis and cell adhesion (Fig 3). We observed that down-regulation of EMMPRIN led to a significant suppression of prostate cancer attachment to fibronectin, a major ECM component (Fig. 4, panel A). Thus a defect in the cytoskeleton organization can be induced by functional loss of EMMPRIN. Furthermore down regulation of EMMPRIN protein led to decreased prostate cancer cell migration. Considering the evidence that cell migration is independent of MMPs and that MMP and EMMPRIN knockout mice (29) have different phenotypes, it is reasonable to postulate that these two proteins may operate in independent pathways functionally converging downstream. EMMPRIN may be engaged in distinct signaling pathways, directly promoting the invasive behavior of prostate cancer cells towards metastasis. This notion gains support from evidence indicating lack of correlation between EMMPRIN expression and MMP activity during adult mouse mammary gland development (30). Moreover, EMMPRIN has been shown to directly promote insoluble fibronectin assembly (21).

In this study, EMMPRIN loss significantly reduced prostate cancer cell filopodia formation on a fibronectin substratum. This defective filopodia formation implies disruption of cytoskeleton organization and actin signaling in cells lacking EMMPRIN. These observations are consistent with reports suggesting that EMMPRIN (D-basigin in *Drosophila*) tightly regulates cytoskeleton rearrangement in *Drosophila melanogaster* (23). Based on the present results and the existing evidence, we propose that EMMPRIN promotes tumor cell metastasis in an MMP-dependent and -independent pathway (Fig. 5, panel C). One must also consider that EMMPRIN has been associated with prominent membrane proteins caveolin-1 and vimentin, implicating its involvement in lipid raft and control of membrane dynamics. Here we show for the first time that silencing EMMPRIN resulted in enhanced cell aggregation (Fig. 5, panel A) and increased the protein expression for several tight-junctions mediators including ZO-1, ZO-2, AF-6 and β -catenin (Fig. 5, panel B). Considering the reported relationship between tight junction proteins and cytoskeletal changes associated with cell aggregation (27, 31) our findings provide new insights into the ability of elevated EMMPRIN to navigate tight junctions and cell-cell adhesion within the tumor microenvironment. The mechanistic scenario discussed above can lead to enhanced prostate cancer invasiveness by EMMPRIN overexpression. Significantly

enough, our group recently demonstrated that talin1, an actin-binding protein that links integrins to actin cytoskeleton in focal adhesion complexes, correlated with prostate cancer progression to metastasis (32). Mechanistically, talin1 binding to β integrin recruits the focal adhesion partners ILK, FAK and SRC, and activates downstream signals, PI3K/Akt and Erk; activation of this signaling promotes cell survival, migration and invasion and resistance to anoikis. EMMPRIN may serve as an upstream partner for talin, facilitating its role in anoikis resistance and actin cytoskeleton remodeling, and consequently promoting metastatic spread.

Mammalian cells ubiquitously adopt a variant splicing strategy to cope with multiple functions and their requirement by diverse physiological processes. At least two different variants of EMMPRIN have been reported. Variant 2 is a ubiquitous expression protein as previously reported and a larger variant 1 is expressed in retinal epithelial cells in a tissue specific fashion. In this study, we identified three distinct EMMPRIN splicing variants: variant 2, 3 and 4 (Fig. 1, panel C). The latter two variants are distinct from the commonly found variant 2. Significantly enough these two variants lack exon 2 where glycosylation occurs (3). Moreover, variant 4 lacks exon 5, where another glycosylation site is also located. The dynamics of the ratio of different isoforms and the mechanisms via which the different splicing variants are engaged to navigate EMMPRIN expression and activity to meet the physiological demands of both ECM remodeling and cancer cell motility are currently being pursued.

The present results are of translational significance as functional exploitation of EMMPRIN in prostate cancer metastasis may lead to new approaches for impairing the metastatic process by a) reversing the ability of tumor cells to resist anoikis (thus enhancing their sensitivity to anoikis-inducing agents); and b) interfering with the tumor cell migration and adhesion to secondary sites. Ongoing studies focus on immunoprofiling EMMPRIN expression in human prostate specimens from patients with primary and metastatic tumors to determine the significance of EMMPRIN as a marker of progression to advanced castration-resistant disease.

In summary, our findings demonstrate that EMMPRIN loss has a major impact on cell membrane re-organization and spatial disruptions that significantly affect prostate tumor cell adhesion, migration and invasion. The present work provides new insights into the function of EMMPRIN as a contributor to prostate cancer cell metastatic behavior and its potential value as a therapeutic target during tumor progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

EMMPRIN	extracellular matrix metalloprotease inducer
shRNA	short hairpin RNA
PBS	phosphate buffer saline
MMP	matrix metalloprotease
ECM	extracellular matrix
BPH	benign prostatic hyperplasia
VEGF	vascular endothelial growth factor

TUNEL	terminal UTP end-labeling
SDS-PAGE	sodium-dodecylsulphate polyacrylamide gel electrophoresis
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium salt

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Figure 1. EMMPRIN expression and alternative splicing in prostate cancer cells

Panel A, EMMPRIN expression levels in total cell lysates of BPH-1, LNCaP and PC-3 cells. Bands of different motilities are likely due to glycosylation. Both LNCaP and PC-3 cell lines exhibited elevated levels of highly glycosylated EMMPRIN compared to BPH-1 cells. Panel B. LNCaP and PC-3 prostate cancer cells exhibited a significantly higher amount of membrane anchored EMMPRIN compared to BPH-1 cells. Membrane fractions (30 μ g protein) were subjected to Western blotting. (C). EMMPRIN transcripts were analyzed by RT-PCR and electrophoresis. Splicing variants 2, 3 and 4 were confirmed by DNA sequencing as Band #3, #1 and #2, respectively.

Figure 2. Suppression of EMMPRIN expression in transient and stable shRNA prostate cancer transfected cells

PC-3 cells were transfected with three EMMPRIN shRNA plasmids or scrambled control and subjected to Western blot analysis to establish EMMPRIN protein expression levels (major band detected at 50 kDa). Panel A, after transient transfection a considerable reduction in EMMPRIN expression was detected in GFP-positive cells transfected with three different shRNA plasmids, while no changes in EMMPRIN levels were observed in the GFP negative control cells. Panel B; stably transfected cells with GFP marker were obtained after selection with G418 (300 μ g/ml). Substantial reduction in EMMPRIN levels was demonstrated in the individual stable shRNA clones.

Figure 3. Consequences of EMMPRIN silencing on prostate cancer cell adhesion to ECM, migration and invasion

EMMPRIIN shRNA transfectant PC-3 cells were seeded on fibronectin-coated (panel A) or laminin (panel B) plates for 30mins and attached cells were fixed and counted. Panel C, Cell migration was assessed by wounding the cell monolayer and determining the number of cells migrating to the wounded area after 24hrs. Panel D, The effect of EMMPRIN silencing on prostate cancer cell invasion was determined using the matrigel assay as described in “Materials and Methods”. The average values from three independent experiments performed in triplicate are shown. Numerical values are expressed as percentage of control Sh scramble cells. Statistical significance is reached at $p < 0.01$.

Figure 4. EMMPRIN loss reduces filopodia formation in prostate cancer cells

Panel A. Cells were plated on fibronectin-coated glass coverslips and after spreading (24hrs), they were exposed to F-actin staining, and subsequently visualized under confocal microscopy, Arrow heads indicate individual filopodia. The insert represents a zoom-in of region indicated in dotted boxed areas. Panel B, The average number of filopodia in each cell was quantified in scramble control and the three shEMMPRIN clones, 98i, 277i and 776i cells. Filopodia from at least 20 cells were counted and representative average values are shown. Approximately 20 cells/field and 10 random fields were examined for each cell line; error bars indicate average values from these measurements (mean) $\pm$ standard error of mean

(SEM) gathered from three independent experiments. Statistical difference is considered significant at $p < 0.01$.

Figure 5. Effect of EMMPRIN on cell aggregation and tight junction proteins

Panel A. Cell aggregation increased with reduced EMMPRIN expression in prostate cancer cell EMMPRIN Sh clones. Panel B, Western blotting indicating expression of gap junction proteins in EMMPRIN silenced PC-3 cells. Panel C, Potential role of EMMPRIN in prostate cancer metastasis. EMMPRIN can stimulate production of MMPs, leading to reduced cell adhesion to ECM. Alternatively, EMMPRIN may directly promote the metastatic potential of prostate cancer cells by enhancing migration and invasion through cytoskeleton reorganization and impairing cell-microenvironment interactions.