ILK overexpression in human hepatocellular carcinoma and liver cirrhosis correlates with activation of Akt

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Abstract. Hepatocellular carcinoma (HCC) is one of the most common life-threatening malignancies in the world. The molecular mechanisms leading to the development of HCC are complex and only recently have they begun to be clarified. Integrin linked-kinase (ILK), a multifunctional signaling and scaffold protein of focal adhesion plaques, has been implicated in the pathogenesis of several human malignancies. In the current study the expression of ILK, ß-catenin and E-cadherin and the phosphorylation of Akt were studied by immunohistochemistry in 69 human HCCs and adjacent normal and cirrhotic liver parenchyma. ILK and phosphorylated-Akt (p-Akt) immunostaining was observed in 100 and 79.7% of HCCs, respectively, and their protein levels correlated significantly. Activation of ß-catenin and downregulation of E-cadherin were frequently observed in HCC, but they were not related to ILK expression. A strong correlation between ILK expression and phosporylation of Akt was also observed in cirrhotic liver. Moreover, downregulation of E-cadherin and membranous ß-catenin were found in cirrhotic tissue suggesting their involvement in the liver tissue remodeling observed in cirrhosis. Our results indicate that ILK overexpression during liver oncogenesis and cirrhosis correlates with activation of Akt but not with other conventional ILK targets.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer-related mortality (1). Hepatocarcinogenesis is a multistage process involving different genetic alterations that ultimately lead to oncogenic transformation of the hepatocyte. Cirrhosis of any aetiology may be complicated by HCC and is the greatest single risk factor. Although the major risk factors of HCC are well defined, the molecular mechanisms that regulate its development remain largely unknown.

Integrin-linked kinase (ILK), a serine/threonine kinase located in focal adhesion plaques, interacts with ß integrins and numerous cytoskeleton-associated proteins (2,3) and regulates signaling pathways involved in cell cycle progression, apoptosis, cell adhesion and tumor invasion (4-7). Overexpression of ILK in epithelial cell lines has been shown to result in nuclear accumulation of ß-catenin, activation of Akt and downregulation of E-cadherin (8-10). Increased expression of ILK has also been reported in many human cancers such as prostate, colon, ovarian cancer and melanoma (11-14).

In vitro studies implicate ILK in the regulation of hepatocyte survival and differentiation (15). ILK has also been found involved in liver wound healing and chemically-induced rat hepatocarcinogenesis (16,17). Recently, ILK was shown to be overexpressed in HCC (18). However, ILK signaling pathways in HCC and liver cirrhosis have not been fully addressed. In a series of hepatocellular carcinomas, surrounding cirrhotic liver and adjacent normal liver tissue, we evaluated by immunohistochemistry the expression of ILK, ß-catenin, E-cadherin and the phosphorylation of Akt at Ser473. Potential correlations between these factors and their relationships to clinical and pathologic parameters of the disease such as tumor grade and presence of underlying cirrhosis were also examined.

Materials and methods

Tissue specimens. The study was performed in accordance with institutional ethical guidelines and was approved by the Committee on Research and Ethics and the Scientific Committee of the University Hospital of Patras, Greece. Formalin-fixed paraffin-embedded tissue specimens from 69 human HCCs were obtained from the Department of Pathology, 'Areteion' Hospital, Athens, Greece. Tumors were graded according to World Health Organization (WHO) criteria, and they included 22 well differentiated (grade I), 24 moderately differentiated (grade II) and 23 poorly differentiated (grade III) HCCs. In 47/69 HCC specimens, hepatitis B virus (HBV)-related cirrhosis was present in liver parenchyma adjacent to the tumor while in the rest of the cases there was no underlying cirrhosis as confirmed by clinical information and histological examination.

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Table I. Antibody characteristics.

Antibody	Type	Source	Dilution	Protein blocking
ILK	P	Upstate Biotechnology, Lake Placid, NY	1:600	TBS-5% milk
ß-catenin	M	BD Biosciences, CA, USA	1:2000	TBS-5% milk
E-cadherin	M	BD Biosciences	1:2000	TBS-5% milk
p-Akt (Ser473)	P	Cell Signaling, Beverly, MA	1:50	TBS-3% BSA

P, polyclonal rabbit; M, monoclonal mouse; TBS, Tris-buffered saline; BSA, bovine serum albumin.

Table II. ILK expression in human hepatocellular carcinomas and correlation with clinicopathological parameters.

		ILK expression[a]								
		0		1		2		3		
	No. of samples	n	(%)	n	(%)	n	(%)	n	(%)	p-value[b]
HCC	69	0	(0)	6	(8.7)	33	(47.8)	30	(43.5)	
Grade										0.257
I	22	0	(0)	1	(4.5)	11	(50.0)	10	(44.5)	
II	24	0	(0)	4	(16.7)	12	(50.0)	8	(33.3)	
III	23	0	(0)	1	(4.3)	10	(43.5)	12	(52.2)	
Cirrhosis										0.237
No	22	0	(0)	2	(9.1)	13	(59.1)	7	(31.8)	
Yes	47	0	(0)	4	(8.5)	20	(42.6)	23	(48.9)	

[a]ILK expression was scored as described in Materials and methods. [b]Kruskal-Wallis or Mann-Whitney test. A p-value <0.05 was considered statistically significant.

Immunohistochemical staining procedure. Representative 4-μm tissue sections were de-paraffinized in xylene and rehydraded in graded ethanols. Antigen retrieval was enhanced by heating the slides in 0.01 M citrate buffer (pH 6.0) in a microwave oven. Endogenous peroxidase activity was blocked by treatment with 1% hydrogen peroxide for 15 min, followed by incubation with protein blocking solution. Sections were subsequently incubated with primary antibodies in appropriate dilutions overnight at 4˚C (Table I). The Envision Detection System (Dako, Carpinteria, CA, USA) was used for visualization according to the manufacturer's instructions. Sections were counterstained with Harris' hematoxylin, dehydrated and mounted permanently for examination. Negative controls were performed in all cases by omitting the primary antibodies.

Evaluation of immunohistochemical staining. All slides were assessed by two pathologists (H.P., J.V.) and two investigators (S.P., V.B.) independently and blinded to the cases. Both the intensity of staining and percentage of positive cells were taken into account. Membranous, cytoplasmic and nuclear staining when observed was evaluated separately. The following scoring system was used: 0, staining in <10% of tumor cells; 1, weak staining in 10-75% of tumor cells or moderate staining in <35%; 2, weak staining in >75%, moderate staining in 35-75% or strong staining in <35% of tumor cells; and 3, strong staining in >35% or moderate staining in >75% of tumor cells.

Statistical analysis. Statistical analysis was performed with the SPSS for Windows, release 12.0 (SPSS Inc., Chicago, IL, USA). Correlations of clinicopathological parameters with protein expression were analyzed with the non-parametric Kruskal-Wallis or Mann-Whitney tests for ordinal data or the Chi-square test for nominal data. Correlations between expression of proteins (immunohistochemical scores) were evaluated by the Spearman rank-order correlation coefficient. All ranking tests were performed with correction for ties. The significance level was defined as $p<0.05$.

Results

ILK is overexpressed in HCC and cirrhotic liver. Immunohistochemical staining for ILK was performed in 69 HCCs and adjacent cirrhotic and normal liver tissue. In normal liver none or weak cytoplasmic expression of ILK was detected in hepatocytes and epithelial cells of bile ductules (Fig. 1A). All HCCs examined (100%) were positive for ILK. Staining was mainly observed in the cytoplasm of tumors cells (Fig. 1B).

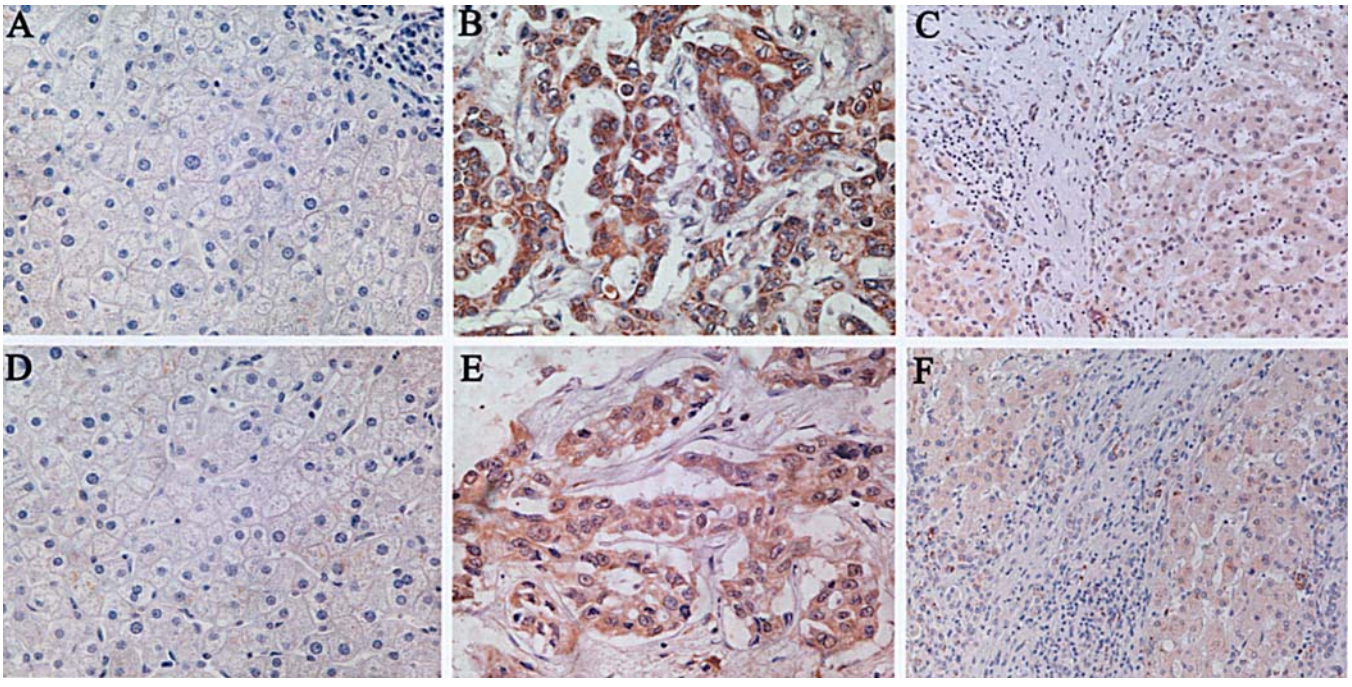

Figure 1. ILK expression and phosphorylation of Akt in normal liver, HCC and liver cirrhosis. (A) Negative staining for ILK in normal liver tissue (x400). (B) HCC showing strong cytoplasmic ILK expression (x400). (C) Weak expression of ILK in hepatocytes of cirrhotic nodules (x200). (D) Normal liver with negative p-Akt staining (x400). (E) Intense cytoplasmic p-Akt immunoreactivity in HCC (x400). (F) Weak staining of p-Akt in hepatocytes of cirrhotic nodules (x200).

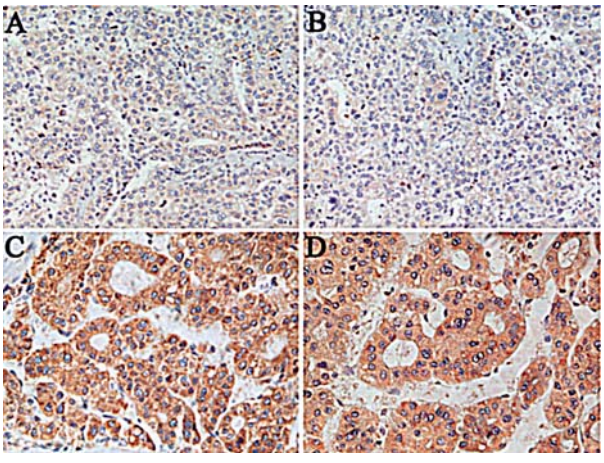

Figure 2. ILK expression correlates with activation of Akt in human HCC. Adjacent sections of an HCC showing weak immunoreactivity for ILK (A) and p-Akt (B) (x200). Adjacent sections of an HCC with strong ILK (C) and p-Akt immunostaining (D) (x400).

In 63/69 tumors (91.3%) ILK expression was moderate or strong and only 6/69 (8.7%) cases showed weak expression. In HCCs there was no statistically significant correlation of ILK expression with tumor grade or the presence of cirrhosis (Table II). Positive ILK staining was also observed in 40/47 (85.1%) cases of cirrhotic liver (Fig. 1C). ILK expression was weak in 20/47 (42.6%), moderate in 17/47 (36.2%) and strong in 3/47 (6.4%) cases of cirrhosis. ILK expression was significantly higher in cirrhosis compared to normal liver ($p<0.001$) and in HCCs compared to cirrhotic and normal liver ($p<0.001$).

Activation of Akt is frequently observed in HCCs and cirrhosis and correlates significantly with ILK expression. Phosphorylation of Akt was examined in 69 tumors and adjacent cirrhotic and normal liver tissue. In normal liver, p-Akt immunostaining was absent (Fig. 1D). In contrast, phosphorylation of Akt was observed in 55/69 HCCs (79.7%) (Fig. 1E). Immunostaining of p-Akt was mainly found in the cytoplasm of cancer cells and only in 17/69 (24.6%) cases was a nuclear localization also noted. There was no correlation of p-Akt with tumor grade. However, phosphorylation of Akt in HCCs was significantly higher in cases without underlying liver cirrhosis ($p=0.017$) compared to the tumors occurring in the setting of cirrhosis (Table III). Thirty-five of 47 (74.3%) cases of cirrhotic liver were positive for p-Akt cytoplasmic staining (Fig. 1F). In 28/47 (59.6%) cases of cirrhosis, p-Akt staining was weak, while 7/47 (14.9%) cases showed moderate staining. In cirrhosis, nuclear p-Akt localization was also observed in 34/47 cases (74.5%). Cytoplasmic and nuclear levels of p-Akt were significantly higher in carcinomas compared with cirrhosis ($p=0.003$ and $p<0.001$, respectively). Notably, phosphorylation of Akt correlated significantly with overexpression of ILK in HCC ($r=0.420$, $p=0.002$) as well as in cirrhosis ($r=0.511$, $p<0.001$) (Figs. 2 and 3).

Activation of ß-catenin during liver oncogenesis does not correlate with ILK expression. The subcellular localization of ß-catenin was investigated in 69 HCCs and adjacent cirrhotic and normal liver tissue. Normal liver showed diffuse membranous staining of ß-catenin in hepatocytes and an even stronger membranous staining in bile duct epithelium (Fig. 4A). In contrast, cytoplasmic and nuclear accumulation of ß-catenin was found in 42 (60.9%) and 33 (47.8%) of 69 HCCs,

Table III. p-Akt expression in human hepatocellular carcinomas and correlation with clinicopathological parameters.

		Cytoplasmic p-Akt[a]									Nuclear p-Akt[a]								
		0		1		2		3			0		1		2		3		
	No. of samples	n	(%)	n	(%)	n	(%)	n	(%)	p-value[b]	n	(%)	n	(%)	n	(%)	n	(%)	p-value[b]
HCC	69	14	(20.3)	9	(13.0)	21	(30.4)	25	(36.2)		52	(75.4)	7	(10.1)	5	(7.2)	5	(7.2)	
Grade										0.141									0.438
I	22	4	(18.2)	0	(0)	9	(40.9)	9	(40.9)		16	(72.7)	3	(13.6)	1	(4.5)	2	(9.1)	
II	24	8	(33.3)	5	(20.8)	4	(16.7)	7	(29.2)		20	(83.3)	2	(8.3)	2	(8.3)	0	(0)	
III	23	2	(8.7)	4	(17.4)	8	(34.8)	9	(39.1)		16	(69.6)	2	(8.7)	2	(8.7)	3	(13.0)	
Cirrhosis										0.017									0.310
No	22	2	(9.1)	0	(0)	9	(40.9)	11	(50.0)		15	(68.2)	2	(9.1)	3	(13.6)	2	(9.1)	
Yes	47	12	(25.5)	9	(19.1)	12	(25.5)	14	(29.8)		37	(78.7)	5	(10.6)	2	(4.3)	3	(6.4)	

[a]p-Akt expression was scored as described in Materials and methods. [b]Kruskal-Wallis or Mann-Whitney test. A p-value <0.05 was considered statistically significant.

Table IV. ß-catenin expression in human hepatocellular carcinomas and correlation with clinicopathological parameters.

		Nuclear ß-catenin[a]									Cytoplasmic ß-catenin[a]								
		0		1		2		3			0		1		2		3		
	No. of samples	n	(%)	n	(%)	n	(%)	n	(%)	p-value[b]	n	(%)	n	(%)	n	(%)	n	(%)	p-value[b]
HCC	69	36	(52.2)	9	(23.0)	20	(29.0)	4	(5.8)		27	(39.1)	11	(15.9)	27	(39.1)	4	(5.8)	
Grade										0.413									0.400
I	22	10	(45.5)	3	(13.6)	7	(31.8)	2	(9.1)		11	(50.0)	3	(13.6)	6	(27.3)	2	(9.1)	
II	24	15	(62.5)	3	(12.5)	5	(20.8)	1	(4.2)		7	(29.2)	4	(16.7)	11	(45.8)	2	(8.3)	
III	23	11	(47.8)	3	(13.0)	8	(34.8)	1	(4.3)		9	(39.1)	4	(17.4)	10	(43.5)	0	(0)	
Cirrhosis										0.081									0.221
No	22	8	(36.4)	3	(13.6)	10	(45.5)	1	(4.5)		11	(50.0)	4	(18.2)	5	(22.7)	2	(9.1)	
Yes	47	28	(59.6)	6	(12.8)	10	(21.3)	3	(6.4)		16	(34.0)	7	(14.9)	22	(46.8)	2	(4.3)	

[a]ß-catenin expression was scored as described in Materials and methods. [b]Kruskal-Wallis or Mann-Whitney test. A p-value <0.05 was considered statistically significant.

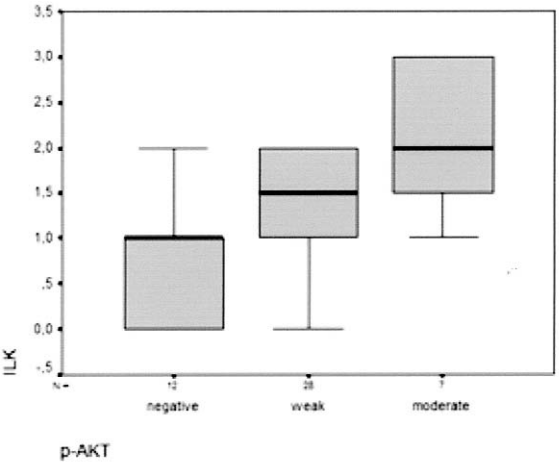

Figure 3. Boxplot chart showing significant correlation between ILK and p-Akt in cirrhosis.

respectively (Fig. 4B). Loss of membranous ß-catenin expression was observed in 53/69 (76.8%) HCCs and only 16/69 (23.1%) tumors showed a focal weak or moderate membranous staining. There was no significant correlation of nuclear, cytoplasmic or membranous ß-catenin expression with tumor grade (Table IV). However, membranous ß-catenin expression was significantly higher in tumors without coexistent cirrhosis, compared to tumors with underlying cirrhotic liver parenchyma (p=0.002). There was also a strong positive correlation between nuclear and cytoplasmic ß-catenin expression (p<0.001). In cirrhotic liver, focal membranous expression of ß-catenin in hepatocytes was observed in only 10/47 (21.3%) cases, while strong membranous expression in proliferative bile ductule epithelium was retained in all cases. No cytoplasmic or nuclear staining of ß-catenin in cirrhosis was noted (Fig. 4C). It is important to note that ILK expression did not correlate with nuclear, cytoplasmic or membranous ß-catenin expression.

Downregulation of E-cadherin in HCC and liver cirrhosis does not correlate with ILK expression. The expression of E-cadherin was examined in 69 HCCs and adjacent cirrhotic and normal liver tissue. In normal liver there was a strong membranous staining of E-cadherin in hepatocytes and bile ductule epithelial cells (Fig. 4D). In contrast HCCs showed complete loss or weak focal expression of E-cadherin in 59/69 (85.5%) cases (Fig. 4E) and negative or weak membranous expression of E-cadherin was also observed in 30/47 (63.9%) cases of cirrhosis (Fig. 4F). However, there was no statistically significant difference in the expression levels of E-cadherin between cirrhosis and HCCs. The expression of E-cadherin in HCCs did not correlate with tumor grade but was statistically significantly lower in tumors without underlying cirrhosis (p=0.007) (Table V). We did not find any correlation between E-cadherin and ILK expression in HCC or cirrhosis.

Discussion

ILK has been shown to regulate signaling pathways that are crucial for cell survival, proliferation, cell adhesion, migration and carcinogenesis (19). In the present study, we examined

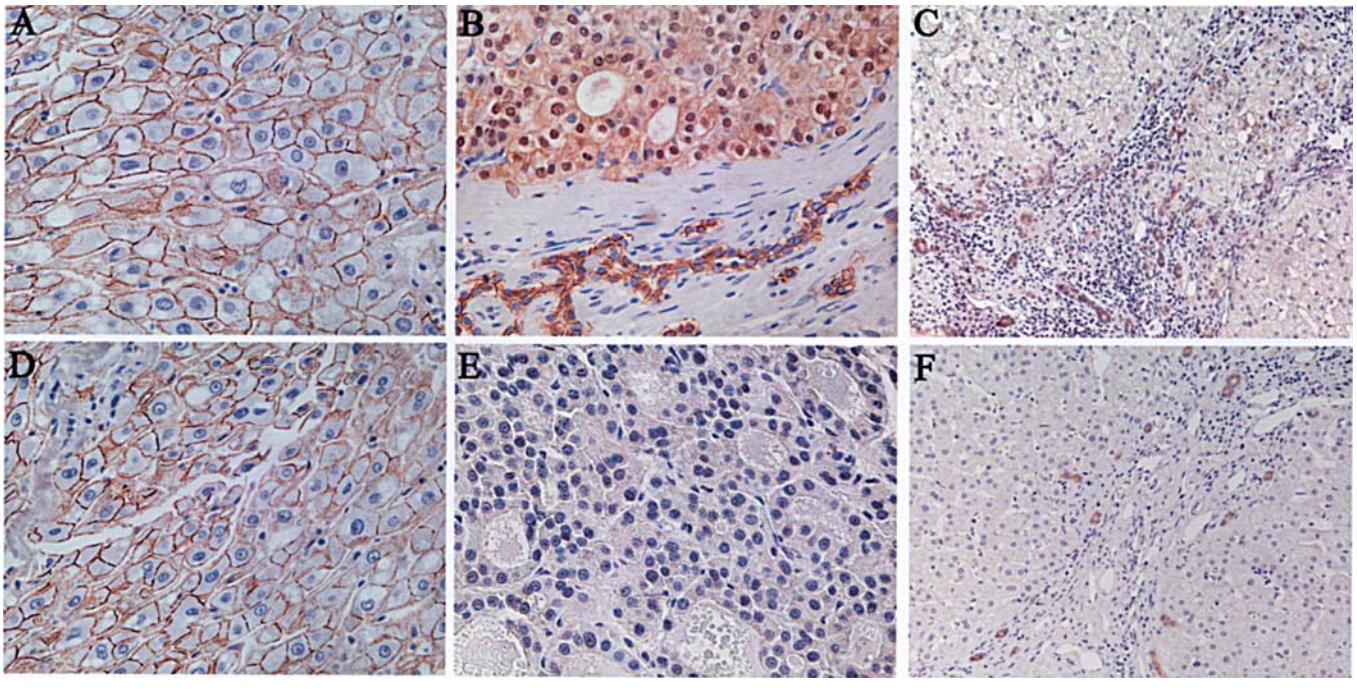

Figure 4. Expression of ß-catenin and E-cadherin in normal liver, HCC and liver cirrhosis. (A) Membranous expression of ß-catenin in normal hepatocytes (x400). (B) Strong nuclear and cytoplasmic staining of ß-catenin in HCC tumor cells while strong membranous expression is retained in adjacent bile ductule epithelium (x400). (C) Focal weak membranous expression of ß-catenin in hepatocytes and strong membranous ß-catenin staining in proliferating bile ductule epithelium of cirrhotic liver (x200). (D) Normal liver showing strong membranous immunopositivity of E-cadherin (x400). (E) HCC demonstrating negative E-cadherin expression (x400). (F) Hepatocytes in cirrhotic liver showing loss of E-cadherin expression while staining is preserved in epithelial cells of proliferating bile ductules (x200).

Table V. E-cadherin expression in human hepatocellular carcinomas and correlation with clinicopathological parameters.

		E-cadherin expression[a]								
		0		1		2		3		
	No. of samples	n	(%)	n	(%)	n	(%)	n	(%)	p-value[b]
HCC	69	51	(75.0)	7	(10.3)	8	(11.8)	2	(2.9)	
Grade										0.896
I	22	16	(72.7)	4	(18.2)	2	(9.1)	0	(0)	
II	24	19	(79.2)	1	(4.2)	4	(16.7)	0	(0)	
III	23	17	(73.9)	2	(8.7)	2	(8.7)	2	(8.7)	
Cirrhosis										0.007
No	22	21	(95.5)	1	(4.5)	0	(0)	0	(0)	
Yes	47	31	(66.0)	6	(12.8)	8	(17)	2	(4.3)	

[a]E-cadherin expression was scored as described in Materials and methods. [b]Kruskal-Wallis or Mann-Whitney test. A p-value <0.05 was considered statistically significant.

the ILK protein expression profile in human hepatocellular carcinomas and liver cirrhosis by immunohistochemistry. We also evaluated the correlation between ILK, p-Akt, E-cadherin and ß-catenin and their relation to clinicopathological parameters.

ILK cytoplasmic overexpression was observed in all tumor specimens in contrast to normal liver where ILK expression was absent or very weak. Consistently, overexpression of ILK has been shown in various human tumors such as melanoma, prostate and colon cancer (11,12,14). *In vitro* data also implicate ILK in multiple aspects of malignant development and progression, including anchorage-independent cell growth and survival, migration and invasion (5,6). Moreover, ILK has been shown to be involved in chemically-induced hepatocarcinogenesis and while preparing our manuscript Intaraprasong *et al* reported overexpression of ILK in human HCC (18). Taken together, these data suggest that ILK overexpression is implicated in liver oncogenesis.

There was no correlation of ILK expression with tumor differentiation of HCC. This finding is in agreement with previous reports in gastric and lung cancer where ILK expression did not correlate with tumor grade (20,21). In contrast, other studies in ovarian tumors, prostate and colon cancer found that the intensity of ILK-specific immunostaining was positively correlated with advancing tumor grade (11-13). Our data support the notion that ILK expression in HCC may not be involved in tumor grade progression.

Importantly, elevated levels of ILK were also found in cirrhotic liver tissue compared to normal liver suggesting a role of ILK in liver cirrhosis. It is known that activation of hepatic stellate cells (HSCs) plays a key role in the development of liver cirrhosis (22). Consistent with our finding of ILK overexpression in human cirrhotic liver specimens, ILK was previously found to be overexpressed in liver from rats with chemical-induced cirrhosis, while culture activation of HSCs was accompanied by a marked upregulation of ILK (23). In the same study, ILK was shown to be critically involved in the regulation of HSC migration, proliferation and apoptosis (23). ILK overexpression has also been observed in renal fibrosis and TGF-ß, a critical inducer of liver fibrosis, has been shown to regulate ILK levels in kidney interstitial cells (24).

Most of the HCCs examined demonstrated activation of Akt. It is well established that the serine/threonine protein kinase Akt plays a key role in carcinogenesis by stimulating cell proliferation and inhibiting apoptosis (25). Consistent with our findings, phosphorylation of Akt that leads to its activation has been observed in a variety of tumors including hepatocellular carcinoma (26). A recent study also demonstrated the critical involvement of Akt phosphorylation in the aggressiveness of HCC and identified p-Akt as a significant prognostic factor (27). However, in our study, there was no correlation of Akt with advancing tumor grade. Notably, phosphorylation of Akt in HCC was higher in tumors in which non-tumoral liver showed no fibrosis, regeneration or other evidence of cirrhosis. Etiological factors of HCC in non-cirrhotic liver are often unidentified, and distinct molecular mechanisms of oncogenesis are probably involved (28). It seems likely that activation of Akt may be related to molecular events that lead to hepatocarcinogenesis in the absence of cirrhosis.

One important finding of our study was the significant correlation of p-Akt with ILK overexpression in human HCC. ILK is known to play a role as an upstream regulator of Akt by stimulating the phosphorylation of Akt on serine residue 473 (10). This interaction has been supported mainly by *in vitro* (9,29,30) and a few *in vivo* studies (12,21). Our results suggest that ILK is probably involved in the activation of Akt during human hepatocarcinogenesis.

Increased phosphorylation of Akt was also observed in liver cirrhosis indicating its potential role in this process. In agreement with our finding, previous *in vitro* studies have reported that activation of the Akt pathway is involved in liver fibrosis by inducing the proliferation, migration and survival

of HSCs (31,32). The PI3K-Akt pathway has been shown to mediate platelet-derived growth factor (PDGF)-induced HSC proliferation (32). Activated Akt has also been demonstrated to result in downregulation of p27kip1 leading HSCs into the S/G2/M phase of the cell cycle and proliferation (31). Additionally, we found a significant correlation of p-Akt with ILK expression in cirrhotic liver tissue suggesting a functional correlation of ILK and Akt signaling in the development of cirrhosis. In line with this hypothesis, *in vitro* studies have shown that ILK is critically involved in the phosphorylation of Akt in activated HSCs (23).

In addition to its role in HSC activation, ILK signaling is involved in inflammatory processes. Specifically, ILK has been shown to regulate inducible nitric oxide synthase and cyclooxygenase-2 expression in an NF-κB-dependent manner and to be implicated in leukocyte trafficking and survival (33,34). Many of these ILK functions are mediated via activation of Akt (33,34). Chronic inflammation significantly accounts for the generation of mutagenic and mitotic stimuli that lead from cirrhosis to HCC (35). Therefore, the implication of ILK signaling in inflammatory responses and liver wound healing suggests that overexpression of ILK and subsequent activation of Akt may represent a molecular link between cirrhosis and HCC.

Activation of ß-catenin and downregulation of E-cadherin were frequently observed in our cases of HCC in accordance with several previous studies (36-39). ILK did not correlate with ß-catenin or E-cadherin in HCC although several studies in epithelial cell lines support an important role of ILK in ß-catenin activation and in the regulation of E-cadherin expression (7,40). It seems likely that in liver oncogenesis mechanisms other than overexpression of ILK account for the observed deregulation of ß-catenin and E-cadherin. In line with this hypothesis, it has been shown that mutations of the Wnt signaling pathway components may be prerequisites for the nuclear accumulation of ß-catenin in hepatocellular carcinomas (37,41). Moreover, loss of E-cadherin in HCC is frequently attributed to genetic or epigenetic alteration of the E-cadherin gene (42).

Reduced membranous ß-catenin and E-cadherin expression was also observed in cirrhotic tissue. However, cytoplasmic/nuclear staining of ß-catenin was not found in cirrhosis consistent with previous reports (36,37). In agreement with our findings, loss of E-cadherin in cirrhotic liver tissue due to promoter methylation has been previously reported (43). These data suggest that loss of cell to cell adhesion as a result of E-cadherin downregulation or decreased membranous ß-catenin may contribute to the abnormal structural reorganization observed in liver cirrhosis.

In conclusion, ILK overexpression and activation of Akt are frequent events in human hepatocellular carcinoma and liver cirrhosis suggesting their involvement in the pathogenesis of these processes. HCC also showed activation of ß-catenin and downregulation of E-cadherin while loss of membranous E-cadherin and ß-catenin was a prominent feature in cirrhosis. While there was no correlation of ILK and ß-catenin or E-cadherin, ILK correlated significantly with activation of Akt in both HCC and cirrhotic liver suggesting an *in vivo* functional association of ILK and Akt during liver oncogenesis and cirrhosis.

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