Expression of Integrin-linked Kinase is not a Useful Prognostic Marker in Resected Hepatocellular Cancer

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Abstract. *Background: Hepatocellular cancer (HCC) is one of the most common malignancies worldwide, and is known to be associated with a poor prognosis. Unfortunately there are no available reliable markers of prognosis. The aim of this study was to determine whether integrin-linked kinase (ILK) expression correlates with post-resection survival from HCC. Patients and Methods: A tissue microarray was constructed using HCC samples, and immunohistochemical analysis for ILK was then carried out and scored by three independent observers. Clinical chart review was performed to determine survival parameters. Results: Of the 52 cases of HCC, 22 cases were associated with hepatitis B (HBV), 18 with hepatitis C (HCV), 2 with HBV and HCV co-infection; 81% of all patients were male and 19% female. Western immunoblotting showed a highly significant correlation between levels of expression of ILK and ser473-PKB phosphorylation, both in control and tumor sections (Spearman rank correlation, r=0.8155, p=0.0004), however, there was no direct correlation between the levels of expression of ILK with patient survival (log-rank test, p=0.864). Conclusion: ILK expression does not appear to have a role in predicting outcome in patients with resected HCC.*

Hepatocellular carcinoma is one of the most common malignancies worldwide, and is being increasingly diagnosed in North America due to two major factors: increasing migration of populations from high-risk (higher incidence of chronic infection with hepatitis B virus) areas, and the increased incidence of chronic hepatitis C infection. HCC is known to be associated with a very poor prognosis, with an average life expectancy of less than a year after diagnosis, and a 5-year survival rate of less than 10% (1-3).

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Liver cirrhosis from chronic viral hepatitis is the strongest risk factor for HCC development (4). For this reason, it is recommended that patients with HBV/HCV-associated cirrhosis undergo routine surveillance for HCC. Currently, the only reasonable available tools for mass population HCC surveillance are serum alpha fetoprotein (AFP) level and hepatic ultrasound (with confirmatory CT/MRI). However AFP levels have a poor sensitivity and specificity for HCC. Newer methods for the early detection of HCC are needed in the form of either biomarkers or radiological tests. It is known that tumors harbor mutations in components of the axin-beta-catenin nexus (5). We are specifically interested in integrin-linked kinase (ILK), a potential modifier of this axis, and a molecule originally discovered in 1996 as a beta-1 integrin subunit cytoplasmic domain interactor. ILK, functioning as a scaffold in forming multiprotein complexes connecting integrins to the actin cytoskeleton and signaling pathways, is essential for embryonic development (6). Overexpression of ILK in epithelial cells induces epithelial-mesenchymal transition (EMT) by inhibiting E-cadherin expression, activates nuclear beta-catenin and induces a transformed, tumorigenic phenotype. ILK overexpression also promotes cell survival by stimulating the phosphorylation of AKT (PKB) on Ser 473; conversely, inhibiting ILK in cancer cells inhibits AKT phosphorylation and cell survival. ILK regulates tumor angiogenesis through vascular endothelial growth factor and exhibits increased expression in many types of cancer such as prostate, colon, ovarian and malignant melanoma (6). Expression of ILK increased activation of Ser473 phosphorylation of PKB and also inhibited glycogen synthase kinase 3 beta (GSK3 beta). Levels of ILK expression have been shown to increase with tumor grade in prostatic cancer and are inversely related to 5-year survival. There is also an increased expression in human colon adenocarcinoma, with increased expression at invasive regions of the tumor correlating with increased phosphorylation of GSK3 beta and nuclear beta-catenin (6). However, the role of ILK in human HCC has not been studied to date.

We hypothesized that increased ILK expression may be associated with patient outcome in patients undergoing resection for HCC. We used a tissue microarray (TMA) and immunohistochemistry (IHC) to evaluate ILK expression in these tumors, and performed chart reviews to obtain corresponding clinical parameters.

Patients and Methods

Tissue microarray. The TMA was constructed from formalin-fixed, paraffin-embedded tissue blocks containing HCC. Fifty-two tissue samples were obtained from patients with HCC undergoing partial hepatectomy at the Vancouver General Hospital during 2001-2003, and 8 control samples from patients with surgical resection for benign liver lesions. The most representative tumor area of each sample was carefully selected and marked. TMAs were assembled using a tissue array instrument essentially as described elsewhere (7, 8). Tissue cores were created from tissue blocks and transferred into recipient blocks. Multiple 4-μm sections were obtained with a Leica microtome. Sections were then transferred to adhesive-coated slides using a routine histology procedure. IHC was carried out as described elsewhere (9). Briefly, antigen retrieval was achieved by placing the slides in 0.01M citrate buffer for 10 min in a microwave oven. The slides were then incubated with the ILK antibody (Santa Cruz Biotechnology Corporation, Santa Cruz, CA, USA), then the biotinylated secondary antibody (Dako, Mississauga, Canada) and then color was developed using DAB (Vector laboratories, Burlingame, CA, USA). For evaluation of immunostaining, ILK expression was examined double-blinded by three independent observers, the average scores were calculated for each staining. The staining intensity of each core was scored as: 0, negative, 1, weak staining, 2, moderate staining, and 3, strong staining. There was consistency of immunohistochemical staining between duplicate cores in the TMA.

Western blot analysis. Equivalent amounts of tissue were placed in homogenization buffer consisting of: 20 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 150 mM NaCl, 50 mM β-glycerophosphate, 5 mM ethylene glycol tetraacetic acid (EGTA), 50 mM NaF, 1 mM dithiothreitol (DTT), 1 mM sodium vanadate, and 1 mM PMSF, and sonicated for 15 s (x 2) and centrifuged at 14,000 RPM for 15 min. The protein concentration in the supernatant was determined by the Bradford assay (Bio-Rad, Mississauga, ON, Canada). A total of 30 μg of protein from each sample were resolved using 10% SDS-PAGE before being transferred to nitrocellulose membranes (Bio-Rad). The blots were blocked in 5% skimmed milk in TBST (20 mM Tris-HCl pH 7.4, 250 mM NaCl, 0.05% Tween-20) for 1 h before probing for 2 h using the appropriate primary antibody. The blots were washed three times with TBST for 10 min, before being incubated with the appropriate secondary antibody for 1 h. Following a further 3 washes in TBST, they were developed using the enhanced chemiluminescence detection system (ECL, Amersham, Montreal, Que, Canada). The antibodies used were: GSK3, ser9-GSK3, ser240/244-S6, ser473PKB, PDK, obtained from NEB (Pickering, ON, Canada), or all MAPKs, GAPDH, PKB and ILK, obtained from Santa Cruz Biotechnology Corporation (Santa Cruz, CA, USA).

Table I. *Clinical characteristics of patients.*

Gender	
Male	42
Female	10
Age (years)	
≤50	12
>50	40
Cirrhosis	
Present	31
Absent	10
Unknown	11
Viral hepatitis	
B	22
C	18
Unknown	10
B+C	2
Tumor differentiation	
Well	24%
Moderate	55%
Poor	21%
Associated conditions	
Autoimmune hepatitis	1
Haemochromatosis	1
Previous TACE	8
Previous rupture	1

Statistical analysis. ILK expression was evaluated in relation to various clinical parameters by means of the Chi-squared test. Survival analysis was performed by the Kaplan-Meier method and the log-rank test for analyzing ILK expression *versus* patient survival, using GraphPad Prism version 4.0b for Macintosh, and Spearman rank correlation was performed using GraphPad InStat version 3.0b for Macintosh (GraphPad Software, San Diego, California USA. www. graphpad.com).

This study was approved by the Research Ethics Review Committee of the University of British Columbia.

Results

In all, fifty-two HCC tissues were obtained from patients with HCC undergoing hepatectomy at the Vancouver General Hospital during the period of 2001-2003. Twenty-two cases were associated with hepatitis B (HBV), eighteen with hepatitis C (HCV), two with hepatitis B and C and the remainder were unknown (relative contributions of other chronic liver conditions: autoimmune hepatitis (AIH), 1; haemochromatosis, 1; chronic alcohol, the remainder: undetermined). Forty-two (80.8%) cases were from male patients and 60% were associated with cirrhosis (Table I); 55% of the samples were moderately differentiated tumors, and 24% and 21% were well-differentiated and poorly differentiated, respectively.

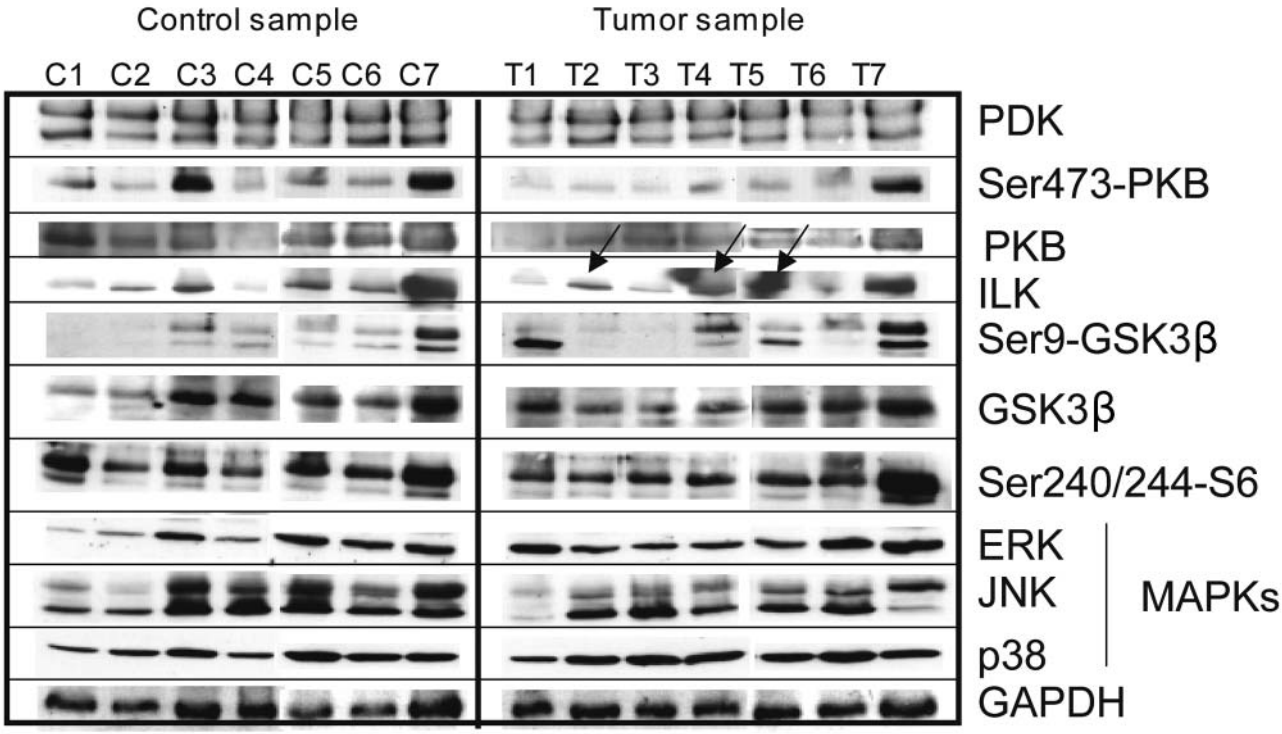

Figure 1. *Initial exploratory Western blot of 7 paired hepatocellular carcinoma samples. Equivalent amounts of control (from the same resection sample) and tumor lysate were resolved on 10% SDS-PAGE, and probed with the antibodies indicated. ILK overexpression was found in only a minority of samples (3 out of 7, arrows). However, a significantly enhanced ILK signal with an increasing ser473PKB signal was noted in the cases analyzed. As previously reported, there was an increased expression of MAPKs in a few cases. (The GAPDH analysis shows equal loading).*

Exploratory analyses. Initially we performed Western immunoblotting on seven paired HCC samples and adjacent tissue (unrelated to the 52 cases above) to obtain an estimate of the range and the approximate frequency of enhanced ILK expression in human HCC. The data indicate (Figure 1) that 3 samples (T2, T4 and T5) exhibited enhanced expression over their control counterparts. Interestingly, there was a large range of ILK expression, with some samples clearly demonstrating impressive levels in the benign lesions (especially C7). Intriguingly, regardless of whether the sample was benign or malignant, there was a significant correlation (Spearman rank correlation) in the level of ILK expression and the intensity of the ser473-PKB signal ($p=0.004$). This finding was confirmed on IHC in one of these lesions probed with the ILK and ser473PKB antibodies (Figure 2A-F). The tumor sample depicted in Figure 2B shows a clearly enhanced signal as compared with its control in Figure 2A (Figure 2C is a close-up of 2B). Similarly the corresponding ser473 signals shown below the ILK stained sections show the same pattern (compare 2E and 2D). These changes were not strongly related to the expression of the other major regulator of PKB, PDK, at least at the protein level. As the data indicate (uppermost panel, Figure 1) PDK appeared to exhibit uniform expression across all samples. In several samples, there was a close agreement between the level of ser9-GSK3 beta phosphorylation and ILK expression. Previous work has indicated that the MAPK family members (especially p42/44ERK) are altered in HCC (10), and as the data indicates this was also observed in this study (see T1, T2, T4, T6 and T7). Based on these findings, especially the lack of a uniform increase in ILK expression across all lesions sampled we decided to construct our tissue micro-array using cancer lesions, in duplicate.

Patient samples. Different levels of ILK staining were observed in the tissue samples examined. Strong intensity was recorded in samples from 2 patients (4%), moderate from 20 patients (38%), weak from 25 (48%), and staining was absent in samples from 5 patients (10%) (Figure 3A). When ILK expression was compared between hepatitis B- and C-derived samples, most of them were of weak intensity (Figure 3B). ILK expression in hepatitis B was generally weak. With hepatitis C, ILK expression was mostly weak to moderate. Using Chi-square analysis, there was no association between HCC in patients with or without

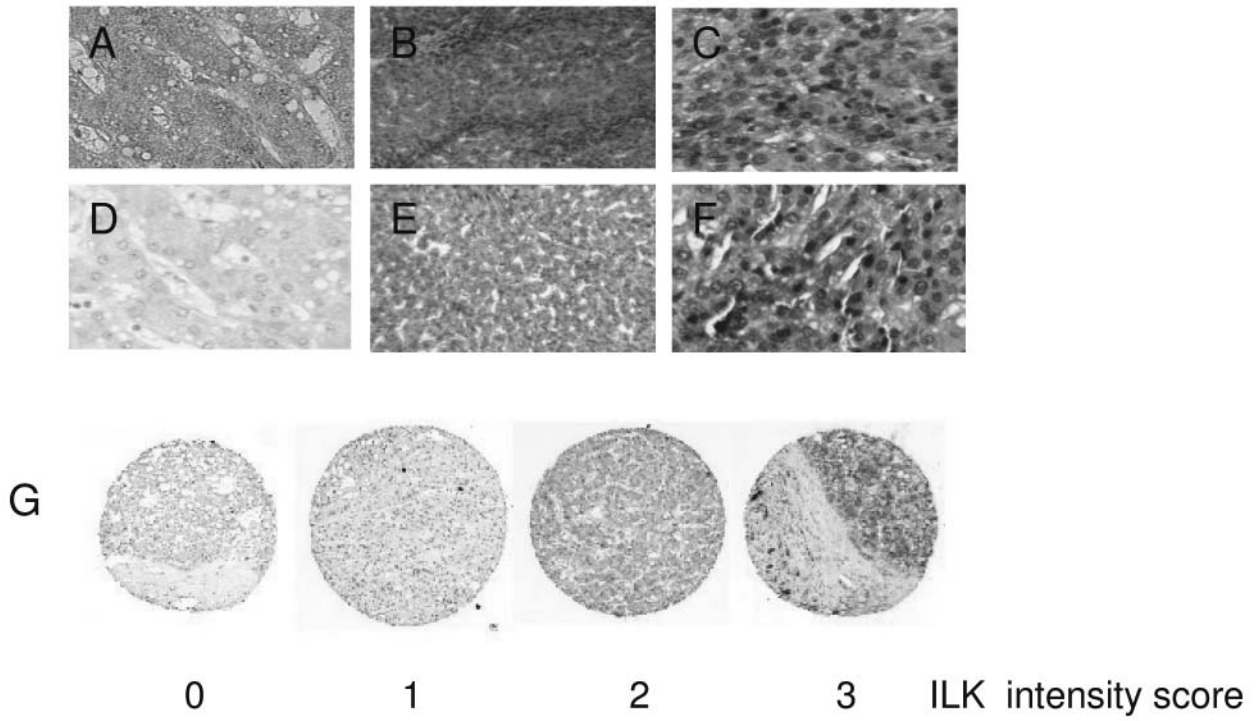

Figure 2. *Enhanced ILK expression and ser473 PKB signal in HCC versus its normal margin. This shows enhanced expression of ILK in the tumor sample B, and its magnified image C, as compared with the normal tissue in the same section A. The ser473 signal for PKB is similarly enhanced (E & F) as compared with the normal (D). The series of sections in G reveal the range of levels of expression of ILK, which were scored between 0-3 depending on the intensity of ILK expression (control staining was also performed using pre-immune serum and the secondary antibody alone, neither of which gave a signal, data not shown).*

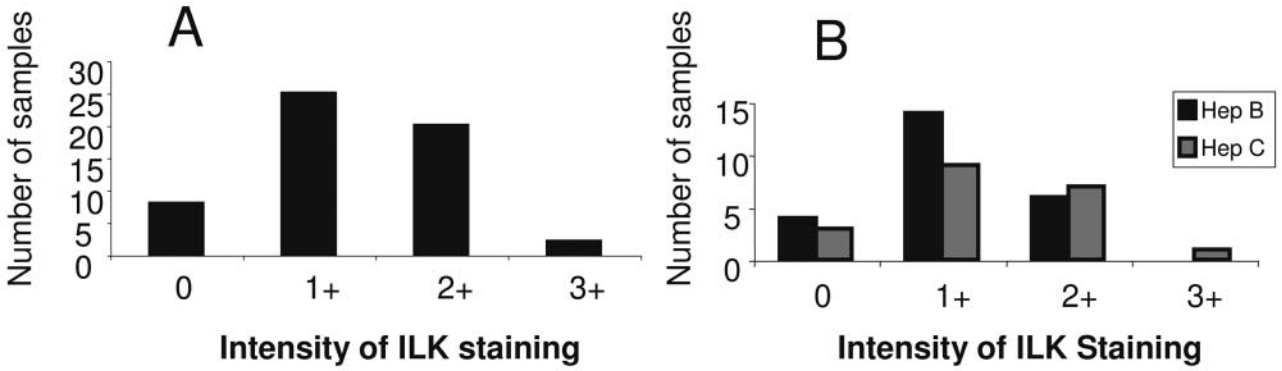

Figure 3. *Distribution of immunohistochemical ILK signal intensity in sampled hepatocellular carcinomas. The staining of the sampled lesions followed a normal distribution as might have been predicted. At the extremes, more lesions were found to be negative for ILK than having intense staining. B. For the lesions where the viral association was known there was a comparable distribution of the intensity of ILK staining.*

cirrhosis and the level of ILK expression. Furthermore, there was no association between the degrees of tumor differentiation (well, moderate and poorly differentiated carcinomas) with ILK expression across all samples analyzed. To evaluate whether increased ILK expression correlated with poor prognosis, we divided the patients into two different groups. The first group included patients whose tumors exhibited low intensity ILK staining (grades 0 and 1), and the second group included patients with high levels of ILK staining (grades 2 and 3). As the data in Figure 4 indicates there was no significant difference in survival between these two groups ($p=0.864$).

Discussion

HCC is one of the most lethal malignancies with a case fatality rate of 1. As there are no clearly defined biomarkers for this disorder besides AFP, this study was performed to assess the possibility that ILK expression could be a candidate marker in this context. This molecule has been shown to correlate with survival from prostate and melanoma lesions previously (6), although levels of ILK expression were found to correlate with tumor behavior in several other cancers also (including colon, gastric and ovarian malignancies (6).

The initial part of the study revealed some interesting findings, most notably the significant correlation between ILK expression and ser473 PKB phosphorylation. As the latter event has been shown to occur *via* ILK, this is intriguing, and supports a role for this signaling pathway in the biology of HCC also. The observation that increased ILK expression was not universally present, likely indicates that only specific lesions occurred in a setting of enhanced signaling through the growth-factor-PI3K axis or possibly *via* enhanced extracellular matrix-derived stimuli. This finding also points to a more complex role for ILK in HCC pathogenesis also.

From the tissue microarray data, we initially sought to determine whether there was any association between intensity of ILK staining and demographic variables. There appeared to be no association between this parameter and either age or gender (although men are more frequently affected than females). Turning next to the main focus of the study, we were unable to demonstrate a significant correlation between ILK expression and HCC outcome post-resection. Interestingly, the spread of ILK intensity of staining appeared to be skewed towards the weaker staining category. From the sample analyzed using formal staining procedures, an excellent enhanced signal could be demonstrated in the tumor lesion as compared with its normal margin counterpart (Figure 3). It is possible that technical limitations in performing IHC are involved when dealing with TMAs. Alternatively, it is entirely possible that because we analyzed only samples post resection, this category may exhibit a more benign tumor biology (*i.e.* reduced ILK staining) when compared with those patients who are not deemed to be surgical candidates. As eight of the patients had undergone previous TACE, this was a potential confounder in this analysis. However, closer inspection of this category revealed a range of staining comparable to the whole group (data not shown). Future work will need to include those tumors exhibiting aggressive behavior (and thus precluded from curative resection), where diagnostic biopsy samples are available.

There is no doubt that factors involved in EMT contribute to tumor invasiveness. This has been demonstrated by Giannelli and colleagues (11) who exposed

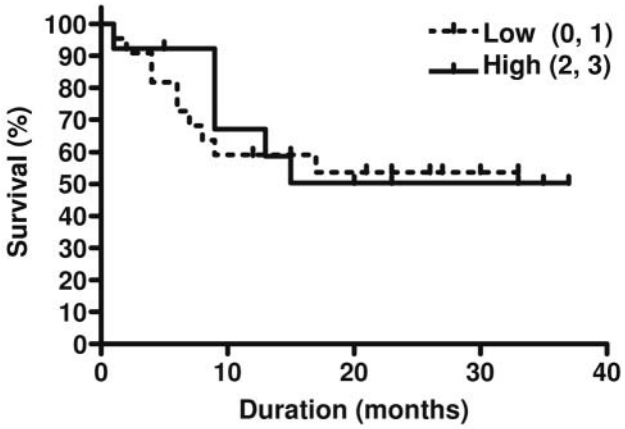

Figure 4. *Kaplan-Meier survival analysis for tumor lesions scored according to ILK staining intensity categorized as low (0-1; broken line) or high (2-3; solid line). The range of survival was between 1-37 months for both groups with 45% of the patients with low ILK scores and 46% of those with high ILK scores dying by the time of analysis. Accordingly, there was no significant difference in survival between the two groups (logrank test, p=0.864).*

'noninvasive' HCC cells to both laminin-5 and TGF beta, and found that these cells upregulated Snail and Slug, as well as down-regulated E-cadherin, whilst exhibiting increased invasive properties. Furthermore, in an experimental model of hepatic carcinogenesis, it was demonstrated that ILK expression correlated with a reduction in the expression of E-cadherin thus providing *in vivo* evidence of ILK related EMT changes (12). Interestingly, Gao and colleagues observed that E-cadherin expression levels exhibited an increase when comparing low-grade neoplasms (grades I and II) with cirrhosis (13). The latter were found to have reduced expression of this marker when compared with normal tissue. In future work we will determine if there is any correlation between these molecules and the level of ILK within the tumors.

Current work indicates that molecules involved in cell adhesion may also impact upon hepatocellular cancer cell line apoptosis. Thus, the effects of a nephrotoxic agent DCVC (S-1,2-dichlorovinyl-L-cysteine) were found to include down-regulation of several molecules including ILK prior to apoptosis occurring (14). Collectively, these observations point to the involvement of ILK in a dynamic tumor cell-matrix interaction which impacts on HCC invasion and response to death-inducing agents.

In summary, our work is the first to assess the relationship between ILK expression and HCC outcome post-surgery. The complexity of HCC biology, together with variability in the extent of epithelial to mesenchymal transition between samples (and stages of HCC) may in part explain our inability to detect a correlation between these two parameters.

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