Integrin-linked Kinase Expression Increases with Prostate Tumor Grade[1]

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ABSTRACT

Purpose: **Integrin-linked kinase (ILK) overexpression can suppress anoikis, promote anchorage-independent cell cycle progression, and induce tumorigenesis and invasion. Inhibition of ILK in prostatic adenocarcinoma (CaP) cells elicits cell cycle arrest and induces apoptosis. Furthermore, ILK expression increases with androgen-independent progression of human CaP cell lines, suggesting that increased ILK expression may be associated with CaP progression.**

Experimental Design: **To assess whether ILK expression may be related to CaP development and/or progression, we have evaluated ILK expression by immunohistochemistry in 100 human prostate tissues.**

Results: **We show that ILK expression increases significantly with CaP progression. ILK immunostaining is specifically increased in high-grade, primary human CaP relative to adjacent benign prostatic hyperplasia ($P < 0.001$), benign prostatic hyperplasia from patients without cancer ($P < 0.002$), and low-grade CaP ($P = 0.003$). ILK overexpression is specifically associated with the increased proliferative index ($P = 0.001$) that typifies CaP progression. Strikingly, intense uniform ILK immunostaining was inversely related to 5-year patient survival ($P = 0.004$).**

Conclusions: **ILK expression increases dramatically with CaP progression. ILK expression is also specifically related to the disproportionately increased proliferative index that contributes to the net gain of CaP cells during progression. Finally, enhanced ILK expression is inversely related to 5-year patient survival. These data therefore implicate increased ILK expression in prostate tumor progression.**

INTRODUCTION

ILK[3] interacts with the β_1 and β_3 integrins and is activated by cell-extracellular matrix interactions (1, 2). ILK overexpression in epithelial cells suppresses anoikis and elicits anchorage-independent cell cycle progression (1–3), tumorigenesis, and tumor invasiveness (1, 4). Recent studies have indicated that ILK facilitates the phosphorylation of AKT at Ser^{473}, which is required for AKT activation (1, 5, 6). By regulating the activity of AKT as well as glycogen synthase kinase 3 (1, 5), ILK facilitates the assembly and activity of the β-catenin/LEF-1 transcription complex (1, 4), enhances cyclin D1 expression and CDK activity (1, 3), and suppresses expression of the invasion suppressor E-cadherin (1, 4, 7).

Recent reports have suggested that ILK may be involved in prostate tumorigenesis and progression. Inhibition of ILK in CaP cell lines suppresses AKT activation, induces cell cycle arrest, and elicits apoptosis (8). Furthermore, we have recently shown that ILK expression increases in androgen-independent human CaP cells and xenografts derived from the androgen-dependent CaP cell line LNCaP (9). Although these reports implicate ILK in experimental models of human prostate cancer, there are currently no data regarding ILK expression in primary human CaPs. To assess whether ILK might be involved in CaP development and/or progression, we have evaluated ILK expression by immunohistochemistry in 100 primary prostate tissues. Our data reveal that ILK expression was significantly increased in the highest grade CaPs compared with BPH from patients without cancer, BPH adjacent to CaP, and low-grade CaP. Moreover, 81% of patients whose tumors overexpressed ILK failed to survive for 5 years. Together, these data implicate increased ILK expression in human prostate tumor progression.

MATERIALS AND METHODS

Prostate Tissue Collection. Archival, formalin-fixed, paraffin-embedded surgical specimens of BPH and CaPs were obtained from St. Elizabeth Medical Center (Covington/Edgewood, KY). Surgical consultation reports and clinical follow-up information were available for all prostate specimens and cancer patients. This study had the approval of the Institutional Review Board of St. Elizabeth Medical Center. A H&E-stained section from each block of prostate tissue studied was reviewed by a board-certified pathologist (H. W. C.) and graded according to the Mostofi grading system (10). According to this grading system, M1 represents well-differentiated CaP, M2 represents

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[3] The abbreviations used are: ILK, integrin-linked kinase; CaP, prostatic adenocarcinoma; BPH, benign prostatic hyperplasia; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

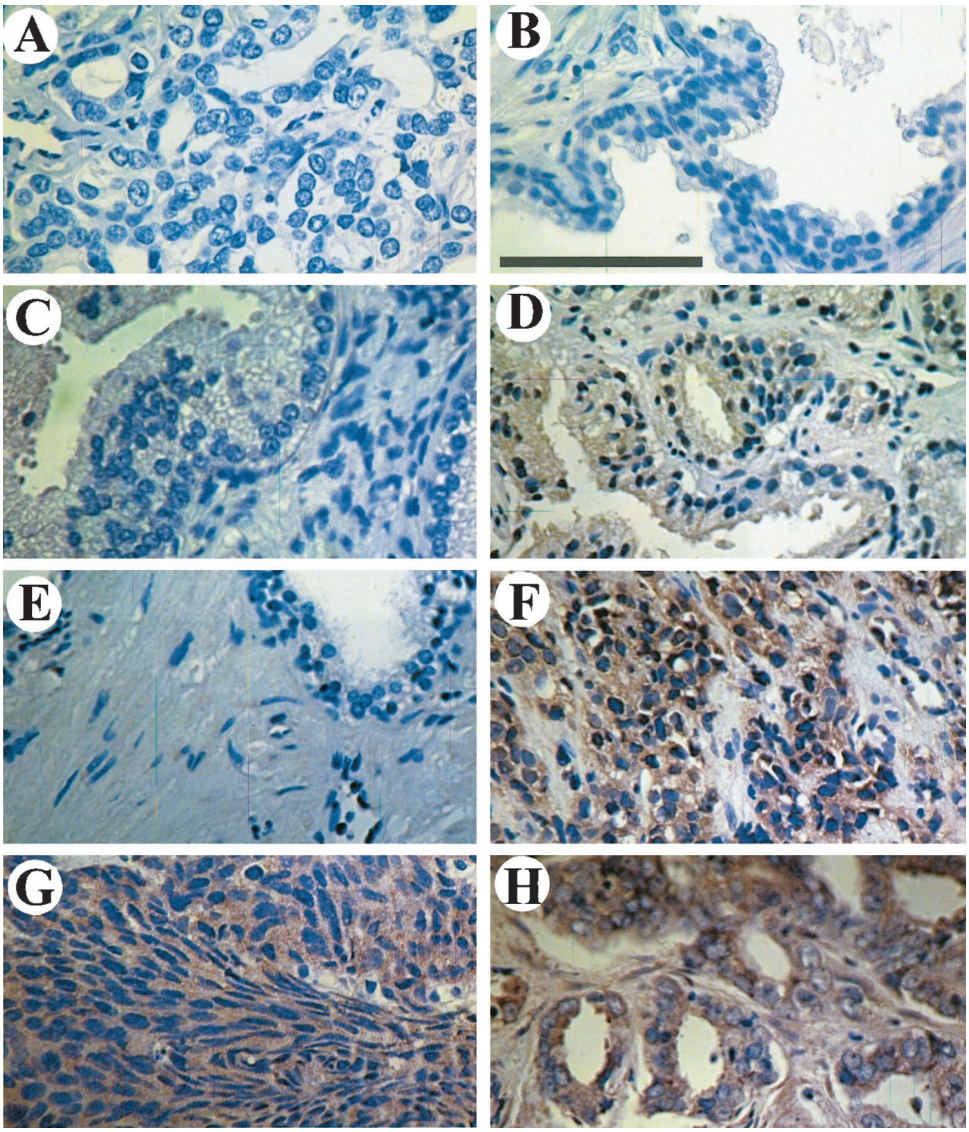

Fig. 1 ILK immunostaining of BPH and CaP. Representative immunostained sections are shown. *A* represents a high-grade CaP (the same as that shown in *H*) stained with rabbit IgG followed by antirabbit secondary antibody as a negative control for staining. *B* depicts a typical stain of BPH from a noncancer patient with little to no staining. *C* shows BPH adjacent to the M1+M2 tumor in *D*. *E* depicts BPH adjacent to the high-grade M2+M3 tumor in *F*. *G* represents a M3 tumor, whereas *H* shows a high-grade M2+M3 tumor. Note the intense, uniform staining in the high-grade CaPs (*F–H*) in contrast to the focal staining characteristic of the low-grade CaPs (*D*). *Black bar* in *B*, 100 μM.

moderately differentiated CaP, and M3 represents poorly differentiated CaP (10). Samples designated M1+M2 and those designated M2+M3 were heterogeneous, with both tissue patterns present. The Mostofi grades assigned correspond to Gleason grades as follows. The M1 grade for well-differentiated tumors corresponds to Gleason grades 1 and 2. The M2 grade tumors correspond to tumors of Gleason grade 3. High-grade, poorly differentiated M3 tumors correspond to the least-differentiated Gleason grade 4 and 5 tumors (11).

Immunohistochemistry. Antibody staining was performed on 5-μm histological sections of formalin-fixed, paraffin-embedded surgical specimens. All sections were deparaffinized in Hemo-De (Fisher, Pittsburgh, PA) and rehydrated through a graded series of alcohols before antigen retrieval (DakoTarget Retrieval Solution; DAKO Corp., Carpinteria, CA). After incubation with 0.3% peroxide in methanol and incubation with normal blocking serum, sections were incubated with primary anti-ILK antibody (0.2 μg/ml; Upstate Biotechnology, Lake Placid, NY) at 4°C overnight. Immunodetection was performed with the Vectastain ABC rabbit IgG kit (Vector Laboratories, Burlingame, CA) using diaminobenzidine as the chromogen. Positive controls for ILK immunostaining were formalin-fixed, paraffin-embedded human prostate cancer xenografts previously shown to be high expressors of ILK by Western blot analysis (LNCaP and LNAI; Ref. 9) and human placenta, the tissue from which ILK was initially cloned (Ref. 2; data not shown). Negative controls included CaP sections stained without antibody (data not shown) and sections stained first with nonspecific rabbit IgG at the same protein concentration as the primary anti-ILK antibody, followed by horseradish peroxidase-linked antirabbit secondary antibody (Fig. 1*A*). All sections were counterstained with Gill's hematoxylin.

Evaluation of Immunohistochemical Stain. Staining patterns including tissue distribution (epithelial/stromal), extent

Table 1 Summary of ILK immunostaining in human prostate tissues

Statistical analyses comparing negative/focal *versus* uniform staining patterns between groups were performed by Fisher's exact 2 two-tailed test. Uniform staining for ILK was significantly different in CaP (groups A and B) *versus* BPH in noncancer patients (group E; $P < 0.002$) and *versus* BPH adjacent to CaP (groups C and D; $P < 0.001$). The incidence of uniform staining was significantly increased in high-grade CaP *versus* adjacent BPH (group C; $P < 0.001$) or *versus* low-grade CaP (group B; $P = 0.003$). Uniform staining was not significantly different between low-grade CaP and adjacent BPH (group B *versus* group D; $P = 0.226$).

Prostate tissue type	Uniform	Focal	Negative
BPH, noncancer patient (group E)	2/27 (7%)	17/27 (63%)	8/27 (30%)
BPH adjacent to low-grade CaP (group D)	0/16 (0%)	11/16 (69%)	5/16 (31%)
BPH adjacent to high-grade CaP (group C)	0/18 (0%)	7/18 (39%)	11/18 (61%)
Low-grade CaP (total; group B)	2/15 (13%)	8/15 (53%)	5/15 (33%)
M1 and M1+M2	2/9 (22%)	5/9 (56%)	2/9 (22%)
M2	0/6 (0%)	3/6 (50%)	3/6 (50%)
High-grade CaP (total; group A)	15/24 (63%)	8/24 (33%)	1/24 (4%)
M2+M3	9/16 (56%)	6/16 (38%)	1/16 (6%)
M3	6/8 (75%)	2/8 (25%)	0/8 (0%)

of stain, and stain intensity were evaluated for each specimen by two experienced investigators [H. W. C. (a board-certified pathologist) and J. H. C.]. In specimens with CaP, the staining pattern was noted for both the neoplasm and the adjacent BPH tissue. The evaluation was categorical, and staining was classified as negative (<10% of the epithelium stained), focal (heterogeneously positive with less than 50% of the epithelium stained in the vast majority of cases), or uniformly positive (involving more than 90% of the epithelium).

Assessment of Proliferation and Apoptosis. Proliferative index was assessed by immunostaining with mouse monoclonal antibody to Ki-67 [2 μg/ml; Oncogene Research Products, Cambridge, MA (12)] after antigen retrieval for 5 min in a microwave oven in 0.1 M citrate buffer (pH 6.0). Ki-67 positivity was detected with the Vectastain ABC mouse IgG kit (Vector Laboratories). These sections were counterstained with methyl green. An average number of 2618 ± 197 cells was counted per section for Ki-67 positivity to determine the percentage of cells undergoing proliferation. Apoptotic index was assessed by TUNEL staining (13). To assess the percentage of cells undergoing apoptosis, an average of 3809 ± 284 cells was counted per section for terminal deoxynucleotidyl transferase positivity. The proliferative or apoptotic index was then calculated by the following equation: (number of stained nuclei:total number of nuclei counted) × 100%.

Statistical Analyses. All statistical analyses were performed with the SAS program (SAS, Cary, NC). For statistical analyses, tissues were divided into five groups: (*a*) group A, high-grade CaP (M3 and M2+M3); (b) group B, low-grade CaP (M2, M1+M2, and M1); (*c*) group C, BPH adjacent to high-grade CaP (*i.e.*, group A); (*d*) group D, BPH adjacent to low-grade CaP (*i.e.*, group B); and (*e*) group E, BPH from patients without cancer. For all statistical analyses, staining patterns compared were negative/focal *versus* uniform. Relationships between staining patterns and each tissue group were evaluated by Fisher's exact two-tailed test. The relationship between staining and 5-year patient survival was also assessed by the Fisher's exact two-tailed test. Differences between groups with respect to proliferative index (*i.e.*, Ki-67 staining; Ref. 12) and apoptotic fraction (TUNEL staining; Ref. 13) were calculated using Student's *t* test.

RESULTS AND DISCUSSION

To assess whether ILK expression may be up-regulated in CaPs relative to benign prostate tissue (*i.e.*, BPH), we examined BPH from patients without cancer ($n = 27$), BPH adjacent to CaP ($n = 34$), and invasive CaP ($n = 39$) by immunohistochemistry (summarized in Table 1). Staining for ILK was largely confined to the epithelial component of the tissues and was cytoplasmic (Fig. 1). The majority of BPH from noncancer patients (63%) and BPH adjacent to CaP (53%) showed no staining or weak, focal staining for ILK (Table 1; Fig. 1, *B*, *C*, and *E*). Uniform ILK immunostaining involving >90% of the epithelium was absent from adjacent BPH (0%; Fig. 1, *C* and *E*) and evident in only 7% of BPH from noncancer patients (Fig. 1*B*; summarized in Table 1) but was evident in 44% of the CaPs (see Fig. 1, *F–H*). The incidence of tumor samples with uniform staining for ILK was significantly different from that of the adjacent BPH (groups A and B *versus* C and D, $P < 0.001$) and BPH from patients without cancer (groups A and B *versus* E, $P < 0.002$). These data indicate that ILK expression is up-regulated in CaPs relative to BPH.

To evaluate whether ILK expression was increased with prostate tumor progression, we compared the ILK staining patterns in low-grade *versus* high-grade CaPs. Only a small fraction of the low-grade CaPs (M1, M1+M2, and M2) showed uniform staining (13.3%; Table 1). Most of these low-grade CaPs showed only weak, focal staining (see Fig. 1*D*). In contrast, the majority (62.5%) of high-grade CaPs (M2+M3 and M3) showed intense, uniform staining throughout the tumor (Fig. 1, *F–H*). These staining patterns in the high-grade CaPs differed significantly from those in low-grade CaPs (group A *versus* group B, $P = 0.003$) and also from those in the adjacent BPH (group A *versus* C, $P < 0.001$). In contrast, low-grade tumors did not differ significantly from the adjacent BPH (group B *versus* D, $P = 0.226$). These data indicate that intense, uniform ILK immunostaining is specifically associated with high-grade CaPs.

Prostate cancer progression involves an increase in both proliferation and apoptosis, with proliferation outstripping apoptosis, thereby contributing to the net increase in cell number that accompanies progression (14–16). Because ILK has been

Table 2 Uniform ILK immunostaining is related to apoptosis and proliferation

Proliferative index was assessed by Ki-67 staining (12), and apoptotic index was assessed by TUNEL staining (13). Values represent the mean values ± SE from the mean. An average of 2618 ± 197 cells was counted per section for Ki-67 positivity. An average of 3809 ± 284 cells was counted per section for terminal deoxynucleotidyl transferase positivity. The proliferative or apoptotic index was then calculated by the following equation: (number of stained nuclei/total number of nuclei counted) × 100%.

Staining pattern	Apoptotic index	Proliferative index
Focal/negative	0.80 ± 0.07	1.46 ± 0.18
Uniform	1.31 ± 0.19	4.74 ± 0.85

defined as an oncogene that promotes cell cycle progression and affects apoptosis (1, 4, 5, 8), we next asked whether ILK expression in these prostate tumors might be related to these parameters. In this set of samples, uniform ILK immunostaining was strongly related to proliferation ($P = 0.001$), as measured by Ki-67 positivity, and to apoptotic index ($P = 0.004$), as measured by TUNEL staining. In CaPs with uniform ILK expression, the proliferative index increased 325%, whereas the apoptotic index increased only 164% relative to CaPs that showed only focal or negative ILK staining (Table 2). Thus, CaPs with uniform ILK immunostaining showed the disproportionate increase in proliferation *versus* apoptosis that drives the net accumulation of cells during prostate cancer progression (14–16).

Because ILK immunostaining was strongest in the most advanced CaPs, we next evaluated whether ILK staining patterns might be related to patient survival. Our data reveal that ILK expression was inversely related to 5-year patient survival ($P = 0.004$). Indeed, 81% (13 of 16) of the patients with prostate cancers having uniform ILK expression failed to survive for 5 years, whereas only 31% (7 of 22) of CaP patients without uniform ILK staining failed to survive for 5 years. These data further support the notion that enhanced ILK expression may be involved in the malignant progression of CaP. Furthermore, these data also suggest that enhanced ILK expression may be related to patient prognosis, particularly in concert with the loss of the tumor suppressor PTEN, which negatively regulates ILK function (1). Indeed, mirroring ILK overexpression, loss of PTEN expression occurs in >50% of human prostate tumors (17–19) and is specifically correlated with high-grade CaP (19).

The data in this report are the first to demonstrate that ILK expression is increased in primary CaPs relative to BPH from noncancer patients and to BPH adjacent to CaP. More significantly, we also show that ILK expression increases dramatically with increasing tumor grade. These data therefore indicate that ILK is up-regulated with prostate tumor progression, as our previous analyses of androgen-independent human prostate cancer cell lines suggested (9). Moreover, our data support the notion that ILK expression may be a key player involved in prostate tumor progression. ILK was uniformly expressed specifically in poorly differentiated CaPs and was directly related to key biological parameters associated with progression (*i.e.*, the disproportionate increase in proliferation over apoptosis). Furthermore, patients whose tumors had the highest ILK expression were the least likely to survive for 5 years, suggesting that ILK overexpression may have implications for prognosis. Taken together with the recent reports implicating ILK in tumor formation and invasion (4, 5, 7) and with reports demonstrating that inhibition of ILK profoundly suppresses prostate cancer cell growth (8), the data in this report implicate ILK in prostate tumor progression and suggest that ILK may be a promising therapeutic target for advanced CaP.

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