

# Integrin-Linked Kinase (ILK) Expression Correlates with Tumor Severity in Clear Cell Renal Carcinoma

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**Abstract** Integrin-linked kinase (ILK) is a unique intracellular serine/threonine kinase and adapter protein. When dysregulated, it has been associated with increased cell proliferation, anchorage-independent cell growth, evasion of apoptosis, angiogenesis, invasion of surrounding tissues, downregulation of E-cadherin expression, nuclear translocation of  $\beta$ -catenin and metastasis, all features of tumoral malignancy. The objective of the present work was to evaluate the expression of ILK in clear cell renal carcinomas (CCRC) as a possible prognostic indicator. ILK immunorexpression was evaluated in a tissue microarray (TMA) with 45 human CCRCs. In addition, the apoptotic and proliferative indices and the immuno-expression of  $\beta$ -catenin and E-cadherin were also evaluated. E-cadherin expression was significantly decreased in tumors with positive ILK expression in relation to those with negative immunorexpression ( $p=0.011$ ). ILK immunostaining was significantly increased in high-grade in comparison to low-grade CCRCs ( $p=0.0008$ ). ILK expression was also associated with increased proliferative index ( $p=0.020$ ), tumor size  $>7.0$  cm ( $p=0.018$ ) and with renal vein and capsule invasion ( $p=0.003$  and  $p=0.00$ ). Finally, tumors stage I and II (noninvasive)

presented significantly reduced ILK immunorexpression when compared to stage III (locally invasive) ( $p=0.0028$ ). ILK immunorexpression in CCRC increases with loss of intercellular adhesion, nuclear grading, increased proliferative index and Robson stage. Altogether, our data suggest a possible role for ILK in the progression of CRCC.

**Keywords** Immunohistochemistry · Tissue array analysis · Carcinoma · Renal cell · Integrin-linked kinase · Human

## Introduction

Integrin-linked kinase (ILK) is a serine/threonine kinase that interacts with  $\beta$ -integrin cytoplasmic domain and functions as a scaffolding protein in the formation of multiprotein complexes connecting integrins to the actin cytoskeleton, therefore modulating intracellular signaling pathways originated by those connections [1, 2]. Its kinase activity is stimulated by cell adhesion to extracellular matrix (ECM) [3] or by growth factors and chemokines through tyrosine kinase receptors in a phosphatidylinositol 3-kinase (Pi3K)-dependent manner [4]. ILK has been shown to directly phosphorylate several proteins, resulting in several oncogenesis-related processes, such as, increased cell proliferation, anchorage and growth-factor independence, evasion of apoptosis, angiogenesis, invasion of surrounding tissues and metastasis [5]. Accordingly, ILK has been shown to modulate the localization and transcriptional activity of  $\beta$ -catenin, the kinase activity of PKB/Akt, the expression of E-cadherin and the activity of several transcription factors [5–10].

ILK expression and activity are increased in many types of cancer, such as prostate, colon, gastric, ovarian cancers, malignant melanomas, Ewing's sarcoma, primitive neuroectodermal tumour, non-small cell lung cancer, basal cell carcinoma, ductal pancreatic adenocarcinoma,

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hepatocellular carcinoma, laryngeal carcinomas and bladder cancer [11–26].

The clear cell renal carcinoma (CCRC) is the most common histologic variant of renal cell carcinoma, accounting for approximately 70 % of cases and is well-known for a variable and unpredictable biological behavior. In order to correlate its histologic findings with outcome, numerous studies on tumour microvascular density, cell proliferation and apoptosis have been performed with conflicting results [27, 28]. The overall survival rate after nephrectomy for patients with CCRC is around 50 %. Numerous pathological variables have been investigated to help predicting survival. Among prognostic factors determining patient outcome, i.e., nuclear grade, tumor size, sarcomatoid differentiation, infiltrative margin and vascular invasion, the tumor stage is the most important. However, CCRCs in the same stage or grade may exhibit distinct biological behavior. Therefore, other indicators that might help the improvement of prognostic evaluation of CCRC are required.

In the present study, we analyzed ILK expression by immunohistochemistry in a series of CCRCs. We also evaluated E-cadherin and  $\beta$ -catenin expression as well as the apoptotic and cellular proliferative indices. Potential correlations among those elements and their relationship to prognostic and progression parameters of those tumors were addressed.

## Materials and Methods

### Renal Cell Carcinoma Tissue Microarray Construction

TMA included archival, formalin-fixed, paraffin-embedded surgical specimens of 45 CCRCs from the Pathology Department of Federal University of São Paulo (UNIFESP/EPM) and Salomão & Zoppi Laboratory, São Paulo city, dated between 1997 and 2001. The study was approved by the institutional Committee on Research and Ethics. Hematoxylin and eosin (H&E) stained microslides were reviewed and graded according to the Fuhrman system. Core biopsies of 1.0 mm in diameter were taken from each donor block and arrayed into a recipient paraffin block in spaces of 1.2 mm using a tissue microarrayer (Beecher Instruments, Sun Prairie, Wisconsin, USA), as described by Kononen et al. [29]. There were two cores for each tumor in the array, resulting in two histological spots on the corresponding slides. Controls were 5 cores of normal renal tissue, which was represented in the array by two cores each.

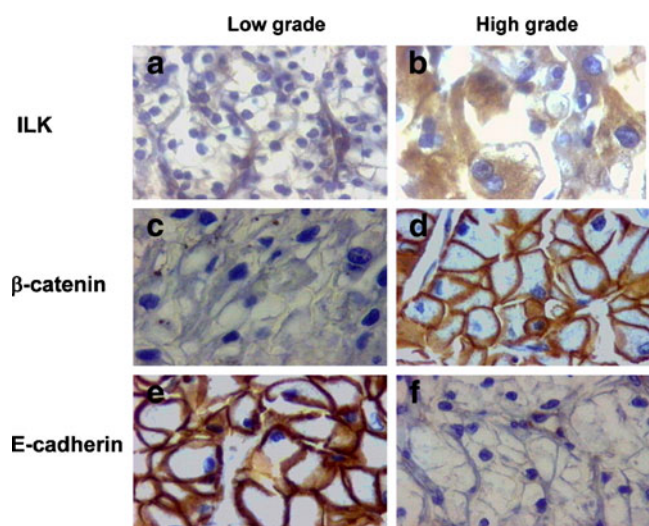
### Immunohistochemistry

Antibody staining was performed on 5  $\mu$ m histological sections of the receptor block (three for each antibody). All sections were deparaffinized in xylene and rehydrated

in graded ethanols before antigen retrieval for 30 min with microwave treatment in 0.1 M citrate buffer (pH 6.0). After incubation with 0.3 % hydrogen peroxide in methanol and incubation with normal blocking serum, sections were incubated with primary antibody: anti-E-cadherin (Dako – clone NCH-38 – cat # M3612) 1:100; anti- $\beta$ -catenin (BD Biosciences – clone 14), 1:600; anti-PCNA (Dako clone PC10 – cat # M 0879), 1:500; anti-ILK (BD Biosciences – cat # 611802) 1:150 at 3 °C overnight. Immunodetection was performed with the LSAB kit+, Peroxidase – Universal, cat # K 0690 (Dako) using diaminobenzidine as chromogen. Positive controls for immunostaining were formalin-fixed, paraffin-embedded specimens of human colonic mucosa (E-cadherin), colon carcinoma ( $\beta$ -catenin), breast carcinoma (PCNA), and kidney (ILK). For negative controls, blocking solution was instead of the primary antibody. All sections were counterstained with Harris's hematoxylin.

### Immunohistochemical Evaluation

Staining patterns, including cell distribution (membrane, cytoplasmic, and nuclear pattern), extent and intensity of stain were evaluated independently for each specimen by three investigators. For ILK, the evaluation was categorical, and staining was classified as negative (<10 % of the neoplasm stained), patchy, (heterogeneously positive with less than 50 % of the neoplasm stained), or uniformly positive (involving more than 90 % of the neoplasm). Comparisons were made between negative/patchy and uniform staining pattern [11]. For E-cadherin e  $\beta$ -catenin, a 0–4 scale was used: negative; focal (<10 %), regional (10–50 %) e diffuse (>50 %). Tumors with negative or patchy staining were considered negative. Tumors presenting regional and diffuse staining were categorized as positive.



**Fig. 1** Immunoexpression of ILK,  $\beta$ -catenin and E-cadherin by immunohistochemistry in low and high grade CCRCs (400 $\times$ )

**Table 1** Correlation between nuclear grade and immunoeexpression of proteins and apoptosis in CCRCs

		Low grade	High grade	<i>p</i>
ILK	Uniform	6/33 (18,18 %)	9/12 (75 %)	* <i>p</i> =0.0008
	Patchy	14/33 (42 %)	3/12 (25 %)	
	Negative	13/33 (39 %)	0/12 (0 %)	
E-cadherin (regional/diffuse)		13/33 (39 %)	1/12 (8.33 %)	<i>p</i> =0.046
β-catenin (regional/diffuse)		14/33 (42.42 %)	7/12 (58.33 %)	<i>p</i> =0.27
Proliferative index		16.26 %±8.77	26.15 %±6.78	<i>p</i> =0.002
Apoptotic index		10.96 %±17.17	1.75 %±3.01	<i>p</i> =0.02

Values represent the mean values ± SD

\**p* high grade vs low grade

### Assessment of Proliferation and Apoptosis

Proliferative index was assessed by immunostaining with mouse monoclonal antibody to PCNA by evaluating all cells in each core. Apoptotic index was assessed by TUNEL staining (kit ApopTag® Plus Peroxidase, Intergen, New York, EUA) by counting in each core all cells showing terminal deoxynucleotidyl transferase positivity. Counting was performed with the Image-tool for Windows program. An average of 1218±197 cells was counted per section for PCNA positivity. An average of 509±84 cells was counted per section for terminal deoxynucleotidyl transferase positivity. The proliferative and apoptotic indices were then calculated by the following equation: (number of stained nuclei/total number of nuclei counted × 100).

### Statistical Analyses

For statistical analyses, the samples were classified in accordance with Fuhrman nuclear grade (low grade = grade 1 and 2, high grade = grade 3 and 4), size of the tumor (<7 cm and >7 cm, <4 cm and >4 cm), presence or absence of capsule invasion, presence or absence of renal vein invasion and Robson stage (I–II and III). Relationships between staining patterns and each tissue group were evaluated by Fisher's exact test. Differences between groups with respect

to proliferative index (*i.e.*, PCNA staining) and apoptotic fraction (TUNEL staining) were calculated using Mann Whitney test. A *p* value inferior to 0.05 was accepted as significant.

## Results

### Correlation between ILK Expression and Tumor Severity

There was a statistically significant correlation between ILK expression and Fuhrman's nuclear grade, with high grade CCRCs presenting higher levels of ILK than low grade tumors (*p*=0.0008) (Fig. 1a and b). Furthermore, there was a simultaneous and significant increase in proliferative index and ILK expression in high grade in comparison to low grade CCRCs (*p*=0.002 and *p*=0.0008) (Table 1). In addition, tumors with ILK expression positive showed increased proliferation index in relation to tumors with ILK expression negative (*p*=0.020) (Table 2).

Renal vein invasive and renal capsule invasive tumors showed significantly higher expression of ILK when compared to those that did not present invasion (*p*=0.00 and *p*=0.003). And tumors larger than 7.0 cm presenting higher levels of ILK in relation CCRCs smaller than 7.0 cm (*p*=0.018). Finally, tumors stage I and II (noninvasive) presented significantly reduced ILK immunoeexpression when compared to stage III (locally invasive) (*p*=0.0028) (Table 3).

### Correlation between ILK Expression and E-cadherin Expression

CCRC group presented significantly reduced E-cadherin expression when compared to controls (*p*=0.005) (Fig. 1e and f, Table 5). When related to Fuhrman's grade, E-cadherin expression was also significantly diminished in high-grade in relation to low grade carcinomas (*p*=0.046) (Table 1). E-cadherin expression was significantly decreased in tumors with positive ILK expression in relation to those with negative immuno-expression (*p*=0.011) (Table 2). Finally, tumors with negative E-cadherin expression showed lower apoptotic index than positive E-cadherin expression (*p*=0.0099) (Table 4).

**Table 2** Correlation among ILK expression and Proliferative and Apoptotic indices, E-cadherin and β-catenin expression in CCRCs

Values represent the mean values ± SD

	Uniform	Focal	Negative	<i>p</i>
Proliferative index	23.49 %±9.42	18.69 %±7.76	13,10 %±8.376	<i>p</i> =0.020
Apoptotic index	6.20 %±9.40	10.58 %±19.79	8.46 %±14.54	<i>p</i> =0.345
E-cadherin (regional/diffuse)	1/15 (6.66 %)	10/17(58.82 %)	3/13 (23.0 %)	<i>p</i> =0.011
β-catenin (regional/diffuse)	11/15 (73.3 %)	9/17(52.94 %)	1/13 (33.3 %)	<i>p</i> =0.012

**Table 3** Correlation between prognostic parameters and ILK expression in CCRCs

		Uniform	Focal	Negative	<i>p</i>
Tumour size	≤7cm	6/29 (20.68 %)	13/29 (44.82 %)	10/29 (34.48 %)	*0.018
	>7 cm	9/16 (56.25 %)	4/16 (25 %)	3/16 (18.75 %)	
Capsule invasion	No	4/26 (15.38 %)	12/26 (46.15 %)	10/26 (38.46 %)	*0.003
	Yes	11/19 (57.89 %)	5/19 (26.31 %)	3/19 (15.78 %)	
Vein invasion	No	4/34 (11.76 %)	16/34 (47.05 %)	14/34 (41.17 %)	*0.0001
	Yes	10/11 (90.90 %)	1/11 (0.90 %)	0/11 (0 %)	
Robson stage	I–II	1/23 (4.34 %)	12/23 (52.17 %)	10/23 (43.47 %)	*0.0001
	III	14/22 (43.47 %)	5/22 (22.72 %)	3/22 (13.63 %)	

Values represent the mean values ± SD

\**p*<0.05

### Correlation between ILK Expression and β-catenin Expression

β-catenin immunoexpression presented membranous pattern of staining and was significantly higher in ILK immunopositive CCRCs in comparison to the negative ones (*p*=0.012) (Fig. 1c and d, Table 4). β-catenin expression was significantly decreased in CCRCs (*p*<0.020) in relation to controls (Table 5).

### Discussion

So far, there is no report in the literature, to our knowledge, using ILK immunoexpression as a prognostic indicator in patients with CCRC. We used the tissue microarray technique, which permit the simultaneous evaluation of a large number of tissue samples and a high level of immunohistochemistry staining standardization, since all tissue samples are pretreated and stained in identical conditions [29–32].

In normal human kidney tubular cell staining, primarily in the distal and proximal tubules, was present, but not in the glomerulus. As a whole, ILK immunoexpression was significantly lower in CCRCs as compared to the control group. Accordingly, Haase et al. [33] concluded that the mean ILK expression in renal cell carcinomas is reduced by 35 % compared with normal kidney tissue, which is statistically significant. The explanation for this apparent contradiction is that

CCRC cells present cytoplasm almost totally occupied with droplets of lipids due to deficient glycogenolysis and lipolysis, making its comparison with other histologic types flawed. Therefore, we decided to compare ILK immunoreactivity in relation to nuclear grade.

More realistically, our results showed that high-grade CCRCs presented ILK expression significantly higher in relation to low grade CCRCs. Consistently, several studies demonstrated increased ILK immunostaining in high-grade primary human prostatic, ovarian, colonic and no small cell pulmonary carcinomas in comparison to low-grade tumors [11, 14, 16, 19].

CCRCs with positive ILK expression presented significant increase in proliferation index (*p*=0,002) in relation to tumors with negative ILK expression. Compatibly, ILK expression was also specifically related to the increase in proliferative index that contributes to the net gain of prostatic carcinomas cells during progression [11].

CCRCs larger than 7.0 cm and CCRCs invading the renal vein and capsule presented ILK immunoexpression significantly higher than tumors smaller than 7.0 cm and that did not invade it, which was suggestive of involvement of ILK in tumor dissemination. Finally, stage III tumors (locally invasive) showed increased ILK expression in relation to stage I and II tumors (noninvasive). In gastric carcinoma significant association was detected between ILK mRNA expression and presence of nodal metastasis and strong expression of ILK protein was significantly associated with deep invasion of tumor

**Table 4** Immuno-expression of proteins and apoptosis in CCRCs and control group

	ILK	E-cadherin	β-catenin	Proliferative index	Apoptotic index
Control	5/5 (100 %)	5/5 (100 %)	5/5 (100 %)	16.96 %±11.54	1.50 %±1.00
CCRC	15/45 (33.33 %)	14/45 (31.11 %)	21/45 (46.66 %)	18.56 %±9.29	8.51 %±15.29
<i>p</i>	* <i>p</i> =0.007	* <i>p</i> =0.005	* <i>p</i> =0.020	0.60NS	0.66NS

Values represent the mean values ± SD



**Table 5** Correlation between E-cadherin immunoexpression and Proliferative and Apoptotic indices in CCRCs

	E-cadherin immuno-expression		<i>p</i>
	Negative	Positive	
Proliferative index	19.28 %±10.02	17.08 %±7.69	0.49
Apoptotic index	4.80 %±9.0	16.71 %±22.34	<i>p</i> =0.0099

Values represent the mean values ± SD

cells in gastric wall [15]. The levels of ILK expression correlated strongly with tumour invasion and stage, and were significantly higher in metastatic human colon cancer [14]. Furthermore, ILK expression is significantly associated with melanoma thickness and with lymph node invasion [17]. In non-small cell lung cancer, ILK expression was significantly associated with T status, lymph node metastasis and stage [19]. Matsui Y et al. reported that ILK is overexpressed in invasive bladder cancer and plays an important role in the EMT of bladder cancer via the control of E-cadherin and MMP-9 expression [26].

Loss of cell–cell adhesion mediated by the  $\beta$ -catenin/E-cadherin complex proteins is well documented in various human malignancies. Down-regulation of E-cadherin is a critical step in the induction of epithelial-mesenchymal transition (EMT), a process considered crucial to cancer invasion and metastasis [34]. In addition, the poor prognostic implications of decreased expression of these proteins have been reported in various carcinomas, including those arising from kidney [35–37]. In the present study, E-cadherin expression was significantly decreased in CCRC in relation to control and was also significantly diminished in high-grade in relation to low grade carcinomas. However it was not correlated with tumor size, capsule or vein invasion as also reported by Slaton et al. [37] in their study of 46 cases of CRCCs.

E-cadherin expression was significantly decreased in tumors with positive ILK expression in relation to those with negative immuno-expression. Normally, loss of cell adhesion leads to apoptosis. However, we found that tumors with negative E-cadherin expression showed lower apoptotic index than positive E-cadherin expression, reflecting both decrease in intercellular adhesion and evasion of apoptosis. These two aspects might be vinculated to ILK activity, since it has been shown that ILK down-regulates E-cadherin expression [10] and may inhibit apoptosis via PKB/Akt signaling pathway. As a matter of fact, inhibition of ILK leads to apoptosis in anaplastic thyroid cancer, pancreatic adenocarcinoma and glioblastomas [38–40]. Recently, Bravou et al. [14] demonstrated that in human colon cancer progression,

ILK overexpression correlates with activation of  $\beta$ -catenin, down-regulation of E-cadherin and activation of the Akt-FKHR pathway. Moreover, ILK overexpression can inhibit MTA-3 expression, resulting in the stimulation of the E-cadherin transcriptional repressor, Snail [10].

$\beta$ -catenin is involved in intercellular adhesion and function as a key molecule in the Wnt signalling pathway [41]. In our study,  $\beta$ -catenin expression in CCRCs presented membrane staining pattern and was significantly lower than in control group. We could not detect nuclear accumulation of  $\beta$ -catenin, suggesting that Wnt pathway activation is not a frequent phenomenon in CRCC. Furthermore,  $\beta$ -catenin expression was significantly higher in ILK immunopositives CCRCs in compariso to negatives CCRCs. Accordingly, Guo et al. [42] concluded that  $\beta$ -catenin expression is generally down-regulated in low nuclear grade CCRCs. Conversely, it appears to be preserved in those with high nuclear grades. Kim et al. [43], studied  $\beta$ -catenin expression in 52 RCCs, and demonstrated that it was mainly localized in cell membrane. Five of 22 CCRCs showed  $\beta$ -catenin accumulation in the cytoplasm. None displayed positive  $\beta$ -catenin immunostaining in the nuclei and only one case presented mutation in  $\beta$ -catenin exon 3. In a recent study, analyzing 38 samples of RCCs, it was demonstrated that  $\beta$ -catenin gene was not overexpressed at the mRNA level and protein level and no alterations was found in  $\beta$ -catenin exon 3 [44].

In relation to apoptosis, the CCRC group presented increased apoptosis index when compared to control group and high grade tumors presented lower apoptosis index and higher proliferative index in relation to low grade. This finding implies an oncogenetic mechanism leading to both stimulation of cellular cycle progression and promotion of apoptosis evasion. As a matter of fact, progression from well to less differentiated renal cell carcinoma requires decrease of apoptotic rate, as well as increase in proliferative activity [45]. ILK could be responsible for this effect, since it is already known that it has anti-apoptotic activity through PKB/Akt signaling pathway as well as proliferative activity through stimulation of the cell cycle [5–10, 14].

Altogether the present data indicate that ILK immuno-expression in CRCCs is related to loss of intercellular adhesion, to the differentiation grade and keeps positive correlation with the proliferative index, renal capsule and renal vein invasion, tumor size and Robson stage. ILK immuno-expression presented a direct correlation with the progression of the tumor in CCRCs, and opens up new perspectives for a targeted molecular therapy of CCRCs with specific pharmacologic inhibitors of members of the ILK pathway.

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