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Survivin overexpression in HCC and liver cirrhosis differentially correlates with p-STAT3 and E-cadherin

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Summary. Survivin, a member of the family of inhibitor of apoptosis proteins, functions as a key regulator of apoptosis and cell proliferation. Overexpression of survivin has been implicated in several human cancers, including human hepatocellular carcinoma (HCC). Although several factors have been shown *in vitro* to upregulate survivin expression in cancer cells, the *in vivo* regulators of survivin in human hepatocarcinogenesis are largely unknown. We studied by immunohistochemistry the protein expression of survivin in relation to cyclin D1, phosphorylated signal transducer and activator of transcription 3 (p-STAT3), ß-catenin, E-cadherin and phosphorylated-Akt (p-Akt) in 69 cases of HCC and adjacent liver cirrhosis. Survivin was expressed in 63/69 (91.3%) cases of HCC and in 40/47 (85.1%) cases of liver cirrhosis. Survivin localization in HCC was exclusively nuclear, while intense cytoplasmic and low nuclear expression of survivin was observed in cases of cirrhosis. Survivin expression in HCC correlated significantly with low grade tumors, expression of cyclin D1 and p-STAT3. Expression of survivin in liver cirrhosis correlated with downregulation of E-cadherin expression. There was no significant correlation of survivin with ß-catenin or p-Akt in HCC or liver cirrhosis. In conclusion, we showed an association of nuclear survivin with well differentiated HCC, as well as with the expression of the cell cycle regulator cyclin D1. Activation of STAT3 and loss of E-cadherin but not ß-catenin or Akt pathways seem to be implicated in survivin upregulation in HCC and liver cirrhosis.

Key words: Cirrhosis, E-cadherin, Hepatocellular carcinoma, p-STAT3, Survivin

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Introduction

Survivin, a member of the family of inhibitor of apoptosis proteins, functions as a key regulator of programmed cell death and cell cycle progression (Johnson and Howerth, 2004; Altieri, 2006). Expression of survivin inhibits cell death in response to several apoptotic stimuli via caspase-dependent and independent mechanisms (Johnson and Howerth, 2004). Apart from its anti-apoptotic function, survivin mediates the proper targeting of chromosomal passenger proteins to kinetochores and it stabilizes the microtubules, contributing to spindle formation and mitotic progression (Altieri, 2006).

Survivin is expressed during embryonic development but its levels are undetectable in most normal adult differentiated tissues (Adida et al., 1998). However, overexpression of survivin has been consistently demonstrated in human cancer (Okada et al., 2000; Kennedy et al., 2003; Kim et al., 2003; Tringler et al., 2004). Increased survivin expression in several malignancies has been considered an unfavourable prognostic marker, correlating with regional lymph node invasion, metastasis and decreased overall survival (Li et al., 2005). Recent studies have also attributed to survivin a significant role in angiogenesis and chemoresistance (Altieri, 2003a; Blanc-Brude et al., 2003).

Survivin expression can be deregulated in cancer by several mechanisms, including gene amplification, hypomethylation and increased transcription (Altieri, 2003b). Several factors are implicated in trancriptional up-regulation of survivin expression, including members of the Ras oncogene family, signal transducer and activator of transcription 3 (STAT3) and Wnt/ ß-catenin and PI3K/Akt pathways (Zhang et al., 2001; Li, 2003; Wang et al., 2008). On the other hand, transcriptional activity of the survivin gene is repressed by wild-type

p53 (Mirza et al., 2003). Loss of E-cadherin from cell adhesions has recently been shown to contribute to survivin overexpression (Torres et al., 2007).

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer mortality (McGlynn and London, 2005). Cirrhosis of any aetiology may be complicated by HCC and is the greatest single risk factor. Expression of survivin has been previously studied in hepatocarcinogenesis (Ito et al., 2000; Fields et al., 2004; Chau et al., 2007). Nevertheless, there are controversies in the literature regarding the expression, cellular localization and prognostic significance of survivin in cirrhotic liver and HCC (Degushi et al, 2002; Moon and Tarnawski, 2003; Fields et al., 2004; Morinaga et al., 2004; Guo et al., 2006; Chau et al., 2007). In addition, although p53 and hepatitis B virus infection have been implicated in the regulation of survivin levels in HCC cells, the mechanisms that regulate survivin expression in HCC and liver cirrhosis have not been fully elucidated (Kannangai et al., 2005; Lu et al., 2005). In this study we examined by immunohistochemistry the protein expression of survivin in relation to cyclin D1, phosphorylated STAT3 at Tyr705 (p-STAT3), ß-catenin, E-cadherin and phosphorylated Akt at Ser473 (p-Akt) in 69 cases of HCC and 47 cases of adjacent liver cirrhosis. The correlation of survivin expression with clinicopathological parameters of the tumors, such as tumor grade, was also examined.

Materials and methods

Tissue specimens

Formalin-fixed, paraffin-embedded tissue specimens from 69 human hepatocellular carcinomas and 4 non-neoplastic, non cirrhotic liver wedge biopsies were obtained from the Department of Pathology "Aretaieion" Hospital, Athens, Greece according to ethical guidelines. H&E slides were examined in a double blinded study by two pathologists and the diagnosis of hepatocellular carcinoma was confirmed. The tumors were graded according to the WHO criteria and included 22 well differentiated (grade 1), 24 moderately differentiated (grade II) and 23 poorly differentiated (grade III) hepatocellular carcinomas. In 47/69 HCC specimens hepatitis B virus (HBV)-related cirrhosis was present in liver parenchyma adjacent to the tumour.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Peroukides et al., 2008). Briefly, representative 4μm tissue sections were de-paraffinized, rehydraded and heat-treated in 0.01M citrate buffer (pH=6) in a MW 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 1). The Envision Detection System (DAKO, Hamburg, Germany) was used for visualization according to the manufacturer's instructions. Sections were counterstained with haematoxylin, dehydrated and mounted. Negative controls were performed in all cases by omitting the primary antibodies.

Evaluation of immunohistochemical staining

All slides were assessed by two pathologists (HP, JV) and two investigators (SP, VB) independently and blinded to the case. Both intensity of staining and percentage of positive cells were taken into account. Cytoplasmic and nuclear staining were evaluated separately when observed. The following scoring system was used: 0; staining in less than 10% of tumor cells, 1; weak staining in 10-70% of tumor cells or moderate staining in <35%, 2: weak staining in >70%, moderate staining in >35% 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

Table 1. Antibody characteristics.

Antibody	Type	Source	Dilution	Protein blocking
Survivin	M	Cell Signaling, Beverly, MA	1:100	TBS-3% BSA
Cyclin D1	M	Spring Bioscience	1:40	TBS-3% BSA
p-STAT3 (Tyr705)	M	Cell Signaling, Beverly, MA	1:25	TBS-3% BSA
ß-catenin	M	BD Biosciences, CA, USA	1:2000	TBS-5%milk
E-cadherin	M	BD Biosciences, CA, USA	1:2000	TBS-5% milk
p-Akt (Ser473)	P	Cell Signaling, Beverly, MA	1:50	TBS-3% BSA

significance level was defined as $p<0.05$.

Results

Survivin is overexpressed in HCC and liver cirrhosis with distinct subcellular localization pattern

Survivin expression in non-neoplastic, non-cirrhotic liver was absent, while positive staining was found in 40/47 (85.1%) cases of liver cirrhosis and in 63/69 (91.3%) cases of HCC. In liver cirrhosis, expression of survivin was cytoplasmic and/or nuclear. Cytoplasmic localization of survivin was found in 32/47 (68.1%) cases of adjacent cirrhotic liver and nuclear expression was observed in 19/47(40.4%) cases. In 8/21 cases of cirrhosis there was only nuclear survivin expression, in 21/47 there was only cytoplasmic localization and in 11/47 cases survivin was localized both in the nucleus

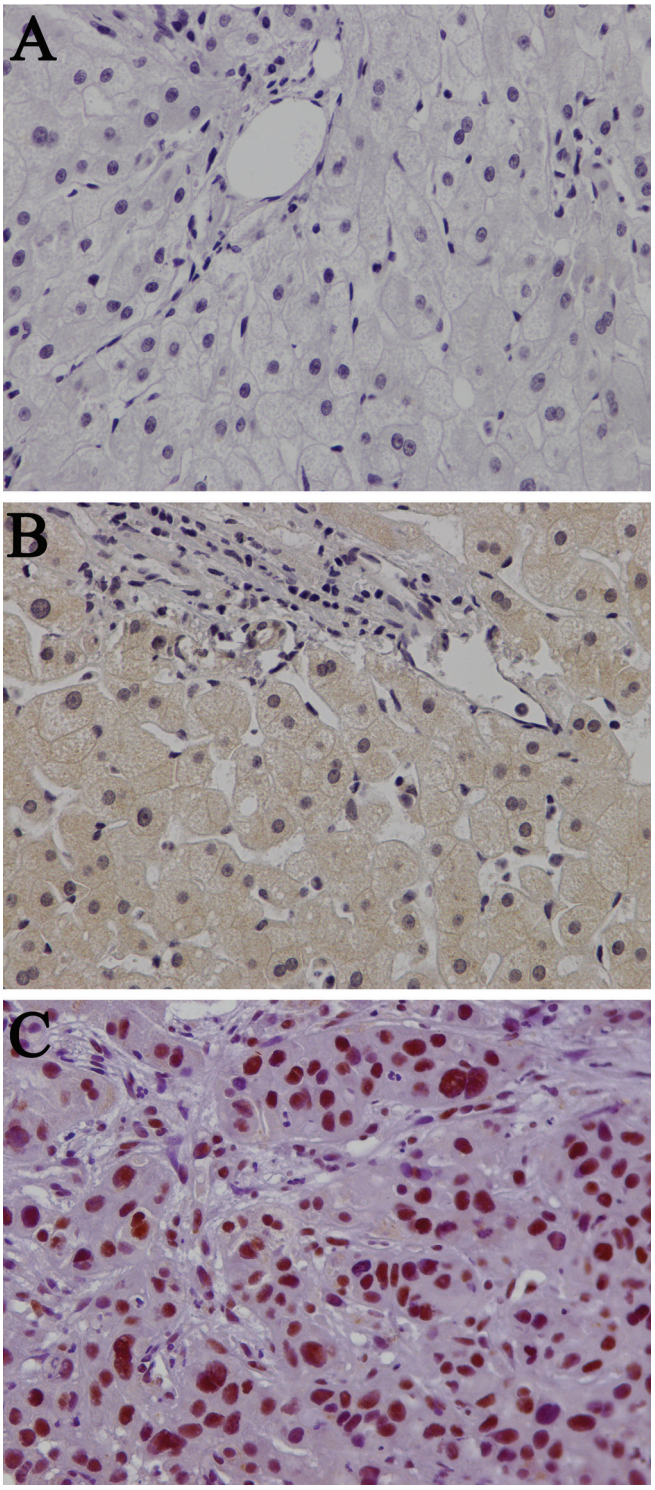

Fig. 1. Survivin is overexpressed in liver cirrhosis and HCC with distinct localization pattern. **A.** Negative survivin expression in non-neoplastic, non cirrhotic liver. **B.** Cytoplasmic and low nuclear expression of survivin in liver cirrhosis. **C.** High nuclear and negative cytoplasmic immunostaining of survivin in a case of HCC. x 400.

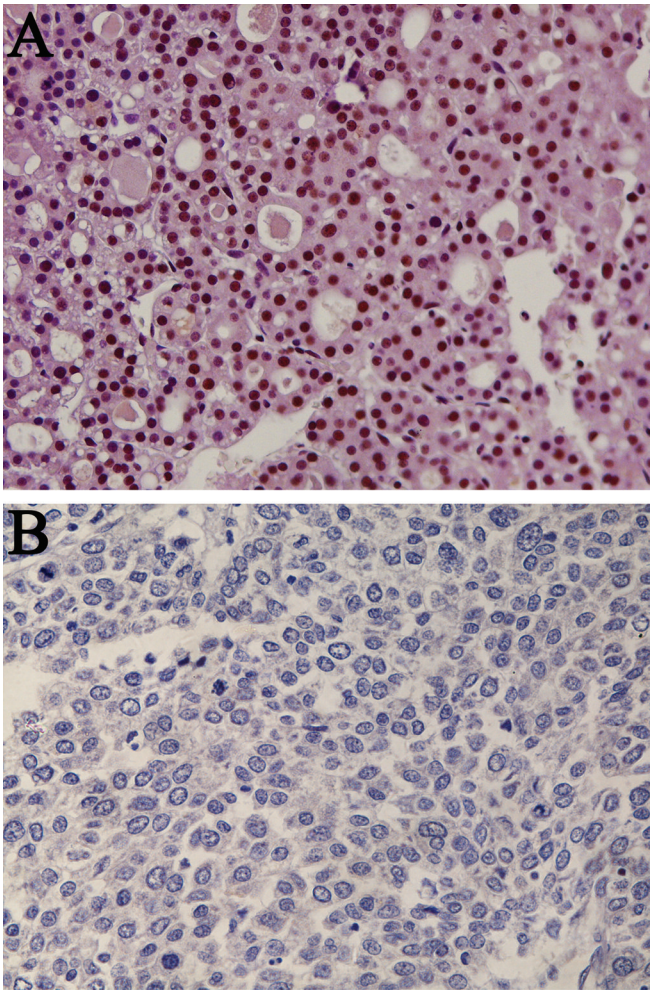

Fig. 2. Survivin expression in HCC correlates with low grade tumors. **A.** High nuclear expression of survivin in a well differentiated tumor. **B.** Negative staining for survivin in a poorly differentiated HCC. x 200.

and cytoplasm of hepatocytes. In HCC, survivin immunopositivity was confined to the nucleus of cancer cells (Fig. 1). Notably, nuclear expression of survivin was significantly higher in HCC compared to cirrhosis ($p<0.001$).

Expression of survivin in HCC correlates with tumor grade, cyclin D1 and p-STAT3

Nuclear survivin expression in HCC significantly correlated with tumor grade being higher in well differentiated tumors ($p<0.001$) (Fig. 2). There was no statistical, significant correlation of survivin with the presence of cirrhosis (Table 2). Nuclear positivity for cyclin D1 and p-STAT3 was observed in 46/69 (66.7%) and 9/55 (16.4%) cases of HCC respectively, while their expression in non-neoplastic, non-cirrhotic liver was absent (Fig. 3). There was no significant correlation of cyclin D1 or p-STAT3 with any of the parameters under evaluation (Table 3). Notably, expression of survivin in HCC correlated significantly with cyclin D1 ($r=0.372$, $p=0.002$) and p-STAT3 ($r=0.328$, $p=0.015$) (Fig. 4) (Table 4). However, there was no statistically significant correlation of survivin expression with ß-catenin, E-cadherin or p-Akt in the tumor specimens examined (Table 4).

Expression of survivin in liver cirrhosis correlates with loss of E-cadherin

Nuclear immunostaining of cyclin D1 and p-STAT3 was observed in 21/47 (44.7%) and 22/37 (59.5%) cases of liver cirrhosis respectively. While cyclin D1 expression was significantly higher in HCC compared to liver cirrhosis ($p=0.011$), immumopositivity of p-STAT3 was higher in cirrhotic nodules compared to HCC

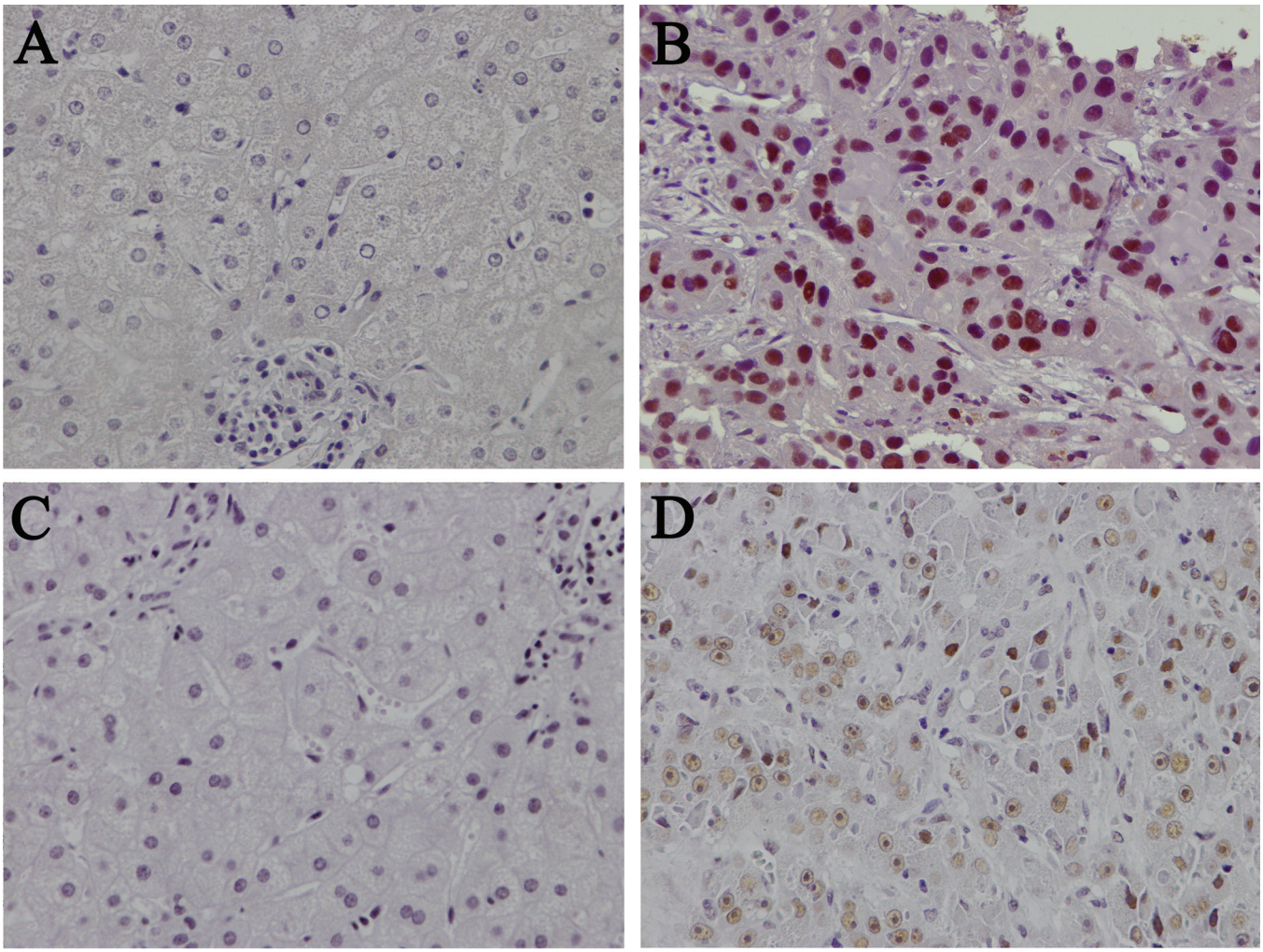

Fig. 3. Cyclin D1 and p-STAT3 are overexpressed in HCC. Negative immunostaining for cyclin D1 **(A)** and p-STAT3 **(C)** in non-neoplastic, non cirrhotic liver. Intense nuclear expression of cyclin D1 **(B)** and p-STAT3 **(D)** in cases of HCC. x 400

Table 2. Survivin expression in human hepatocellular carcinoma. Correlation with clinicopathological parameters.

		N	Survivin expression[a]								p value[b]
			0		1		2		3		
			n	(%)	n	(%)	n	(%)	n	(%)	
HCC		69	6	(8.7)	19	(27.5)	24	(34.8)	20	(29)	
Grade	Grade I	22	0	(0)	0	(0)	12	(54.5)	10	(45.5)	0.000
	Grade II	24	3	(12.5)	6	(25)	6	(25)	9	(37.5)	
	Grade III	23	3	(13)	13	(56.5)	6	(26.1)	1	(4.3)	
Cirrhosis	no	22	1	(4.5)	7	(31.8)	8	(36.4)	6	(27.3)	0.973
	yes	47	5	(10.6)	12	(25.5)	16	(34)	14	(29.8)	

[a]: Expression of survivin was scored as described in Materials and Methods. [b]: Kruskal-Wallis or Mann-Whitney test. P value<0.005 was considered statistical significant.

Table 3. Cyclin D1 and p-STAT3 expression in human hepatocellular carcinoma. Correlation with clinicopathological parameters.

		N	Cyclin D1[a]				p value[b]	N	p-STAT3[a]				p value[b]
			0	1	2	3			0	1	2	3	
			n (%)	n (%)	n (%)	n (%)			n (%)	n (%)	n (%)	n (%)	
HCC		69	23 (33.3)	11 (15.9)	22 (31.9)	13 (18.8)		55	46 (83.6)	8 (14.5)	1 (1.8)	0 (0.0)	
Grade	Grade I	22	4 (18.2)	2 (9.1)	11 (50)	5 (22.7)		15	12 (80)	3 (20)	0 (0)	0 (0)	
	Grade II	24	9 (37.5)	3 (12.5)	6 (25)	6 (25)	0.257	21	16 (76.2)	4 (19)	1 (4.8)	0 (0)	0.255
	Grade III	23	10 (43.5)	6 (26.1)	5 (21.7)	2 (8.7)		19	18 (94.1)	1 (5.3)	0 (0)	0 (0)	
Cirrhosis	no	22	5 (22.7)	5 (22.7)	7 (31.8)	5 (22.7)	0.358	18	15 (83.3)	2 (11.1)	1 (5.6)	0 (0)	0.9
	yes	47	18 (38.3)	6 (12.8)	15 (31.9)	8 (17)		37	31 (83.8)	6 (16.2)	0 (0)	0 (0)	

[a]: Expression of cyclin D1 and p-STAT3 was scored as described in Materials and Methods. [b]: Kruskal-Wallis or Mann-Whitney test. P value<0.005 was considered statistical significant.

Table 4. Correlation of survivin expression with cyclin D1, p-STAT3, nuclear ß-catenin, E-cadherin and p-Akt in HCC.

		Survivin				r/p-value*
		0	1	2	3	
Cyclin D1	0	5 (7.2%)	6 (8.7%)	9 (13)	3 (4.3%)	0.372/0.002
	1	0 (0%)	6 (8.7%)	3 (4.3)	2 (2.9%)	
	2	1 (1.4%)	5 (7.2%)	8 (11.6)	8 (11.6%)	
	3	0 (0%)	2 (2.9)	4 (5.8)	7 (10.1%)	
p-STAT3	0	6 (10.9%)	14 (25.5%)	18 (32.7%)	8(14.5%)	0.328/0.015
	1	0 (0%)	2 (3.6%)	1 (1.8%)	5 (9.1%)	
	2	0 (0%)	0 (0%)	0 (0%)	1 (1.8%)	
Nuclear ß-catenin	0	4 (5.8%)	8 (11.6%)	13 (18.8%)	11 (15.9%)	0.011/0.928
	1	0 (0%)	5 (7.2%)	3 (4.3)	1 (1.4%)	
	2	1 (1.4%)	6 (8.7%)	7 (10.1%)	6 (8.7%)	
	3	1 (1.4%)	0 (0%)	1 (1.4%)	2 (2.9)	
E-cadherin	0	4 (5.8%)	15 (21.7%)	18 (26.1%)	15 (21.7%)	-0.014/0.909
	1	0 (0%)	2 (2.9)	3 (4.3)	2 (2.9)	
	2	2 (2.9)	1 (1.4%)	2 (2.9)	3 (4.3)	
	3	0 (0%)	1 (1.4%)	1 (1.4%)	0 (0%)	
p-Akt	0	2 (2.9)	4 (5.8%)	4 (5.8%)	5 (7.2%)	0.004/0.971
	1	2 (2.9)	7 (10.1%)	7 (10.1%)	6 (8.7%)	
	2	1 (1.4%)	5 (7.2%)	9 (13%)	8 (11.6%)	
	3	1 (1.4%)	3 (4.3)	4 (5.8%)	1 (1.4%)	

* Spearman rank-order correlation coefficient.

(p<0.001). Nuclear expression of survivin in cirrhosis correlated significantly with expression of cyclin D1 (r=0.297, p=0.042). However, in contrast to HCC, survivin expression in cirrhosis was not correlated with p-STAT3. Interestingly, there was a significant correlation between nuclear survivin and downregulation of E-cadherin (r=0.405, p=0.005) in cases of cirrhosis (Fig. 5). No significant correlation was noticed between survivin and p-Akt (Table 5).

Table 5. Correlation of nuclear survivin expression with cyclin D1, p-STAT3, E-cadherin and p-Akt in liver cirrhosis.

		Nuclear Survivin			r/p-value*
		0	1	2	
Cyclin D1	0	18 (38.3%)	6 (12.8%)	2 (4.3%)	0.297/0.042
	1	6 (12.8%)	2 (4.3%)	2 (4.3%)	
	2	4 (8.5%)	3 (6.4%)	4 (8.5%)	
p-STAT3	0	11 (29.7%)	4 (10.8)	0 (0%)	0.193/0.252
	1	6 (16.2%)	2 (5.4%)	3 (8.1%)	
	2	4 (10.8)	0 (0%)	1 (2.7%)	
	3	3 (8.1%)	1 (2.7%)	2 (5.4%)	
E-cadherin	0	12 (25.5%)	10 (21.3%)	6 (12.8%)	-0.405/0.005
	1	1 (2.1%)	1 (2.1%)	0 (0%)	
	2	2 (4.3%)	0 (0%)	1 (2.1%)	
	3	13 (27.7%)	0 (0%)	1 (2.1%)	
p-Akt	0	8 (17%)	3 (6.4%)	1 (2.1%)	0.058/0.697
	1	4 (8.5%)	2 (4.3%)	1 (2.1%)	
	2	14 (29.8%)	6 (12.8%)	6 (12.8%)	
	3	2 (4.3%)	0 (0%)	0 (0%)	

* Spearman rank-order correlation coefficient.

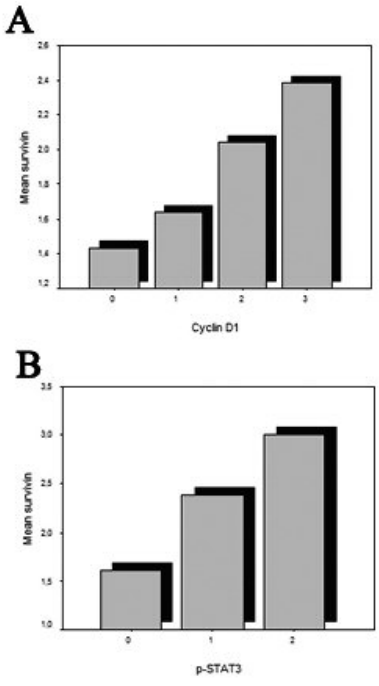

Fig. 4. Bars showing significant correlation of survivin expression in HCC with cyclin D1 **(A)** and p-STAT3 **(B)**.

Discussion

Survivin regulates apoptosis and cell proliferation and it has been critically implicated in human carcinogenesis (Altieri, 2003a,b). In our study, survivin was overexpressed in HCC and liver cirrhosis with a significant difference in its subcellular localization pattern. Cirrhotic liver showed high cytoplasmic and low nuclear survivin staining, while high nuclear survivin expression was observed in HCC. It is known that survivin exists in two different cytoplasmic and nuclear pools, with distinct functions of the two compartments; cytoplasmic survivin suppresses apoptosis while nuclear survivin regulates mitosis (Li et al., 2005). Consistent with our findings, increased survivin mRNA and protein expression has been previously demonstrated in chronic hepatitis, liver cirrhosis and HCC (Deguchi et al., 2002; Moon and Tarnawski, 2003; Takashima et al., 2005; Chau et al., 2007). Additionally, previous studies have shown that survivin localizes in the nucleus of HCC cells, correlates with the proliferation index of the tumors and promotes cell proliferation of hepatoma cells (Ito et al., 2000; Fields et al., 2004). It has also been reported that cytoplasmic survivin is associated with apoptosis of non-malignant hepatocytes (Moon and Tarnawski, 2003). These findings suggest that the predominant role of survivin in HCC is promotion of cell proliferation, while in liver cirrhosis survivin exerts mainly cytoplasmic anti-apoptotic functions (Ito et al., 2000; Moon and Tarnawski, 2003; Fields et al., 2004; Llovet et al., 2006).

We also showed that high nuclear survivin in HCCs was correlated with low tumor grade, possibly indicating a favourable prognostic role of survivin in HCC. The prognostic significance of nuclear survivin in HCC is controversial (Fields et al., 2004; Morinaga et al., 2004; Guo et al., 2006). In support of our findings, nuclear survivin correlated with favourable prognostic parameters in other human malignancies (Okada et al., 2000; Kennedy et al., 2003; Tringler et al., 2004).

We demonstrated a significant correlation of nuclear survivin with the expression of the cell-cycle regulator cyclin D1, consistent with the proposed predominant role of nuclear survivin in cell proliferation (Li et al., 2005). In line with our findings, survivin has been shown to regulate cyclin D1 expression in endometrial cancer cells (Zhihong et al., 2006). In addition, survivin and cyclin D1 are known to be target genes of common regulatory pathways such as ß-catenin and STAT3. (Yoshida et al., 2002; Gotoh et al., 2003; Gritsko et al., 2006; Sakoguchi-Okada et al., 2007).

Although several mechanisms may account for

survivin overexpression in cancer, the *in vivo* regulators of survivin in hepatocarcinogenesis are largely unknown. We demonstrated overexpression of p-STAT3 in HCC according to previous studies, and we additionally showed a significant correlation of activated STAT3 with nuclear survivin expression (Fuke et al., 2007). Consistent with our findings, several in vitro studies have implicated p-STAT3 in the transcriptional regulation of survivin, and inhibition of STAT3 has been demonstrated to suppress survivin expression in HCC cell lines (Gritsko et al., 2006; Li et al., 2006).

We also found significantly higher levels of p-STAT3 in cirrhosis compared to HCC, in agreement with previous studies suggesting a significant role of p-STAT3 in chronic liver disease (Costa et al., 2003; Mangnall et al., 2003; Starkel et al., 2003; Taub, 2003). However, in contrast to HCC, we found no significant correlation of p-STAT3 with survivin expression in cirrhosis. This finding may be explained by previous data suggesting that although p-STAT3 expression is increased, its transcriptional activity is inhibited in liver cirrhosis (Mangnall et al., 2003; Starkel et al., 2003, 2007).

We also showed that there was no significant correlation between survivin expression and ß-catenin or Akt in HCC and liver cirrhosis, although several *in vitro* studies have implicated ß-catenin and Akt pathways in the regulation of survivin levels (Zhang et al., 2001; Kim et al., 2003; Wang et al., 2008). Activation of ß-catenin and Akt in the same subset of tumors has been previously reported (Peroukides et al., 2008). This finding suggests that in hepatocarcinogenesis mechanisms other than activation of ß-catenin or Akt, such as p53, hepatitis B virus infection and p-STAT3,

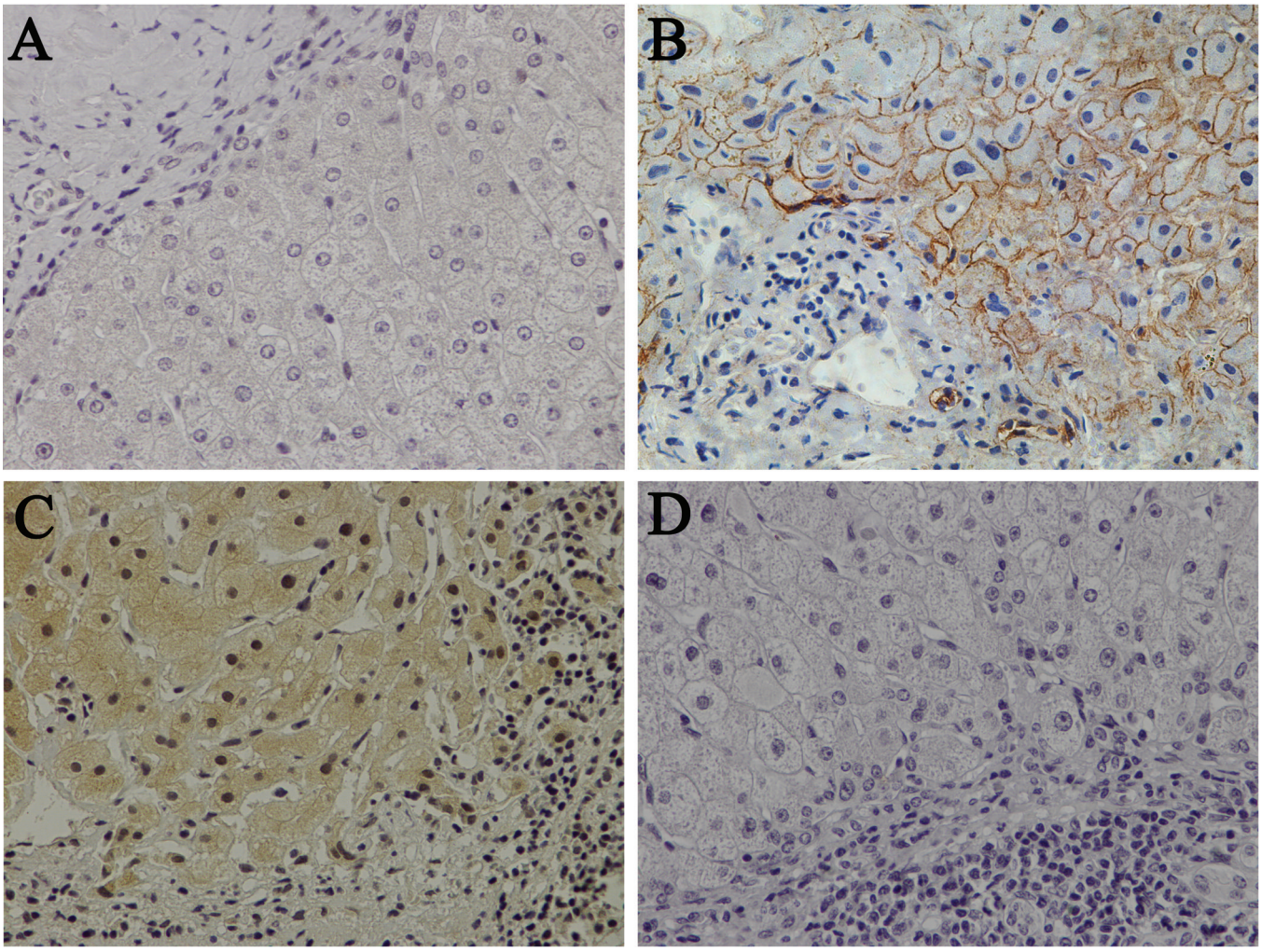

Fig. 5. Survivin expression in liver cirrhosis correlates with downregulation of E-cadherin. **A, B.** A case of liver cirrhosis showing negative survivin expression **(A)** and intense membranous expression of E-cadherin **(B). C, D.** A case of liver cirrhosis with increased nuclear expression of survivin **(C)** and negative membranous E-cadherin immunostaining **(D).** x 400

may account for the observed survivin overexpression (Kannangai et al., 2005; Lu et al., 2005; Li et al., 2006).

Interestingly, while in HCC there was no correlation of survivin with E-cadherin, nuclear survivin in liver cirrhosis significantly correlated with downregulation of E-cadherin. The implication of decreased E-cadherin expression in HCC and liver cirrhosis has been previously reported (Lee et al., 2003; Peroukides et al., 2008). In support of our findings it has been recently shown that E-cadherin is required for caveolin-1 mediated downregulation of survivin, and VE-cadherin has been reported to inhibit survivin expression in endothelial cells (Iurlaro et al., 2004; Torres et al., 2007). In addition, downregulation of E-cadherin has been demonstrated to correlate with survivin expression in epidermal and pancreatic cell lines, as well as in ovarian tumors, indicating a non-cell type-specific role of cadherins in regulating survivin expression (Iurlaro et al., 2004).

In conclusion, we demonstrated increased protein levels and distinct subcellular localization of survivin in HCC and liver cirrhosis. Nuclear survivin associated with well differentiated HCC as well as with overexpression of cyclin D1. Furthermore, survivin overexpression in HCC and liver cirrhosis differentially correlated with p-STAT3 and E-cadherin but not with activation of ß-catenin or Akt.

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