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Survivin expression in hepatocellular carcinoma: correlation with proliferation, prognostic parameters, and outcome

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Survivin **is a novel inhibitor of apoptosis. It is detected in fetal and neoplastic adult tissue, but not in normal tissues. Several recent studies have shown that *survivin* not only inhibits apoptosis, but also accelerates cancer cell proliferative activity. Expression of the protein may be of prognostic significance and therapeutic relevance in many cancers. We investigated survivin expression in hepatocellular carcinoma, correlating results with proliferation (MIB-1), prognostic factors, and outcome. Paraffin-embedded sections of 72 hepatocellular carcinoma were immunostained for survivin and MIB-1 using tissue microarray technology. Expression was evaluated in nuclei and cytoplasm as intensity (0–3 +), and percentage of positive cells scored on a four-tiered system with less than 10% = negative; 10–25% = 1; 26–50% = 2; 51–75% = 3; and 76–100% = 4. Frequency of nuclear survivin expression was 43%. There was a significant correlation between nuclear survivin expression and nuclear grade (*P* = 0.0271), microvascular invasion (*P* = 0.0064), mitotic rate (*P* = 0.0017), and MIB-1 (*P* = 0.0001), as well as local recurrence (*P* = 0.0487), and disease-free survival (*P* = 0.0098). Histologic grade (*P* = 0.0544) and stage (*P* = 0.0548) tended to correlate with survivin expression, which did not correlate with cirrhosis, tumor necrosis, multiple tumors, metastatic disease, or overall survival. Survivin expression correlates with poor prognostic parameters (high nuclear and histologic grade, microvascular invasion, increased proliferation (mitotic count, MIB-1)), local recurrence, and shorter disease-free survival, but does not correlate with overall survival. An important role is suggested for survivin in progression, recurrence, and treatment of hepatocellular carcinoma.**
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Survivin, a member of the inhibitor-of-apoptosis proteins (IAP) family[1,2] is not present in most normal adult differentiated tissues. Its mRNA and the protein are present in large amounts in fetal tissue and most cancers.[3,4]

Survivin protein suppresses apoptosis (programmed cell death) and stimulates cell division.[5] The mechanism whereby it blocks apoptosis is assumed to be via an effect on caspase-9 (causing dissociation of the Apaf-1–caspase-9 complex), which is activated through extrinsic and intrinsic pathways.[2,6] In the intrinsic cell death pathway, upstream stimuli (such as activation of p53) induce expression of proapoptotic bcl-2 family proteins such as bax. The membrane-permeabilizing effects of bax and other proapoptotic proteins are inhibited by antiapoptotic proteins, such as bcl-2 and bcl-X_L, which also may inactivate caspase-9 by binding and inactivating the adapter protein Apaf-1.[7,8] When, as a result, caspase-3 is not activated, apoptosis is inhibited.[1,9]

Survivin is expressed during the G_2/M phase of the cell cycle. It associates with microtubules of the mitotic spindle, at the beginning of mitosis. Disruption of survivin–microtubule interaction results in loss of survivin antiapoptotic function, and during mitosis, caspase-3 activity increases.[10,11] Thus, *survivin* seems to inhibit apoptosis of tumor cells, and accelerate their mitotic activity.[12] In support of this stimulatory effect on proliferation, survivin expression is shown in hepatocellular carcinoma cell lines, to result in a decrease in the G0G1 phase with an increase in the S phase.[13] This promotion of cell

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proliferation by survivin was due to its interaction with cyclin-dependent kinase 4 (Cdk4) with release of p21 (WAFI/Cip 1) from Cdk4.[13] In human hepatocellular carcinoma, nuclear survivin by immunofluorescence and immunohistochemistry strongly correlated with proliferation index (Proliferation Nuclear Cell Antigen) although not with apoptotic index (Tunel method).[13,14] In other studies,[12,15,16] survivin messenger RNA (mRNA) quantitated by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) (divided by amount of glyceraldehyde-3-phosphate dehydrogenase [GAPDH] mRNA to obtain normalized amounts of transcripts), correlated significantly with proliferation (MIB-1), but with a significant negative correlation with apoptotic index (single-stranded DNA).

In hepatocellular carcinoma, a high survivin mRNA GAPDH ratio by RT-PCR is shown to correlate significantly with decreased disease-free 5-year survival and increased recurrence rate.[12,15] Also, the mean mRNA survivin:GAPDH ratio is significantly higher in patients with recurrence than in those without recurrence,[12] a high ratio correlating with a high recurrence rate,[16] and being a strong independent parameter of tumor recurrence after hepatectomy.[12,16]

We studied survivin expression by immunohistochemistry in a tissue microarray of hepatocellular carcinoma, correlating results with prognostic parameters, proliferation (MIB-1), recurrence rates, and outcome (overall and disease-free survival).

Materials and methods

Tissue Specimens

In total, 87 cases of hepatocellular carcinoma diagnosed between 1985 and 2002 were studied. These included 28 liver explants, 28 lobectomies, 14 segmentectomies, 12 wedge resections, and five autopsy specimens. All cases with pre- and post-operative treatment were excluded. The non-neoplastic liver studied, included six in cirrhotic liver adjacent to hepatocellular carcinoma, one in non-cirrhotic liver with hepatocellular carcinoma, and one from a normal liver without cirrhosis or carcinoma. The hepatocellular carcinoma were histologically graded as I–III on the basis of cellular atypia and architectural complexity, according to Edmondson and Steiner.[17] Clinical and macroscopic and/or microscopic features of adjacent non-neoplastic livers were used to diagnose cirrhosis. Nuclear grade was assessed as mild, moderate, or severe cytologic nuclear atypia, mitotic count was per 10 high-power fields $\times 400$ magnification (total area 1.5 mm^2, with a single field of 0.152 mm^2 ($\times 40$ objective, 0.44 mm field diameter)), tumor necrosis, and microvascular invasion as present or absent. Staging (AJCC),[18] tumor size, mode of therapy, and survival data were obtained by review of surgical pathology and/or autopsy reports, and from the Winship Cancer Center database, with approval of the Investigational Review Board. The mean follow-up time was 23 $\pm$20 months.

Targeted tissue areas of 87 hepatocellular carcinoma and eight normal livers were marked on hematoxylin- and eosin-stained sections. Corresponding formalin-fixed paraffin-embedded tissue blocks were obtained from the archives of the Division of Anatomic Pathology, Department of Pathology and Laboratory Medicine, Atlanta, GA, with Institutional Review Board approval. Two tissue cores, 1.0 mm in diameter and 3–4 mm in thickness, were removed from each block, using a manual microarray device (Beecher Instruments, Silver Spring, MD, USA), with a total of 190 tissue cores inserted into the recipient paraffin block. The tissue microarray was sectioned at 4 μm thickness and placed on charged slides.

Immunohistochemistry was performed after steam heat-induced epitope retrieval in a pressure cooker, using polyclonal survivin (1:80) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and monoclonal MIB-1 (clone MIB-1) (1/160) (DAKO Corporation, Carpinteria, CA, USA). An avidin–biotin complex kit (LSAB + System; DAKO) was used with the DAKO Autostainer. Hematoxylin was the counterstain. Sections of colonic adenocarcinoma were positive controls for survivin, and tonsil for MIB-1. Negative controls had primary antibody replaced by buffer. The survivin antibody used has been shown to react specifically with survivin of human origin by Western blotting and immunohistochemistry. Immunostain was recorded as 0–3+ according to stain intensity, distribution in cytoplasm and/or nucleus, and percentage of cancerous cells that stained positively. Tumors were scored on a four-tiered system, with $<10\%$ of carcinoma cells staining called negative, 10–25% positively scored as 1, 26%–50% scored as 2, 51–75% scored as 3, and 76–100 scored as 4.

Statistical Analysis

The data were analyzed using a combination of Fisher's exact test, *t*-tests, analysis of variance (ANOVA), Pearson's correlation, and Kaplan–Meier survival curves followed by logrank tests and Cox proportional hazards modes.

When comparing two categorical factors such as nuclear grade *vs* cirrhosis, Fisher's exact tests were used. When comparing a dichotomous factor *vs* a continuous factor such as nuclear stain *vs* tumor size, *t*-tests were used. When comparing a categorical factor *vs* continuous factor such as nuclear stain at three levels *vs* tumor size, ANOVA followed by Tukey's pairwise comparisons were used. Pearson's correlation was used to test the relationship between two continuous factors such as MIB-1 and nuclear stain percent.

Finally, overall and disease-free survival were compared across other factors using Kaplan–Meier curves and logrank tests. For those factors that were continuous, such as size, Cox proportional hazards model was used instead.

Results

In all, 72 (83%) of the 87 cases of hepatocellular carcinoma were adequate for evaluation. The median age was 59 years (range 24–84). Totally, 77% of patients were male and 23% female. Cirrhosis was present in 61%. The median tumor size was 4.5 cm (range 0.4–19); the mean tumor size 5.5 ± 4.4 cm. In total, 72% of the hepatocellular carcinoma were solitary tumors, 28% multiple. Regional nodes were positive for metastatic hepatocellular carcinoma in three of the 72 cases. Metastases were identified in 15%. Overall, 28% were Stage I, 43% Stage II, 12% Stage III, and 18% Stage IV. Local recurrences occurred in 15%.

Histopathologic types were 69, not otherwise specified, two fibrolamellar and one mixed hepatocellular carcinoma/cholangiocarcinoma. Histologic grade was I in 12.5%, II in 75%, and III in 12.5%. Nuclear grade was 1 in 18%, 2 in 50%, and 3 in 32%. Tumor necrosis was present in 54%, microvascular invasion in 54%. Nuclear survivin expression was present in 43% of 87 hepatocellular carcinoma (Figure 1a), but in none of the eight normal livers (Figure 1b). The mean mitotic activity was 2.8 per 10 high-power fields. Mean MIB-1 labeling index was 6.1% in hepatocellular carcinoma (Figure 1c), and <2% in normal livers (Figure 1d).

Table 1 shows the correlation of nuclear survivin immunopositivity in the hepatocellular carcinoma with clinicopathologic parameters. Survivin immunoreactivity in the hepatocellular carcinoma correlated significantly with nuclear grade ($P = 0.0271$), microvascular invasion ($P = 0.0064$), and local recurrence ($P = 0.0487$). There was a tendency for Stage with Stages III and IV grouped together ($P = 0.0548$), and histologic grade ($P = 0.0544$) to correlate with nuclear survivin expression. Other parameters including tumor necrosis, showed no correlation with survivin expression.

Comparing the mean number of mitoses per 10 high-power fields and the mean MIB-1 labeling index in hepatocellular carcinoma with and without nuclear survivin expression (Table 2), there was as significant correlation with the presence of survivin ($P = 0.0017$ and 0.0001, respectively). Figure 2 graphs nuclear survivin expression against MIB-1 labeling index ($P = 0.0001$, correlation coefficient $R = 0.526$).

Disease-free survival was significantly better in patients with hepatocellular carcinoma lacking survivin expression, as compared with those whose hepatocellular carcinoma expressed survivin ($P = 0.0098$) (Figure 3a). This improvement in disease-free survival is also noted when comparing patients with no (<10% positive) *vs* low *vs* high survivin immunoreactivity ($P = 0.026$) in their neoplasms (Figure 3b). The breakdown into survivin expression low (10–15% positive) and high (>15% positive) was based on median split. The overall survival did not correlate with survivin expression ($P = 0.4994$).

By multivariate analysis, tumor size was the only parameter related to overall survival ($P = 0.0033$), while MIB-1 was the most important factor related to disease-free survival ($P = 0.0006$).

Discussion

Hepatocellular carcinoma is the fifth most common malignancy in men, and the eighth in women, accounting for 1 million deaths in the world annually.[19,20] Risk factors include cirrhosis, hepatitis B and C infections, alcohol abuse, aflatoxin exposure, hemochromatosis, and alpha-1-antitrypsin deficiency.[19,21] The only definitive treatment is surgical resection or transplantation,[19,20] although over half the patients have disease recurrence after surgical resection.[19] Attempts have been made to identify markers that can predict disease recurrence. Poor clinicopathologic prognostic factors include TNM staging (size, multiplicity, nodal status, metastasis, microvascular invasion)[18,20] and nuclear grade and microvascular invasion.[22]

Survivin expression, nuclear in distribution by immunohistochemistry, and/or by RT-PCR is reported to be associated with unfavorable histology and aggressive disseminated disease in endometrial and ovarian carcinoma.[10,23] The overall survival in pulmonary non-small-cell carcinoma,[24] esophageal carcinoma,[25,26] gliomas,[27] soft-tissue sarcoma,[28] squamous laryngeal carcinoma,[29] and endometrial carcinoma[10] is significantly decreased in patients with neoplasms expressing survivin as compared to those with no expression. Disease-free survival is significantly decreased in patients with malignant melanoma,[30] and recurrence rate is increased in patients with neuroblastoma[31] when survivin is present in the neoplasm.

In hepatocellular carcinoma patients, disease-free survival is decreased,[12] and the recurrence rate increased,[12] when survivin is demonstrated in the carcinoma by RT-PCR. We, using immunohistochemistry, show nuclear survivin expression in hepatocellular carcinoma to correlate with poor prognostic parameters (nuclear grade, microvascular invasion, and proliferation as mitotic rate and MIB-1 labeling index) and worse outcome (shorter disease-free survival, increased recurrence rate).

Survivin expression is reported in 41% hepatocellular carcinoma by mRNA[12] concordant with immunohistochemistry. Our 43% frequency of nuclear survivin by immunohistochemistry in

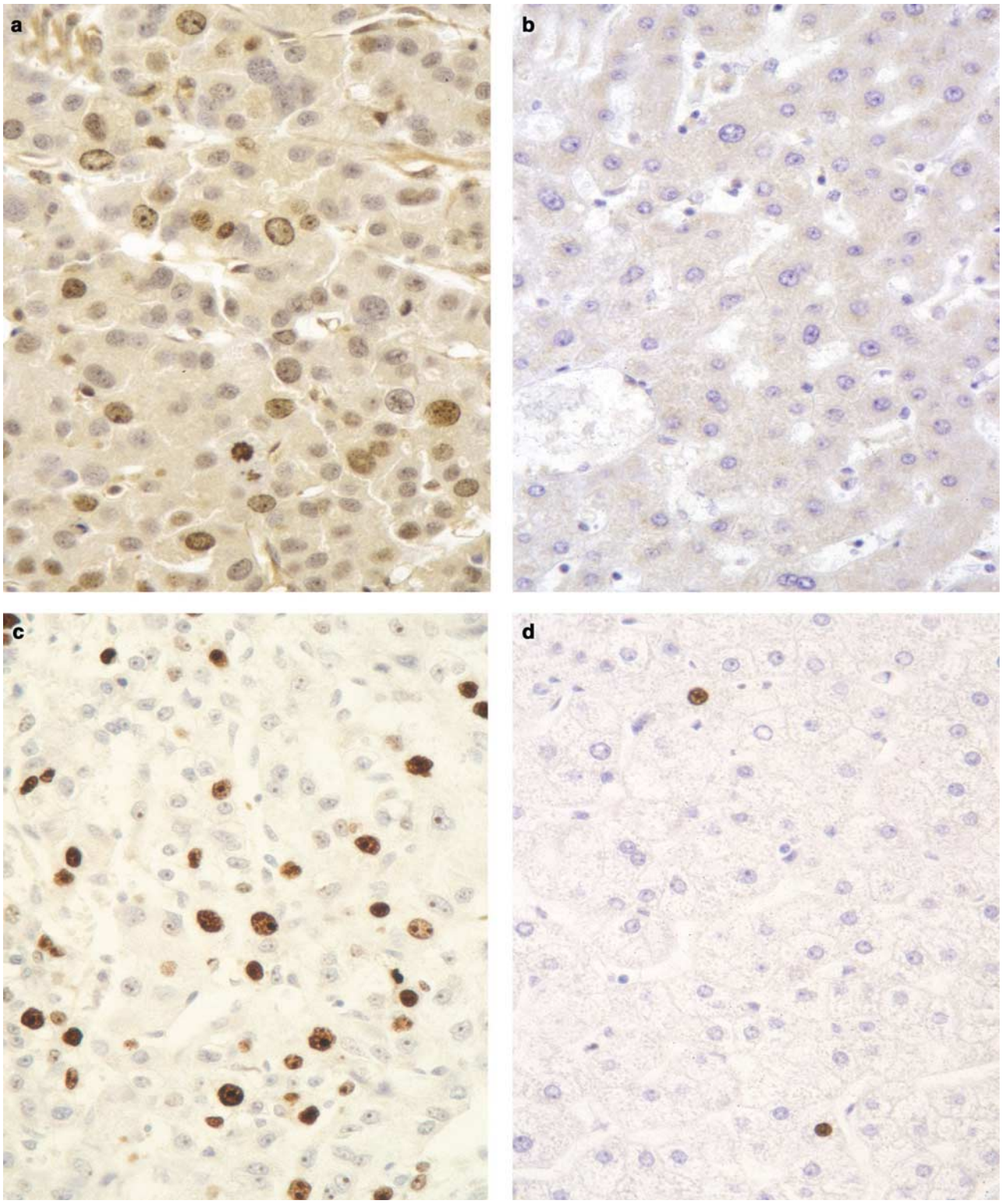

Figure 1 Nuclear survivin expression in hepatocellular carcinoma (**a**), but its absence in non-neoplastic liver (**b**), high-proliferation (nuclear MIB-1) in hepatocellular carcinoma (10%) (**c**), and low-proliferation (<2%) in non-neoplastic liver (**d**).

hepatocellular carcinoma is similar, although both are lower than the 63% expression by immunohistochemistry[32] and the 70% expression by immunofluorescence previously reported.[13] None of the non-neoplastic livers in our study showed survivin expression, similar to the 0% in six normal livers and 4% (two of 51) in noncancerous liver tissue adjacent to hepatocellular carcinoma reported by

Table 1 Correlation of nuclear survivin positivity in 87 hepatocellular carcinoma according to clinicopathologic parameters

Variable	*Category*	*Survivin pos (%)*	P-*value*
Gender	Male	21/55 (38%)	0.4550
	Female	10/17 (59%)	
Cirrhosis	Present	17/44 (39%)	0.4220
	Absent	14/28 (50%)	
Tumor number	Solitary	22/54 (41%)	0.5794
	Multiple	9/18 (50%)	
TNM stage	Stage I	5/19 (26%)	0.0695
	Stage II	13/29 (45%)	
	Stage III	4/8 (50%)	
	Stage IV	9/12 (75%)	
TNM stage	Stage I	5/19 (26%)	0.0548
	Stage II	13/29 (45%)	
	Stage III, IV	13/20 (65%)	
Histologic grade	1	1/9 (11%)	0.0544
	2	24/54 (44%)	
	3	6/9 (67%)	
Nuclear grade	1	2/13 (15%)	0.0271
	2	15/36 (42%)	
	3	14/23 (61%)	
Tumor necrosis	Present	11/22 (50%)	0.4360
	Absent	20/50 (40%)	
MVI	Present	21/37 (57%)	0.0064
	Absent	10/35 (28%)	
Local recurrence	Present	9/11 (82%)	0.0487
	Absent	22/61 (36%)	

MVI = microvascular invasion.

Table 2 Correlation of mitotic count and proliferation (MIB-1) with nuclear survivin expression in hepatocellular carcinoma

	Survivin (+)	*Survivin (−)*	P-*value*
Mitosis per 10 HPF, mean	4.8 ($n=31$)	1.2 ($n=41$)	0.0017
MIB-1 (%)	9.6% ($n=30$)	2.7% ($n=41$)	0.0001

HPF = high-power fields; MIB-1 (%) = mean labeling index.

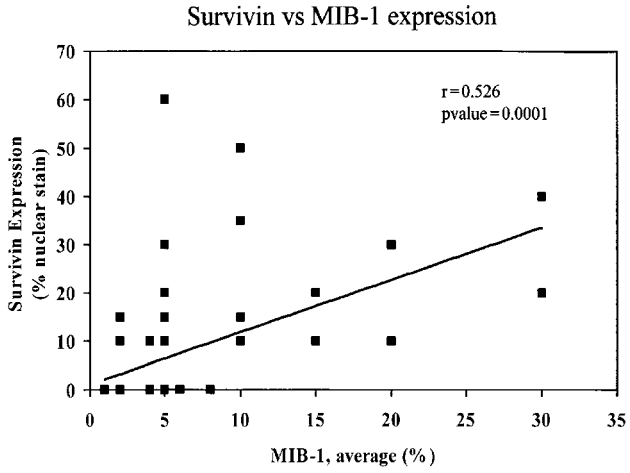

Figure 2 Nuclear survivin expression in hepatocellular carcinoma correlates significantly ($P=0.0001$) with proliferation (MIB-1) with correlation coefficient $R=0.526$.

Ikeguchi *et al*[12] with mRNA, and confirmed by immunohistochemistry, and the 0% in eight nontumorous livers with mRNA by Ito *et al.*[13] Moon and Tarnawski[32] report only cytoplasmic stain in nonmalignant hepatocytes. Similar to the mean 6.1% MIB-1 labeling index in hepatocellular carcinoma and the <2% mean in normal livers, we previously found by quantitative image cytometry, a mean MIB-1 of 5.14% in low-grade and 8.06% in high-grade hepatocellular carcinoma. This is compared to 1.64% in normal liver without hepatocellular carcinoma and 2.17% in cirrhotic liver with hepatocellular carcinoma.[33] Similarly, Pizem *et al*[14] report significantly different mean Proliferation Nuclear Cell Antigen and Ki-67 proliferative indices of 7.41 and 9.67% in hepatocellular carcinoma, and 0.21 and 0.19% in non-neoplastic liver. They find significant differences in proliferation (Proliferation Nuclear Cell Antigen index) between different grades of hepatocellular carcinoma.

In the literature, cytoplasmic expression of survivin by immunohistochemistry is reported to be a poor prognostic parameter in neuroblastoma,[34] laryngeal squamous cell,[29] colorectal,[2,5,35,36] and urothelial[37] carcinoma. For pancreatic,[38] gastric,[39,40] esophageal,[26] and urothelial[41] carcinomas, on the other hand, no association was found between cytoplasmic survivin and patient survival. The translocation of survivin from cytoplasmic in the normal to cytoplasmic and nuclear in high-grade dysplasia and squamous cell carcinoma is noted in the esophagus.[26] The differential nuclear and cytoplasmic localization of survivin is shown to be due to differences in the amino-acid sequence of its carboxy-terminal domain.[26] In hepatocellular carcinoma, the predominant function of *survivin* is its cell cycle nuclear distribution, and not the cytoplasmic caspase-3-dependent antiapoptotic effect.[13] Thus, mean proliferation labeling index (Ki-67, Proliferation Cell Nuclear Antigen) is shown to correlate significantly with survivin, whereas apoptotic index (single-stranded DNA immunohistochemistry, Tunel) does not.[13–16] This suggests that, as in hepatocellular carcinoma, the prognostic significance in other cancers of survivin immunostain in nuclei relates to its cell cycle, and not to its cytoplasmic antiapoptotic, effect. Moon and Tarnawski[32] suggest that cytoplasmic localization of survivin in quiescent nonmalignant cells suppresses apoptosis, while its nuclear translocation in hepatocellular carcinoma may be important in regulation of proliferation and differentiation.

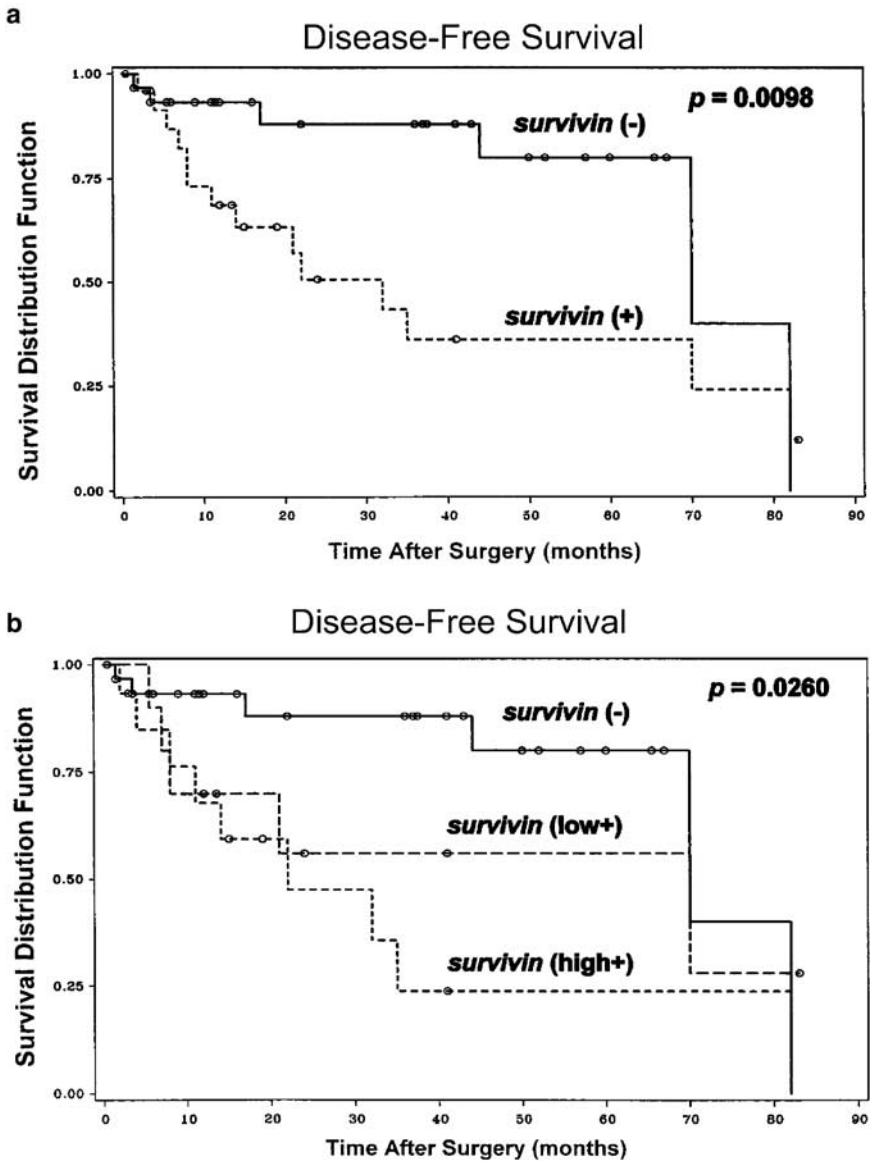

Figure 3 Disease-free survival is significantly less ($P = 0.0098$) in patients with hepatocellular carcinoma immunopositive ($\geq 10\%$) for nuclear survivin (**a**); this significance ($P = 0.0260$) remains when patients are subdivided according to high ($> 15\%$) and low (10–15%) survivin expression in their tumors, with disease-free survival worse when survivin expression is high (**b**).

Ikeguchi *et al*[16] show a significant difference in mean Ki-67 labeling index according to histologic differentiation (grade), but not with size or stage of the hepatocellular carcinoma. They, Ito *et al*[13] and Pizem *et al*[14] report that, in hepatocellular carcinoma, the mean proliferation index did correlate significantly with survivin, which did not correlate with apoptotic index. In several other cancers (ovarian,[42] esophageal,[26] pancreatic,[38] colorectal[43]), survivin overexpression correlates with increased proliferation, although the expression was cytoplasmic in the pancreatic and colorectal carcinomas. *Survivin* gene targeting inhibits cell proliferation, and induces apoptosis in *in vitro* cell lines.[44]

Our immunohistochemical study in hepatocellular carcinoma confirms the presence of nuclear survivin in 43% of the neoplasms, its expression correlating with poor clinicopathologic parameters (stage, histologic and nuclear grade, microvascular invasion, mitotic activity, MIB-1 proliferation index), local recurrence and disease-free survival. Anti-survivin therapies may not only potentiate chemotherapies that stimulate apoptosis, but, by removal of the antiapoptotic effect of survivin, may also exert an anti proliferative effect.

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