ORIGINAL RESEARCH

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Over-expression of extracellular matrix metalloproteinase inducer in prostate cancer is associated with high risk of prostate-specific antigen relapse after radical prostatectomy

Abstract

Purpose: The prognostic efficiency of clinical grading and staging in patients with confined or moderately differentiated prostate cancer (PCa) has been markedly improved, which underscores the importance of new prognostic markers. Extracellular matrix metalloproteinase inducer (EMMPRIN) has been demonstrated to be involved in cancer angiogenesis, metastasis and invasion. EMMPRIN expression was evaluated by measuring mRNA and protein levels in a large cohort of patients with PCa following prostatectomy and the findings were compared with clinico-pathological parameters, including prostate-specific antigen (PSA) relapse time.

Methods: EMMPRIN mRNA levels in 20 pairs of normal and cancerous prostate tissues were determined by quantitative real-time PCR. Protein expression in paraffin-embedded specimens of prostates gathered from 300 patients with PCa was detected by immunohistochemistry using a monoclonal antibody against EMMPRIN. The associations of EMMPRIN protein expression with the clinico-pathological parameters and PSA relapse-free time after radical prostatectomy were subsequently assessed.

Results: Both EMMPRIN mRNA and protein levels were higher in PCa tissue, compared with adjacent normal tissue. In addition, the positive expression rates of EMMPRIN in PCa tissues were significantly associated with preoperative PSA levels ($p=0.008$), AJCC stage ($p=0.006$) and Gleason Score ($p<0.001$), Risk classification ($p<0.001$), lymph node status post-surgery ($p<0.001$) and surgical margin status ($p<0.001$) were also determined. Multivariate analysis, using the Cox proportional hazards model, revealed that positive EMMPRIN expression was an independent prognostic factor for an increased risk of PSA relapse.

Conclusion: Over-expression of EMMPRIN correlated with the aggressiveness of PCa, and the PSA relapse-free time, and may be a novel and useful biomarker for follow-up and treatment decisions for PCa.

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Prostate cancer (PCa) is the second most frequent cause of cancer-related death for Western men. Its incidence is continuously rising, with over 200,000 new cancers and 35,000-40,000 deaths per year [1,2]. Carcinogenesis is a multi-step process, involving both genetic insults to epithelial cells and changes in epithelial-stromal interactions [3]. Despite current improvements in diagnostic and therapeutic methods, many patients with PCa develop metastases and the prognosis rate of this disease varies greatly for patients with different clinical stages and pathological grades. The conventional prognostic markers, such as clinical stage, tumor grade and preoperative prostate-specific antigen (PSA), show limited ability to predict the clinical outcome. Thus, the search for novel biomarkers that are capable of discriminating PCa with low and high aggressiveness and recurrence potential is an important goal in this field.

The degradation of the extracellular matrix (ECM) surrounding primary tumors and metastases is critical for the invasion and metastasis of epithelial tumor cells [4]. Matrix metalloproteinases (MMPs) expression by stromal [5] and endothelial cells [6] are regulated by various soluble or cell-bound factors, such as extracellular Matrix Metalloproteinase Inducer (EMMPRIN), which is a member of the immunoglobulin family. EMMPRIN has been demonstrated to be involved in tumor invasion and metastasis via stimulating matrix metalloproteinase synthesis in neighboring fibroblasts, in malignant cell proliferation via the activation of ERK1/2 and p38 mitogen-activated protein kinases, in enhancing angiogenesis via vascular endothelial growth factor, in inducing chemoresistant tumor cells via the production of hyaluronan, and in resistance of cancer cells to anoikis through inhibition of Bim [7-11]. Some research has demonstrated that EMMPRIN is associated with the outcome of breast cancer [12] and that EMMPRIN expression is associated with the overall survival of patients with PCa [13-14]. In view of these results, the aim of this study was to determine the expression of EMMPRIN at the transcriptional and translational levels, and to clarify the correlation between EMMPRIN expression, in a large cohort of PCa patients, and PSA relapse-free time after radical prostatectomy to further evaluate the validity of EMMPRIN as a diagnostic or prognostic marker.

Materials and Methods

Patients and Tissue Samples

This study was approved by the Ethics Committee of Guangzhou First Municipal People's Hospital, Affiliated Guangzhou Medical College. Informed consent was obtained

TABLE 1. Patient cohort characteristics

Clinical features	N
Mean age (minimum-maximum)	66.2 (11.3) (45-86)
Preoperative PSA (ng/mL)	
≤4	82
>4	218
Gleason score	
≤6	116
=7	125
≥8	59
AJCC pathologic (pT) stage	
2a	64
2b	30
2c	123
3a	49
3b	34
Risk classification	
Low-risk	118
Intermediate risk	123
High-risk	59
Surgical margin status	
Negative	196
Positive	104
Lymph node status post surgery	
Negative	270
Positive	30

from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

Three hundred patients with PCa, whose detailed clinical and pathological information was available, were selected from Department of Pathology at Massachusetts General Hospital (MA, USA) and Department of Urology at Guangzhou First Municipal Peoples' Hospital in Guangzhou Medical College (Guangdong, China). The clinical characteristics of these patients were listed in Table 1. None of the patients recruited in this study had chemotherapy or radiotherapy before the surgery. All of the tissue samples were obtained during transurethral resection prostate and suprapubic prostatectomy. Surgically-resected tissues were paraffin-embedded, sectioned at 3 μm thickness and stained. The pathological diagnosis was performed preoperatively and confirmed postoperatively. Two or more pathologists classified their stages according to American Joint Committee on cancer (AJCC) guidelines [15]. For 20 of the subjects, both cancerous and adjacent normal prostate tissue samples were taken and the normal tissue used as control samples for the immunohistochemical analysis.

The follow-up time was defined as the interval from the date of surgery to the latest measurement of PSA levels. The study end point was the time of PSA relapse and was defined as two consecutive rising serum PSA values greater than 0.2 ng/ml [16].

Quantitative real-time PCR

Total RNA of 20 pairs of adjacent normal and cancerous prostate tissues was prepared and reverse transcribed, and quantitative real-time PCR (QRT-PCR) was carried out as previously described [17]. The primers 5'- TGG CCT TCA CGT TCC TGA GT -3' and 5'- GTC ATC TGC ATC CAC CGT GT -3' were used to amplify 215 bp transcripts of EMMPRIN, and the primers 5'-GTG CCA CCA GAC AGC ACT GTG TTG -3' and 5'-TGG AGA AGA GCT ATG AGC TGC CTG -3' were used to amplify 202 bp transcripts of β -actin. Product-specific amplification was confirmed by melting curve analysis and agarose gel electrophoresis. Serial dilutions were made using previously generated PCR products, assigned arbitrary values corresponding to the dilutions, and used to construct relative standard curves for EMMPRIN. Targets were normalized using β -actin as internal standards.

Immunohistochemistry analysis

HAb 18G/CD147 (EMMPIN) monoclonal antibody was collected by the Biological Research Center (Fourth Military Medical University, Xi'an, China). The antibody specificity had been demonstrated in our previous studies [13,14,17,18].

FIGURE 1. EMMPRIN mRNA analysis. Expression of EMMPRIN mRNA relative to the housekeeping gene β -actin in 20 matched non-cancerous and cancerous prostate tissues. Increased EMMPRIN mRNA in tumor tissue was statistically significant (* $p < 0.001$, normal prostate tissues vs. PCa tissues).

The specimens were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin. The paraffin-embedded tissues were cut at 3 μ m and then deparaffinized with xylene and rehydrated for further H&E or peroxidase (DAB) immunohistochemistry staining employing DAKO EnVision System (Dako Diagnostics, Zug, Switzerland). Following a simple proteolytic digestion and a peroxidase blocking, the tissue slides were incubated with the primary antibody against respective target protein at a dilution of 1:1000 overnight at 4°C. After washing, peroxidase labeled polymer and substrate-chromogen were then employed in order to visualize the staining of the interested proteins. In each immunohistochemistry run, negative controls were carried out by omitting the primary antibody. EMMPRIN overexpression, confirmed by Western blotting, was used as a positive control.

Following hematoxylin counterstaining, the results from the immunostaining was scored by two independent experienced pathologists, who were blinded to the clinicopathological data and clinical outcomes of the patients. The scores of the two pathologists were compared and any discrepant samples were re-examined by both pathologists to achieve a consensus score. The number of positive-staining tumor cells showing immunoreactivity on the cell membranes and cytoplasm in ten representative microscopic fields was counted and the percentage of positive cells was calculated. The immunostaining was assessed for staining intensity (grades 0-3) using light microscopy. The criteria used for assessment were as previously reported [19], where: 0 (negative, $\leq 5\%$); 1+ (positive, $> 5\%$) of the tumor cells stained.

Statistical analysis

The software of SPSS version 13.0 for Windows (SPSS Inc, IL, USA) and SAS 9.1 (SAS Institute, NC, USA) was used for statistical analysis. Continuous variables were expressed as mean $\pm$ standard error. Statistical analysis was performed with Student's *t* test, Fisher's exact test for any 2 $\times$ 2 tables, Pearson's χ^2 test for non-2 $\times$ 2 tables, chi-square trend test (the Log Rank, Mantel-Cox) for ordinal datum, Kaplan-Meier and Cox Regression methods for the question of survival analysis. Differences were considered statistically significant when *p* was less than 0.05.

Results

EMMPRIN mRNA expression

Twenty pairs of PCa and adjacent normal prostate tissues were examined by quantitative RT-PCR (median age: 65.3, range: 46-72 years; mean preoperative PSA level: 11.8 ng/mL, range

TABLE 2. Relationship of EMMPRIN expression with clinicopathologic features of PCa patients (n, %)

Clinicopathologic features	N	EMMPRIN		<i>p</i>
		1+	0	
Age (mean ± std. dev.)	300	65.9±9.0	67.1±8.3	0.4
Preoperative PSA (ng/mL)	300	93.9±10.3	12.5±6.9	<0.001
Gleason score				
≤6	116	53(45.7)	63(54.3)	<0.001
=7	125	83(66.4)	42(33.6)	
≥8	59	50 (84.7)	9(15.3)	
AJCC pathologic (pT) stage				
2a	64	30(46.9)	34(53.1)	0.006
2b	30	15(50.0)	15(50.0)	
2c	123	75(61.0)	48(39.0)	
3a	49	36(73.5)	13(26.5)	
3b	34	30(88.2)	4(11.8)	
Risk classification				
Low-risk	118	53(44.9)	65(55.1)	<0.001
Intermediate risk	123	80(65.0)	43(35.0)	
High-risk	59	53 (89.8)	6(10.2)	
Surgical margin status				
Negative	196	89(45.4)	107(54.6)	<0.001
Positive	104	97(93.3)	7(6.7)	
Lymph node status post surgery				
Negative	270	159(58.9)	111(41.1)	<0.001
Positive	30	27(90.0)	3(10.0)	

3.6-20.8 ng/mL). The RNA quality was the same in the matched samples. The mean absorbance ratio at 260 to 280 nm was 2.03 (range: 1.86-2.08). A significant increase (Student's t test of paired data, $p < 0.001$) in the EMMPRIN/ β -actin ratio in cancerous versus non-cancerous prostate samples was observed (Figure 1). A relationship between EMMPRIN expression and clinicopathologic features was not seen, possibly because of the small sample size and the rather similar clinico-pathologic features.

Expression and location of EMMPRIN in PCa tissues

The expression and distribution of EMMPRIN protein in the tissue samples from 300 patients with PCa (both normal and PCa tissue) were determined using immunohistochemical analysis. EMMPRIN immunoreactivity was detected on all cell membranes and cytoplasm. It was positively, though weakly, stained on a few stromal cells. The positive expression rate of EMMPRIN in PCa patients (186/300, 62.0%) was significantly higher than that in normal prostate tissues (1/20, 5.0%,

FIGURE 2. Immunohistochemical staining for EMMPRIN in PCa and normal prostate tissues. A, EMMPRIN negative staining in normal prostate tissues; EMMPRIN low (B), moderate (C) and high (D) positive expression in PCa were found in cell membrane and cytoplasm at various levels in PCa tissues. Original magnification $\times 400$.

$p < 0.001$). Representative pictures of immunohistochemistry staining of EMMPRIN are shown in Figure 2.

Correlation between EMMPRIN expression and the clinicopathologic characteristics of PCa

Table 2 displays the correlation among EMMPRIN positive expression rate and various clinicopathologic features of patients with PCa. Patients with a high preoperative PSA level (> 4 ng/mL) had significantly higher EMMPRIN positive expression rate than those with a low PSA level (≤ 4 ng/mL) ($p = 0.008$). Patients with high clinical stage ($p = 0.006$ for AJCC pathologic (pT) stage) and high-risk tumors ($p < 0.001$) had more intensive EMMPRIN expression. The elevated expression of EMMPRIN was correlated positively with the Gleason score ($p < 0.001$). EMMPRIN staining in primary tumors was significantly increased in PCa tissues with positive surgical margin ($p < 0.001$) and positive lymph node ($p < 0.001$).

EMMPRIN expression and PSA relapse after radical prostatectomy

To evaluate whether EMMPRIN expression could be used as a potential indicator of clinical outcome after radical prostatectomy, the available PSA follow-up data from 300 patients after radical prostatectomy (Table 1) was examined. The PSA

FIGURE 3. *Kaplan-Meier curves.* (A) PSA relapse-free survival was significantly shorter in patients with positive EMMPRIN expression than those without EMMPRIN expression ($P < 0.001$; Kaplan-Meier analysis). (B) In low risk group, PSA relapse-free survival was significantly shorter in patients with positive EMMPRIN expression than those without EMMPRIN expression ($P = 0.002$; Kaplan-Meier analysis). (C) In intermediate-high risk group, PSA relapse-free survival was significantly shorter in patients with positive EMMPRIN expression than those without EMMPRIN expression ($P < 0.001$; Kaplan-Meier analysis).

relapse-free time was used to indicate progression-free survival. The univariate analyses of the substantial clinico-pathologic parameters proved that the tumor cohort was representatively reflected by a higher risk of PSA relapse time with increased EMMPRIN expression ($p = 0.003$), higher AJCC pathologic (pT) stage ($p = 0.02$), Gleason Score ($p = 0.008$), higher risk classification ($p < 0.001$), positive lymph node ($p < 0.001$) and positive surgical margin status ($p = 0.03$, Figure 3 and Table 3). Multivariate Cox regression analyses, including the significant variables AJCC pathologic (pT) stage ($p = 0.01$), Gleason Score ($p = 0.01$), surgical margin status ($p = 0.02$), risk classification ($p < 0.001$) and EMMPRIN expression, confirmed EMMPRIN as an independent predictor of PSA recurrence.

Discussion

In concordance with previous observation, increased expression of EMMPRIN was observed in a larger number of PCa patients, compared with matching normal prostate tissues, which corresponds to findings in other tumors such as breast cancer [20], lung adenocarcinoma [21], gastric carcinoma [22] and carcinoma of gallbladder [23]. Sample pairs of tumor tissue and normal adjacent tissue of the same patient were also studied and translational confirmation of the transcriptional regulation of EMMPRIN expression was observed. In addition, EMMPRIN staining was observed primarily in the cell membrane and cytoplasm, with a granular pattern and no localization differences according to the morphologic status of

the prostate glands. The heterogeneous staining in tumor tissue was also confirmed. The protein encoded by the EMMPRIN gene is a plasma membrane protein that is important in spermatogenesis, embryo implantation, neural network formation and tumor progression. As a member of the immunoglobulin superfamily, the EMMPRIN protein consists of two IgSF domains, a transmembrane sequence containing a charged residue (Glu) and a cytoplasmic domain of 40 residues, which is why EMMPRIN is localized on the cell membrane and in the cytoplasm. EMMPRIN staining in both the cell membrane and cytoplasm was also described for tissue of breast cancer [20], lung adenocarcinoma [21], gastric carcinoma [22] and carcinoma of gallbladder [23] as well. A significant increase of EMMPRIN protein expression was seen in 62.0% of patients with PCa, which corresponds to our recently published data of other tumors of urinary system [17,18].

This is the first study that describes EMMPRIN mRNA and protein expression in such a large cohort of patients with PCa, and confirmed the tumor-dependent upregulation of EMMPRIN expression. The hypothesis of EMMPRIN serving as a marker of neoplasia was supported by the findings of higher EMMPRIN expression in PCa than in adjacent normal tissues. EMMPRIN has characteristics of an adhesive molecule and mediates cell-to-cell or cell-to-matrix adhesion [24-27]. Some other studies [28-32] showed that the molecule facilitates the production of MMPs from fibroblasts and tumor matrix cells; the latter can degrade most components in basal

membranes and extracellular matrixes. MMPs may also participate in late events during cancer metastatic spread, when cancer cells enter, survive and exit blood vessels or lymphatics. EMMPRIN also can affect expressions of vascular endothelial growth factor (VEGF) at RNA and protein levels, as well as promote the growth of the tumor vascular system [33,34]. Moreover, EMMPRIN is a multifunctional protein whose functional diversity may relate to distinct post-translational modifications. Differential modification through glycosylation may be cell-type specific or associated with malignancy [35].

The correlation between EMMPRIN expression and the clinico-pathologic parameters of patients with PCa supports the suggestion that the over-expression of EMMPRIN is important for the development of this disease. Preoperative PSA levels and AJCC stage were positively correlated with EMMPRIN expression, which clearly increased with increasing Gleason scores. The similar correlation was seen in other tumors of urinary system with clinical stage. Thus, the association of EMMPRIN over-expression and tumor aggressiveness is considered a general phenomenon.

An accurate prediction of the probability of disease recurrence is essential for proper therapy selection. Our finding of EMMPRIN as an independent predictor of PSA recurrence suggests that its expression could be used as an indicator to recognize patients with a high risk of tumor recurrence. In comparison with previous investigations, this study specifically analyzed the correlation between EMMPRIN expression and PSA recurrence-free survival. The positive EMMPRIN expression was associated with poor PSA recurrence-free survival in both univariate and multivariate analyses. In univariate analysis, EMMPRIN expression could predict the PSA recurrence-free survival in patients with both low-risk and intermediate-high risk according to the risk classification of defined by D'Amico *et al.* [36]. In multivariate analysis, no correlation was found between PSA recurrence and high AJCC pathologic (pT) stage, positive surgical margins or lymph node metastasis. In contrast, Gleason score, preoperative PSA level, EMMPRIN expression status and risk classification were demonstrated to be independent predictors for PSA recurrence-free survival in PCa patients.

In conclusion, over-expression of EMMPRIN, correlated with the aggressiveness of PCa and the PSA relapse-free time, and may be a novel biomarker of benefit in the follow-up and treatment decisions for PCa. Further studies are recommended to validate the efficiency of EMMPRIN in inhibiting the progression of PCa and to develop drugs that directly target EMMPRIN for the management of PCa.

TABLE 3. Univariate and multivariate Cox regression analysis of the effect on PSA relapse-free survival

	Hazard ratio (95% CI)	p
Univariate analysis		
AJCC pathologic (pT) stage	2.53(1.35-3.78)	<0.001
Gleason Score	1.96(1.34-2.53)	<0.001
Surgical margin status	2.09(1.05-3.15)	<0.001
Preoperative PSA	1.26(1.08-1.92)	<0.001
EMMPRIN expression	6.64(3.78-11.09)	<0.001
Risk classification	1.58(1.34-2.01)	<0.001
Lymph node status post surgery	2.56(1.98-2.95)	<0.001
Multivariate analysis		
AJCC pathologic (pT) stage	1.67(0.78-2.59)	0.653
Gleason Score	2.05(1.24-2.67)	<0.001
Surgical margin status	0.84(0.34-1.35)	0.341
Preoperative PSA	1.38(1.03-2.01)	<0.001
EMMPRIN expression	5.92(3.05-9.07)	<0.001
Risk classification	1.58(1.34-1.72)	<0.001
Lymph node status post surgery	1.61(1.95-0.92)	0.278

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