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Extracellular Matrix Associated Protein CYR61 is Linked to Prostate Cancer Development

Katherine B. D'Antonio, Antoun Toubaji, Roula Albadine, Alison M. Mondul, Elizabeth A. Platz, George J. Netto, and Robert H. Getzenberg*

Brady Urological Institute (KBDA, EAP, RHG), Sidney Kimmel Cancer Center (EAP, RHG) and Department of Pathology (AT, RA), The Johns Hopkins University School of Medicine (GJN) and Department of Epidemiology, The Johns Hopkins Bloomberg School of Public Health (AMM, EAP), Baltimore, Maryland

Abstract

Purpose—The cancer cell microenvironment includes complex interactions between the cell and the extracellular matrix. Expression of the CCN family of extracellular matrix associated proteins is often modified in disease states. Depending on cancer type these changes are linked with enhanced or inhibited tumor growth. We characterized Cyr61 in prostate cancer. Cyr61 is an integrin binding matricellular protein with altered expression in many cancer types.

Materials and Methods—Cyr61 expression in prostate cancer, benign prostatic hyperplasia and normal tissues was evaluated by microarray analysis, quantitative real-time polymerase chain reaction and tissue microarray. Immunoblots were analyzed to assess endogenous protein expression in prostate cancer cell lines.

Results—On genomic analysis Cyr61 up-regulation was observed in prostate cancer tissue and in normal prostate tissue adjacent to tumor vs that in prostate donor tissue. In 174 matched tumors and normal prostate tissues adjacent to tumor tissue microarray revealed significantly up-regulated Cyr61 protein expression in cancer tissue vs normal prostate tissue adjacent to tumor. Also, increased Cyr61 expression correlated with Gleason sum 8 or greater cancer. Staining in high grade prostatic intraepithelial neoplasia was moderately up-regulated vs that in normal prostate tissue adjacent to tumor but generally less intense than in carcinoma tissue.

Conclusions—In addition to the correlation with more advanced disease, the strong association between Cyr61 expression and prostate cancer supports the potential usefulness of Cyr61 as a novel biomarker for prostate cancer. This warrants further analysis to determine the mechanisms by which Cyr61 may contribute to prostate cancer development and progression.

Keywords

prostate; prostatic neoplasms; cysteine-rich protein 61; biological markers; microarray analysis

In the United States an estimated 27,630 patients will die of prostate cancer this year, making it the second leading cause of cancer death in men,¹ and more than 6½ times that

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*Correspondence: The Brady Urological Institute, The Johns Hopkins Hospital, Marburg 121, 600 North Wolfe St., Baltimore, Maryland 21287 (telephone: 410-502-3137; FAX: 410-502-9336; rgetzen1@jhmi.edu).

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number will be diagnosed with the disease. Despite the prostate specific antigen revolution, which greatly improved the prostate cancer early detection rate, many aspects of diagnosis would be improved by identifying and implementing specific biomarkers. No marker in clinical use reliably differentiates indolent from aggressive prostate cancer. Such a marker would minimize overtreatment and radically alter existing prostate cancer treatment practices.²

Although increasing prostate specific antigen suggests prostate cancer, more likely these increases result from aging and other common prostatic diseases, including BPH and prostatitis. Finally, the standard means of diagnosing prostate cancer is on biopsy by morphology, and hematoxylin and eosin stain but even this is not always definitive. In about 20% of cases additional analysis involving IHC staining for basal cell markers and AMACR is done to improve diagnostic accuracy. A subset of cases remain categorized as atypical due to lack of definitive features of malignancy or the limited amount of cancer representation on needle biopsy.³ We evaluated Cyr61 expression in prostate carcinoma, PIN and benign prostatic tissue, and assessed the ability of Cyr61 to discriminate prostate cancer from BPH or healthy prostate tissue.

Cyr61, also named CCN1, is an extracellular matrix protein involved in growth factor transduction, hormone signaling and mechanical stress response mediation. Cyr61 has been shown to keep cells apprised of changes in the cellular environment. The CCN protein family, named after the earliest identified members Cyr61, CTGF and Nov, is characterized by 4 common conserved domains, including a von Willebrand's factor type C repeat, a thrombospondin type 1 repeat, an insulin-like growth factor binding protein motif and a cysteine-knot motif. CCN proteins have highly regulated expression, and influence growth and development. Cyr61 participates in regulating many pathways, including cell adhesion, migration, proliferation, differentiation and survival.⁴⁻⁹ The specific function of Cyr61 remains largely unknown but its biological activity is believed to be contextual and cell type dependent.¹⁰ Cyr61 signals through interaction with integrins but downstream effects vary greatly depending on the combination of integrins bound.¹¹⁻¹⁴

Numerous genomic studies show altered Cyr61 expression in various cancers, including breast, ovarian, hepatocellular, lung and colorectal cancer, and depending on cancer type Cyr61 may enhance or inhibit tumor growth.¹⁴⁻¹⁸ A previous study showed that Cyr61 mRNA was down-regulated in prostate cancer tissue vs normal tissue adjacent to cancer.¹⁹ Our more recent study revealed moderately increased Cyr61 in symptomatic BPH samples vs that in healthy donor prostates. In a previous study we found greater Cyr61 expression in prostate cancer samples than in BPH or donor prostates.²⁰ Sakamoto et al noted on in situ hybridization that Cyr61 localizes to basal cells in normal prostate tissue.²¹ Additional evidence substantiating the importance of Cyr61 for prostate cancer indicates that transfection with Cyr61 enhances prostate cancer cell migration, invasion and proliferation, and Cyr61 regulates Rac1 signaling, a mechanism by which Cyr61 may exert its influence on growth and motility.²² We more thoroughly characterized Cyr61 expression in prostate cancer tissue.

MATERIALS AND METHODS

RNA Preparation and Quantitative PCR Analysis

Tissues were homogenized by rheostat homogenizer. Total RNA was extracted by the RNeasy® Mini Kit according to manufacturer instructions and treated with deoxyribonuclease (Ambion®) to remove genomic DNA. Hex-reverse transcription was done on 1 µg total RNA, 10U/µl Moloney murine leukemia virus-reverse transcriptase enzyme (Epicentre®), 40U/µl RNase Inhibitor (Promega®), 1.25 mM hexamer primers, 25

mM deoxynucleoside triphosphates, 10 μ l 10 $\times$ PCR buffer and 75 mM MgCl₂. Cyr61 expression (probe 5'-AGCAAGACCAAGAAATCCCCGAACC-3', and primers 5'-GCAGCCTGAAAAAGGGCAA-3' and 5'-AACATCCAGCGTAAGTAAACCTGAC-3') was normalized against the housekeeping gene glucuronidase- β (probe 5'-TGAACAGTCACCGACGAGA-3', and primers 5'-CTCATTTGGAATTTTGCCGATT-3' and 5'-CCGAGTGAAGATCCCCTTTTAA-3'), 5'-FAMTM fluorescent dye and 3'-TAMRATM quencher dye. Each 50 μ l reaction contained 1 $\times$ TaqMan[®] Universal PCR Mastermix composed of AmpliTaq Gold[®] DNA polymerase, 25 mM deoxynucleoside triphosphates and optimized buffer components, including 3.5 mM MgCl₂, with 5 μ l cDNA, 200 nM primers and 100 nM probe. Amplification was done at 95C for 12 minutes for 40 cycles (15 seconds at 95C and 1 minute at 60C) on the ABI Prism[®] 7700 sequencer. Reactions were done at 1 $\times$ and 1/4 $\times$ concentrations with a no-template control containing water and a cDNA positive control. Results were analyzed by the $\Delta\Delta$ Ct method (Δ Ct sample – Δ Ct value calibrator) to determine relative Cyr61 expression per sample.

Western Blot Analysis

Total protein lysates were obtained from cell pellets by repeat shearing using a 26 gauge needle in Triton-X100 buffer, composed of 1% Triton-X100, 10 mM tris (pH 7.4), 150 mM NaCl and 1 mM ethylenediaminetetraacetic acid, and containing aprotinin, polymethyl sulfoxide and protease inhibitor cocktail tablets (Roche, Indianapolis, Indiana). Lysates were centrifuged at 13,000 rpm for 10 minutes at 4C. Protein lysate concentrations were determined by Bradford assay. Protein (30 μ g) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Membranes were probed with anti-Cyr61 primary antibody (1:200) and secondary antibody (1:3,000), and detected by echochemiluminescence (Thermo Fisher Scientific, Rockford, Illinois). Equal protein loading was determined by Ponso S (Sigma[®]).

Cells and Tissues

The study was approved by our institutional review board. Prostate cell lines derived from epithelium (267B1, BPH1 and BRF55T), cancer (LAPC4, LNCaP, CWR22RV1, VCAP, DUCaP, PC3 and Du145) and stroma (6S) were evaluated. TMAs were obtained from the department of pathology TMA facility, The Johns Hopkins School of Medicine. Tissue specimens used for TMA were obtained from 200 consecutive RRP performed at The Johns Hopkins Hospital from 2000 to 2001. Specimens were dissected immediately after surgical removal, immersed in 10% neutral buffered formalin before paraffin embedding and processing for IHC. Five high density TMAs were used to determine the cellular distribution of Cyr61 staining in NAT, high grade PIN tissue and prostate cancer tissue. A total of 13 BPH tissues obtained by transurethral prostate resection due to BPH were analyzed. Control breast cancer (positive) and lung tissue (negative) were evaluated for Cyr61 expression. Tissue for real-time PCR analysis was obtained from elsewhere. Samples (greater than 500 mg) were excised, snap frozen in liquid nitrogen within 30 minutes of excision and stored at -80C until RNA isolation. All samples underwent pathological evaluation to verify classification.

Immunohistochemistry

Cyr61 rabbit polyclonal antibody, epitope 163–240 (sc-13100, Santa Cruz Biotechnology, Santa Cruz, California) was used to stain slides and TMAs. Immunostaining for Cyr61, including deparaffinization and antigen retrieval, was done on a Benchmark XTTM autostainer using the iVIEWTM diaminobenzidine tetrahydrochloride detection kit. Slides were incubated for 90 minutes with ethylenediaminetetraacetic acid buffer and then with primary antibodies (1:200) for 32 minutes at room temperature. The reaction was visualized

by peroxide/diaminobenzidine tetrahydrochloride and slides were counterstained with hematoxylin.

Scoring and Data Analysis

Due to the diffuse, uniform intensity of Cyr61 expression by all glandular epithelial cells in a given TMA spot in tumor, PIN or NAT assessment of the staining area (percent of cells stained) in each TMA spot was not required. A visual intensity score was assigned for each spot by a urological pathologist using a 3-tier intensity system, including 3—strong, 2—moderate, 1—weak and 0—no staining. BPH slides were analyzed by the same pathologist and method.

To validate visual scoring method accuracy these semiquantitative visual intensity score results for 1 of the 5 TMAs were compared to quantitative analysis of Cyr61 staining intensity done by image analysis. The latter was done by capturing TMA core images using the ScanScope® XT scanner. Images were imported into TMAJ Image application (<http://tmaj.pathology.jhmi.edu>), as described previously.²³ Subsequently average intensity and area of staining in each TMA core image was calculated using Frida custom open source image analysis software (<http://sourceforge.net/projects/fridajhu/>), as described by Gurel et al.²⁴ To eliminate potential bias due to variation in epithelial-to-stromal components among cores the total area of epithelial cells in each spot was first calculated and the ratio of the sum of intensity of Cyr61 expression to total epithelial cell area served as the final Frida analysis intensity score for each TMA spot.

Statistical Analysis

To test the correlation between Frida analysis and pathologist assigned visual intensity scores we calculated a correlation coefficient by pairwise correlation analysis using Stata® 9.0. Median staining intensity by spot type was determined by calculating median staining intensity of all spots of a given type in an individual and calculating the median across all individuals. Paired analysis of median staining intensity between spot types were performed using the Wilcoxon signed rank test. Paired analysis of whether at least 1 spot per individual met a threshold value when comparing spot types was done by McNemar's test. Paired analysis was also done using a repeated measures modeling approach with a robust variance, which yielded similar results (data not shown). Analysis comparing median staining intensity in cancer spots in men with Gleason sum 8 or greater vs less than 8 were performed by the Wilcoxon rank sum test.

RESULTS

Increased Cyr61 mRNA in Prostate Cancer Tissue

To confirm gene expression microarray analysis previously reported by a group from our laboratory²⁰ and more fully characterize prostate Cyr61 expression we performed quantitative gene expression analysis to compare gene expression in healthy donor prostate tissue and tissue from prostates with cancer foci. Cyr61 expression was increased in 10 of 11 prostate cancer tissues (91%) vs 10 donor tissues (9.05 ± 5.44 vs 1.84 ± 1.34 , $p < 0.05$, fig. 1). To support the notion of altered Cyr61 expression in prostate cancer tissue we queried the Oncomine™ database. In 2 studies Cyr61 was up-regulated in prostate cancer tissue vs healthy prostate and PIN tissues, and in 9 studies in which expression was compared in primary prostate cancer vs metastatic tissues Cyr61 expression was lower in metastases than in the primary lesion.²⁵

Cyr61 Protein Expression

Cyr61 protein expression in prostate cell lines, including primary, immortalized epithelial and prostate cancer derived lines, was present in 10 of 11 prostate lines (fig. 2). All except VCaP cells expressed Cyr61.

Initial Cyr61 IHC was done in routine prostate tissue sections from 18 RRP specimens to characterize prostate Cyr61 localization. Cyr61 protein appeared significantly up-regulated in prostate cancer compared with adjacent histologically normal glands (data not shown). Expression was consistently intense in each lesion, had a diffuse, finely granular nature and was cytoplasmic in location. No evidence of Cyr61 expression was observed in the stroma.

Based on these findings a tissue microarray of 1,366 spots representing 197 prostates from consecutive RRPs for prostate cancer were analyzed for Cyr61 protein expression and localization (table 1 and fig. 3), consisting of 174 matched tumor/NAT, 25 matched PIN/NAT and 23 matched PIN/tumor tissue samples. Similar cytoplasmic staining was observed in TMAs as in the described tissue sections. Only weak Cyr61 staining was detected in histologically normal prostate tissue while cancer areas generally showed more intense Cyr61 staining. On a scale of 0 to 3 median staining intensity across individuals was 0 for normal tissue, 1 for atrophy, 2 for PIN and 2 for cancer (p trend <0.0001 , table 2 and fig. 4, A). Area under the ROC curve was 0.928 when comparing median staining intensity for cancer and normal spots (fig. 4, B). Staining intensity was 1 or greater, 2 or greater and 3 or greater in 98.3%, 85.1% and 60.9% of cancer spots, and in 60.3%, 21.3% and 1.7% of normal spots, respectively ($p <0.0001$). In men diagnosed with prostate cancer with a Gleason sum of 8 or greater median Cyr61 staining intensity was higher in cancer spots than in men with a Gleason sum of less than 8 ($p = 0.01$, fig. 4, A). However, no threshold value statistically differentiated higher vs lower Gleason sum cancers (data not shown). The incidence of staining intensity 3 was significantly higher in cancer areas than in PIN areas (table 3). Comparison of Frida intensity and visual intensity measurements in 1 of the 5 TMAs revealed a strong correlation on pairwise correlation analysis (correlation coefficient 0.7166, $p <0.0001$).

Cyr61 expression in BPH tissue sections from 13 transurethral prostate resection specimens was analyzed by IHC to characterize Cyr61 localization in benign prostatic lesions. No evidence of increased Cyr61 expression was observed in any BPH tissue in glands or stroma (fig. 3).

DISCUSSION

A proposed mechanism to contribute to prostate cancer initiation is that the balance established during organogenesis between stroma and epithelium, reciprocally regulating prostate growth and development, becomes altered by or during cancer pathogenesis. Disruption of epithelial-stromal interactions most likely involves aberrant signaling between cell types and is an important component of prostate cancer. Current understanding of epithelial-stromal crosstalk remains limited and further study is needed to identify key proteins in this dynamic relationship. With this dynamic in mind the extracellular matrix associated protein Cyr61 holds unique potential in that it gauges the extracellular environment, transmitting signals that alter downstream pathways involved in tumorigenesis, including proliferation and migration.

Most prostate cancers are diagnosed by histological analysis of hematoxylin and eosin stained biopsy tissue but a subset of diagnostically challenging cases require further characterization for accurate diagnosis. In these cases IHC is done for AMACR and basal cell markers, such as high molecular weight cytokeratins or p63, to help differentiate

prostate cancer from benign disease. AMACR, also known as racemase, is characteristically up-regulated in 80% to 100% of prostate cancers.²⁶ This marker also stains up to 21% of benign prostatic glands and 79% of partial atrophy lesions, potentially leading to biopsy sample misinterpretation.²⁶ Supplementing hematoxylin and eosin staining with these IHC markers clearly improves the accuracy of prostate cancer diagnosis. However, additional prostate cancer specific markers are needed to complement current markers, which differentially stain benign prostate glands that may be misdiagnosed, or identify prostate cancer that is not detected by current methods. Unlike current prostate cancer IHC markers, an ideal marker would also have prognostic value.

Our study shows similarities between Cyr61 and AMACR, particularly increased expression of the respective proteins in prostate cancer vs histologically normal adjacent tissue or normal donor prostate tissue. Cyr61 and AMACR are primarily expressed in cancer and in some PIN tissues. Although we did not specifically address it, based on expression and staining pattern resemblances between Cyr61 and AMACR we propose that IHC evaluation for Cyr61 expression has similar potential to aid in diagnosing problematic atypical cases. The uniform pattern of cytoplasmic Cyr61 staining in a given cancer focus has a potential advantage over the variable intensity of AMACR staining in individual cancer glands in a given focus.

Although our study was not specifically designed to address this point, using Gleason sum as a prognostic indicator Cyr61 appears to differentiate the most aggressive prostate cancer cases from less aggressive cases. Oncomine studies comparing primary prostate cancer to metastatic lesions show higher Cyr61 expression in primary tumors.²⁵ This suggests that Cyr61 is likely important for prostate cancer development and/or maintenance rather than metastasis or invasion. Despite the benefits of studying Cyr61 expression in a series of consecutive RRP cases, providing a snapshot of Cyr61 expression in the average man who presents with prostate cancer, our study provides limited prognostic information. Further analysis of Cyr61 expression in a population with longer followup and a higher incidence of disease progression is needed to assess Cyr61 prognostic potential. The potential of Cyr61 to serve as a blood or urine based biomarker may be limited by its lack of prostate specificity.

In distinct tissues Cyr61 influences various signaling pathways involved in the development and regulation of normal physiological functions. The biological mechanisms by which Cyr61 exerts its influence on the cellular environment remain elusive and may in part be a consequence of context dependent signaling by Cyr61, resulting in the activation of different downstream pathways in response to varying stimuli. Clearly further study is necessary to define the pathways upstream and downstream of Cyr61. Developing a better understanding of the role of this protein is particularly vital, given the number of studies documenting its altered expression in diseases ranging from cardiovascular disease to arthritis to cancer.^{9,16-18,27,28} For example, Cyr61 up-regulation in prostate cancer in our study focuses the need for further analysis of the role of Cyr61 in prostate cancer and normal prostate tissues. Analysis of Cyr61 by Sun et al showed that Cyr61 can enhance prostate cell proliferation and suggested that Rac1 signaling may be a mechanism by which Cyr61 exerts its influence.²² Further analysis of the impact of Cyr61 over expression and evaluation of the pathways affected is needed to define how Cyr61 contributes to prostate cancer development and/or progression.

The presented Cyr61 expression pattern in BPH does not agree with previous series showing increased expression in BPH, particularly the stroma, in which we detected no staining in any of our donor, BPH or cancer tissue samples. Most previous studies in BPH tissue involved mRNA analysis^{21,29,30} or were done in cells stimulated by fetal bovine serum.^{21,29} Thus, differences in previously published data may indicate altered mRNA expression that

does not translate to increased protein expression. The lack of consistency in Cyr61 expression in BPH tissue warrants further analysis.

CONCLUSIONS

In addition to recent findings, our study of Cyr61 expression in prostate distinguishes Cyr61 as an exciting new target in prostate cancer. TMA expression suggests a potential role for Cyr61 in the characterization of hard to diagnose prostate cancer cases. Extensive analysis of Cyr61 expression is needed to study its relationship to disease progression and prognosis, and thoroughly evaluate its clinical usefulness.

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Abbreviations and Acronyms

AMACR	α -methylacyl-coenzyme A racemase
BPH	benign prostatic hyperplasia
CCN	family Cyr61
Ct	cycle threshold
Cyr61	cysteine-rich angiogenic inducer 61
Frida	framework for image data set analysis
IHC	immunohistochemistry
NAT	normal prostate tissue adjacent to prostate cancer
PCR	polymerase chain reaction
PIN	prostatic intraepithelial neoplasia
RRP	radical retropubic prostatectomy
TMA	tissue microarray analysis

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Figure 1.

Cyr61 gene expression. Circles indicate relative mRNA abundance as Cyr61 message normalized to glucuronidase- β (*Gus*) in tissues, and LNCaP, PC3, BPH1 and 267B1 cell lines. In LNCaP cells expression was notably lower than in other cell lines.

Figure 2. Western blot analysis of Cyr61 protein. Total protein was analyzed in various prostate cell lines.

Figure 3.

IHC reveals Cyr61 protein expression in prostate tissues. *A* and *B*, NAT. *C* and *D*, BPH. *E* and *F*, high grade PIN. *G* and *H*, tumor. *I* and *J*, tumor with NAT. Reduced from $\times 100$ (*A*, *C*, *E*, *G* and *I*) and $\times 200$ (*B*, *D*, *F*, *H* and *J*).

Figure 4.

A, Cyr61 expression by TMA spot IHC score showing median staining intensity in matched pairs by spot type. *ref*, referent. *B*, Cyr61 ROC curve to discriminate prostate cancer tissue vs NAT.

Table 1

Demographic characteristics of 200 patients with RRP in whom spots from highest grade PCa and benign tissue were sampled

No. pts	200	
% Race:		
White	86	
Black	9.50	
Other	4.50	
Age at RRP:		
Mean $\pm$ SD	57.89 $\pm$ 6.88	
Median (range)	58 (37–74)	
No. stage (%):		
pT2	131	(65.5)
pT3a	52	(26)
pT3b	12	(6)
N1	4	(2)
No. Gleason sum (%):		
5–6	110	(55)
7	67	(33.5)
8–10	23	(11.5)
Followup (yrs):		
Mean $\pm$ SD	2.9 $\pm$ 1.84	
Median	2	

Table 2

Paired comparisons of atrophy, PIN and cancer with normal staining

	Normal	Atrophy	PIN	CA
Median No. spots/pt	4	1	1	3
No. pairs	—	40	25	174
Median staining intensity	0	1	2	2
p Value (Wilcoxon signed rank test)	Referent	0.0009	<0.0001	<0.0001
% Spots staining intensity:				
1 or Greater	60.3 [*]	70.00	96.00	98.30
p Value (McNemar's test)	Referent	0.21	0.003	<0.0001
2 or greater	21.3	15.00	64.00	85.10
p Value (McNemar's test)	Referent	0.32	0.0009	<0.0001
3 or Greater	1.7 [*]	5.00	20.00	60.90
p Value (McNemar's test)	Referent	0.56	0.18	<0.0001

^{*} Total of 174 pairs comparing normal vs cancer tissue.

Table 3

PIN and cancer staining paired comparisons

	PIN	Ca
Median No. spots/pt	1	3
No. pairs	—	23
Median staining intensity	2	2.5
p Value (signed rank test)	Referent	0.06
% Spots staining intensity:		
1 or Greater	100	95.70
p Value (McNemar's test)	Referent	—
2 or Greater	65.20	78.30
p Value (McNemar's test)	Referent	0.32
3 or Greater	17.40	60.90
p Value (McNemar's test)	Referent	0.01