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ORIGINAL ARTICLE

Survivin expression in hepatocellular carcinoma. Correlation with clinicopathological characteristics and overall survival

Ioannis Kapiris[1], Konstantinos Nastos[1], Andreas Karakatsanis[1], Theodosios Theodosopoulos[1], Despoina Karandrea[2], Agatha Kondi-Pafiti[2], John Contis[1]

[1]2nd Department of Surgery, and [2]Pathology Laboratory, Aretaieion University Hospital, Athens, Greece

Summary

Purpose: *Survivin expression is a potential prognostic indicator in various carcinomas. The prognostic value of Survivin for survival in hepatocellular carcinoma, (HCC) however, remains controversial. The aim of the study is to examine the expression of the inhibitor of apoptosis Survivin in HCC and investigate the correlation with the clinicpathologic characteristics and overall survival (OS) following surgical resection.*

Methods: *Specimens from patients with resected HCC were examined by Immunohistochemical staining for Survivin and BCL-2expression. Clinical and histopathological data were retrieved from medical and pathology records, while OS was determined by reviewing records from the department of Oncology and personal communication with survivors. Bivariate analysis was performed using the Chi-square and Mann-Whitney U tests, while survival was estimated by Kaplan Meier method and compared with log-rank test.*

Results: *Sixty patients were included in the study. Survivin was expressed in 26 patients (43.3%). Survivin expression was significantly correlated to OS (p=0.014). A statistically significant negative correlation between Survivin and BCL-2 was also noted (p<0.001).*

Conclusions: *Survivin expression reflects aggressive histological and clinical behavior of HCC and correlates with poorer OS. Further studies are required to confirm if Survivin can be used as a predictive biomarker to evaluate prognosis and target treatments for HCC.*

Key words: *apoptosis, hepatocellular carcinoma, survival, survivin*

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancy worldwide [1] and it is responsible for over 1 million deaths annually. Although surgical resection and liver transplantation are the treatments of choice, recurrence is frequent, estimated to be 50% at 2 years after surgical treatment [2]. Prognosis after surgical treatment depends mainly on tumor stage and the biological behavior of the tumor [3].

HCC is not particularly sensitive to chemotherapeutic agents. Advances in the treatment of HCC are focused on targeted therapies which include immunotherapeutic vaccines, small molecule inhibitors/antagonists, nucleic acid-based and gene ablation approaches to block the function of inhibitors of apoptosis proteins (IAPs) antagonists and regulate cell cycle and apoptosis [4]. Similarly to other tumors, rapid malignant cell proliferation and impaired apoptosis are major ominous characteristics of HCC . Improvement in the understanding of the biology of HCC is necessary to identify potential prognostic factors and clinically relevant molecular targets for therapy. Numerous biomarkers have been identified for early diagnosis, prognosis, monitoring and assessment of recurrence of HCC. These biomarkers include alpha fetoprotein

Corresponding author: Ioannis Kapiris, MD, MSc, PhD. 2nd Department of Surgery, Aretaieion University Hospital, 78 Vassilisis Sofias Ave, 11528, Athens, Greece.
Tel: + 30 6944454546, Fax: + 30 2107789152, Email: ioanniskapiris@hotmail.com
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(AFP), AFP-L3%, gamma-glutamyl transferase (g-GT), glypican-3 (GPC3), des-gamma-carboxy prothrombin (DCP), alpha-1-fucosidase (AFU), human carbonyl reductase, Golgi phosphoprotein 2 (GOLPH2), transfroming growth factor-beta (TGF-Beta), tumor-specific growth factor (TSGF), epidermal growth factor receptor (EGFR), hepatocyte growth factor (HGF) and micro RNAs [5].

Overexpression of antiapoptotic factors like the inhibitors of apoptosis proteins (IAPs) has been documented in many solid tumors in several studies [6].

Survivin is a member of the IAP family [7]. It is known as "baculoviral inhibitor of apoptosis repeat containing 5 (BIRC5)". It is a protein which suppresses apoptosis and stimulates cell division. In transformed cells, it inhibits apoptosis by interacting with microtubules of the mitotic spindle, inhibiting the terminal effector cell-death proteases, caspase-3 and caspase-7 [8]. It also enhances proliferation and promotes angiogenesis. It is highly expressed in fetal tissue but is absent in terminally differentiated cells [9]. A significant difference in the expression between normal and malignant tissue has also been described. As a result, Survivin has been evaluated as a potential prognostic indicator in various malignancies.

Overexpression of Survivin is associated with cancer development in several carcinomas and hematologic malignancies. It is also associated with worse overall survival of different kinds of carcinomas, such as gastric [10], colorectal, lung [11], breast and esophageal cancers [12]. However, the prognostic value of Survivin in HCC remains controversial.

BCL-2 is a proto-oncogene which blocks the apoptotic process by inhibiting the release of cytochrome C from mitochondria and blocking the destruction of the cell by oxidation. Similar to Survivin, BCL-2 is an essential regulator of apoptosis [13]. Paradoxically, overexpression of BCL-2 suggests better prognosis in some solid tumors such as breast and pancreatic cancer [14,15]. In breast cancer, the powerful association between BCL-2 and hormone receptor expression is likely to have a prognostic power that overshadows the antiapoptotic effect of the oncogene [14]. In pancreatic cancer, higher BCL-2 immunoexpression was observed in early-stage and well-differentiated tumors [15]. Several authors reported a relationship between Survivin and BCL-2 in gastric and cervical cancer [16,17].

The present study examined the prognostic role of Survivin expression in HCC as an independent parameter of survival and investigated the correlation with clinical, histological, and apoptotic factors.

Methods

Patients and clinicopathological data

A database of prospectively collected data was retrospectively analyzed. All patients underwent hepatic resections in a single tertiary center for HCC. Only patients

Table 1. Demographic and histopathological characteristics and survival significance in 60 patients with HCC

Characteristics	*n (%)*	*p value*
Age (years), mean±SD	67±12.6	
Tumor size (cm), mean±SD	8±4	
Sex		0.818
Male	48 (80)	
Female	12 (20)	
Complications		0.670
Complication	24 (40)	
No complication	36 (60)	
Tumor size (cm)		0.513
3-6	17 (28.3)	
6-10	24 (40)	
>10	16 (26.7)	
Survivin		**0.014**
Positive	34 (56.7)	
Negative	26 (43.3)	
BCL-2		0.329
Positive	37 (61.7)	
Negative	23 (38.3)	
Grade		0.321
1	20 (33.3)	
2	22 (36.7)	
3	15 (25)	
4	3 (5)	
F score		0.095
0	24 (40)	
1	36 (60)	
Surgical margins		**<0.0001**
Positive	15 (25)	
Negative	45 (75)	
Extent of resection		**0.026**
Major hepatectomy	39 (65)	
Minor hepatectomy	21 (35)	
Vascular invasion		**0.030**
No vascular invasion	13 (21.6)	
Minor vessel invasion	23 (38.3)	
Major vessel invasion	24 (40)	
Transfusion		0.849
Yes	42 (70)	
No	18 (30)	
Viral infection (B,C)		0.933
Yes	29 (48.3)	
No	31 (51.7)	

Boldface numbers denote statistical significance

with complete demographic, clinical and histological data were included in the study.

The study was approved by the Hospital's Ethical Committee and the Code of Ethics of the World Medical Association.

Sixty patients were included in the study. Pathology blocks were retrieved for the selected patients for immunochemistry study examination of Survivin and BCL-2 expression.

Clinical data (gender, age, tumor stage, type and extension of surgery, major postoperative complications, transfusions) and pathological features (tumor size, grading, location, disease extension, cirrhosis stage, fibrosis index, vascular invasion) were retrieved from patient medical records. Stage of disease was based on TNM classification according to AJCC/UICC (8th edition). Records from their regular oncologic follow up were accessed to extract data regarding survival, which was also confirmed by phone interviews with patients and families. Overall survival was calculated from the time of operation until death or last patient communication, and survival in 12, 24, 36, 48 and 60 months was calculated. Mean follow-up was 64±5.244 months. No patient was lost to follow-up.

Immunohistochemistry

All specimens were routinely fixed by formalin, paraffin-embedded and sectioned (4μm). The sections were incubated at 56^{0}C overnight, dewaxed in xylene twice, hydrated by passage through a graded series of ethanol (100, 95 and 85%) and then washed with distilled water. After antigen retrieval by pressure cooking, sections were washed with phosphate-buffered saline (PBS), endogenous peroxidase activity was blocked with 3% hydrogen peroxide and the sections were washed with PBS.

For immunohistochemistry, the sections were obtained and examined by Ventana automatic immunostain method using the Strept AB Complex Duet (DACO). Then the sections were covered with rabbit anti-Survivin monoclonal antibody RbmAb (Ab76424) ABCAM Survivin (USA) clone EP2880Y (1:100 dilution) and mice anti-BCL-2 monoclonal antibody BCL-2-100, GeneMed (USA) (1:25 dilution).

The immunoreaction was evaluated semi-quantitatively depending on the percentage of positively stained cells to the total number of cells examined (at least 100 cells/case). The score was considered negative (-) when the percentage of positively-stained cells was <10% of the total cells examined, and positive (+) when >10% of cells were stained. The positive immunoreaction was observed at the cytoplasm in both BCL-2 and Survivin.

Statistics

Statistical analysis was performed using SPSS V.22 (IBM, USA) software. Continuous variables were expressed as means±standard deviation and compared using the t-test when variables had a normal distribution and there were no significant differences in variances. When these conditions were not met, Mann-Whitney U test was used for continuous variables. Categorical data were analyzed using the chi-square test. Survival data were compared using the Kaplan-Meier method and significance was determined using the Log-rank test. Independent predicting factors for survival were evaluated using the Cox proportional analysis hazards model. The results were defined as significant when $p<0.05$.

Results

Sixty patients with HCC operated in our Department of Surgery were included in the study. Forty-eight patients were male (80%) and 12 (20%) female. Age ranged from 23 to 96 years (67.7±12.63). None of the patients had received any preoperative treatment for HCC. Tumor size ranged from 2 to 18 cm (8±4) and approximately a third of the patients had tumors over 10 cm. Demographic and histopathological characteristics and differences in survival (Kaplan-Meier, $p<0.05$) are summarized in Table 1.

Survivin and BCL-2 expression

Both Survivin and BCL-2 were immunohistochemically expressed in the cytoplasm when positive (Figure 1).

Twenty-six patients (43.3%), were positive for Survivin staining and 20 (33.3%) were positive for BCL-2. Data are summarized in Figure 2.

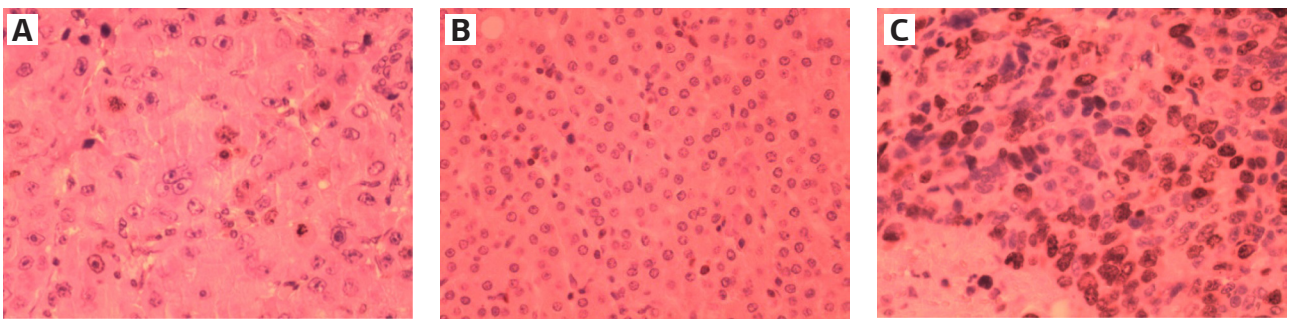

Figure 1. Negative **(A)** and Positive **(B)** immunohistochemical cytoplasmic staining of Survivin in HCC (x200). Positive **(C)** immunohistochemical cytoplasmic staining of BCL-2 in HCC. Positively stained cells>10% of total cells examined. Ventana automatic immunostain method. Counterstaining with hematoxylin.

In Figure 3, the immunohistochemical expression of Survivin and BCL-2 according to grading is presented.

Survivin expression was found to have statistically more frequent positive expression in patients with positive BCL-2 expression (p<0.001), advanced tumor grade (p<0.001) and when microvascular and major vascular invasion was present (p=0.003). No significant correlation with sex, F-score, tumor size, clear surgical margins, the extent of hepatectomy, complications, viral infection and patient age was noted. BCL-2 expression did not have significant differences across any of these factors, except for Survivin expression and age. Data are summarized in Table 2.

Survival analysis

Univariate analysis

Mean follow-up was 64±5.244 months. In univariate analysis using Kaplan-Meier curves (Figure 4), overall survival was found to be significantly higher in patients with negative margins (Figure 4a), negative Survivin expression (Figure 4b), less extensive operations (Figure 4c) and no vascular invasion (Figure 4d). Data are summarized in Table 1. No significant correlation was noted between survival outcomes and the other independent variables (tumor size, BCL-2 staining, sex, grading, F-score, complications, HBV history, transfusion blood units and age). In Figure 5, Kaplan-Meier

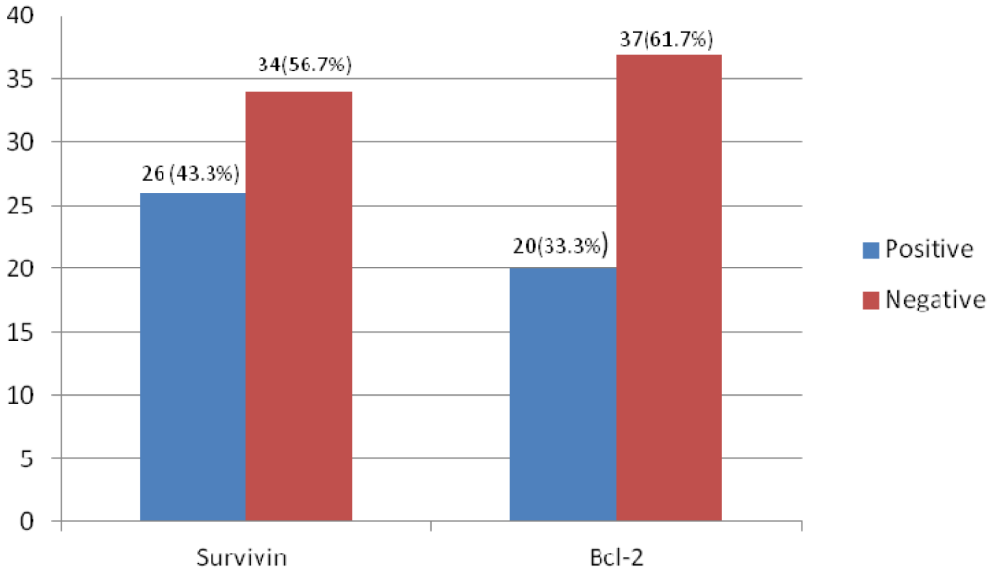

Figure 2. Expression ratio of Survivin and BCL-2 in HCC patients.

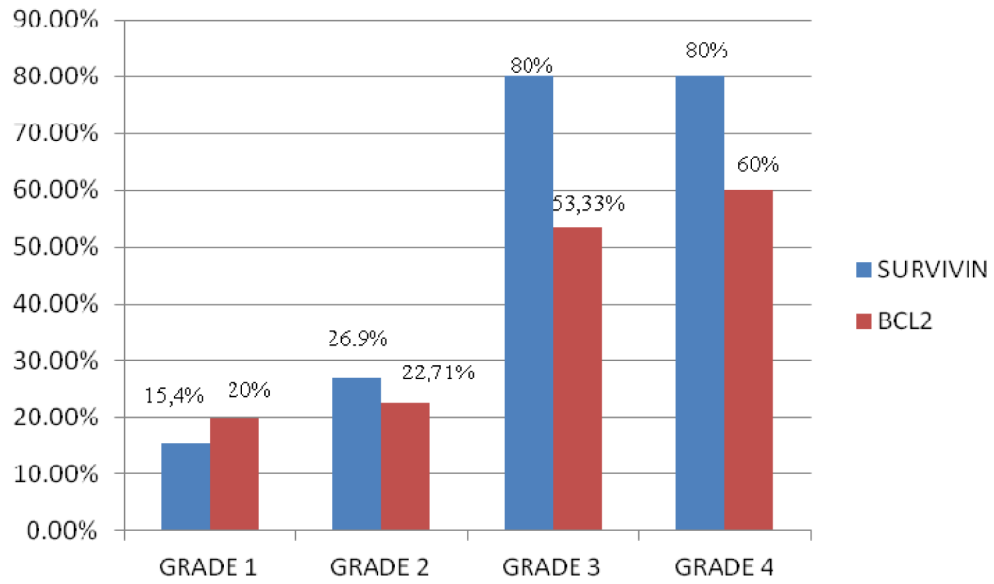

Figure 3. Immunohistochemical expression of Survivin and BCL-2 according to HCC grading.

Table 2. Demographic and histopathological characteristics and expression of Survivin and BCL-2 in 60 patients with HCC

Characteristics	*Survivin expression*			*BCL-2 expression*		
	Positive n (%)	*Negative n (%)*	*p value*	*Positive n (%)*	*Negative n (%)*	*p value*
Age (years), mean±SD	67±10.7	68.3±13.9	0.702	62.3±15.2	70.2±10.9	0.033
Tumor size (cm), mean±SD	8.1±4.8	8±3.4	0.967	7.5±3.7	8.2±3.9	0.546
Sex			0.068			0.591
Male	18 (69.2)	30 (88.2)		15 (75)	30 (81)	
Female	8 (30.8)	4 (11.8)		5 (25)	7 (19)	
Complications			0.750			
Yes	11 (42.3)	13 (38.2)		7 (35)	15 (40.5)	
No	15 (57.7)	21 (61.8)		13 (65)	22 (59.5)	
Tumor size (cm)			0.516			0.225
<3	2 (7.7)	1 (2.9)		0	3 (8.1)	
3-6	9 (34.6)	8 (23.5)		7 (35)	9 (24.3)	
6-10	8 (30.8)	16 (47.1)		10 (50)	13 (35.1)	
>10	7 (26.9)	9 (26.5)		3 (15)	12 (32.4)	
Survivin						
Positive	n/a	n/a	n/a	15 (75)	9 (24.3)	**<0.001**
Negative	n/a	n/a		5 (25)	28 (75.7)	
	15 (62.5)	5 (15.2)	**<0.001**	n/a	n/a	n/a
	9 (37.5)	28 (29)		n/a	n/a	
Grade			**<0.001**			0.284
1	4 (15.4)	16 (47.1)		6 (30)	14 (37.8)	
2	7 (26.9)	15 (44.1)		5 (25)	15 (40.5)	
3	12 (46.2)	3 (8.8)		7 (35)	7 (18.9)	
4	3 (11.5)	0		2 (10)	1 (2.7)	
F score			0.457			0.327
0	9 (34.6)	15 (44.1)		6 (30)	16 (43.2)	
1	17 (65.4)	19 (55.9)		14 (70)	21 (56.8)	
Positive margins			0.367			0.71
Positive	8 (30.8)	7 (20.6)		4 (20)	9 (24.3)	
Negative	18 (69.2)	27 (79.4)		16 (80)	28 (75.7)	
Extent of resection			0.770			0.589
Major	(36)	11 (32.4)		8 (40)	11 (29.7)	
Minor	39 (64)	23 (67.6)		12 (60)	26 (70.3)	
Vascular invasion			**0.003**			0.056
No	1 (4.5)	9 (29)		1 (6)	9 (27.2)	
Minor	14 (63.6)	6 (19.4)		10 (58.8)	9 (27.2)	
Major	7 (31.8)	16 (51.6)		6 (35.2)	15 (45.6)	
Transfusion			0.438			0.905
Yes	20 (80)	22 (71)		15 (75)	25 (73.5)	
No	5 (20)	9 (29)		5 (15)	9 (26.5)	
Viral infection (B,C)			0.205			0.311
Yes	15 (57.7)	11 (42.3)		8 (40)	20 (54)	
No	14 (41.2)	20 (58.8)		12 (60)	17 (45.9)	

Boldface numbers denote statistical significance

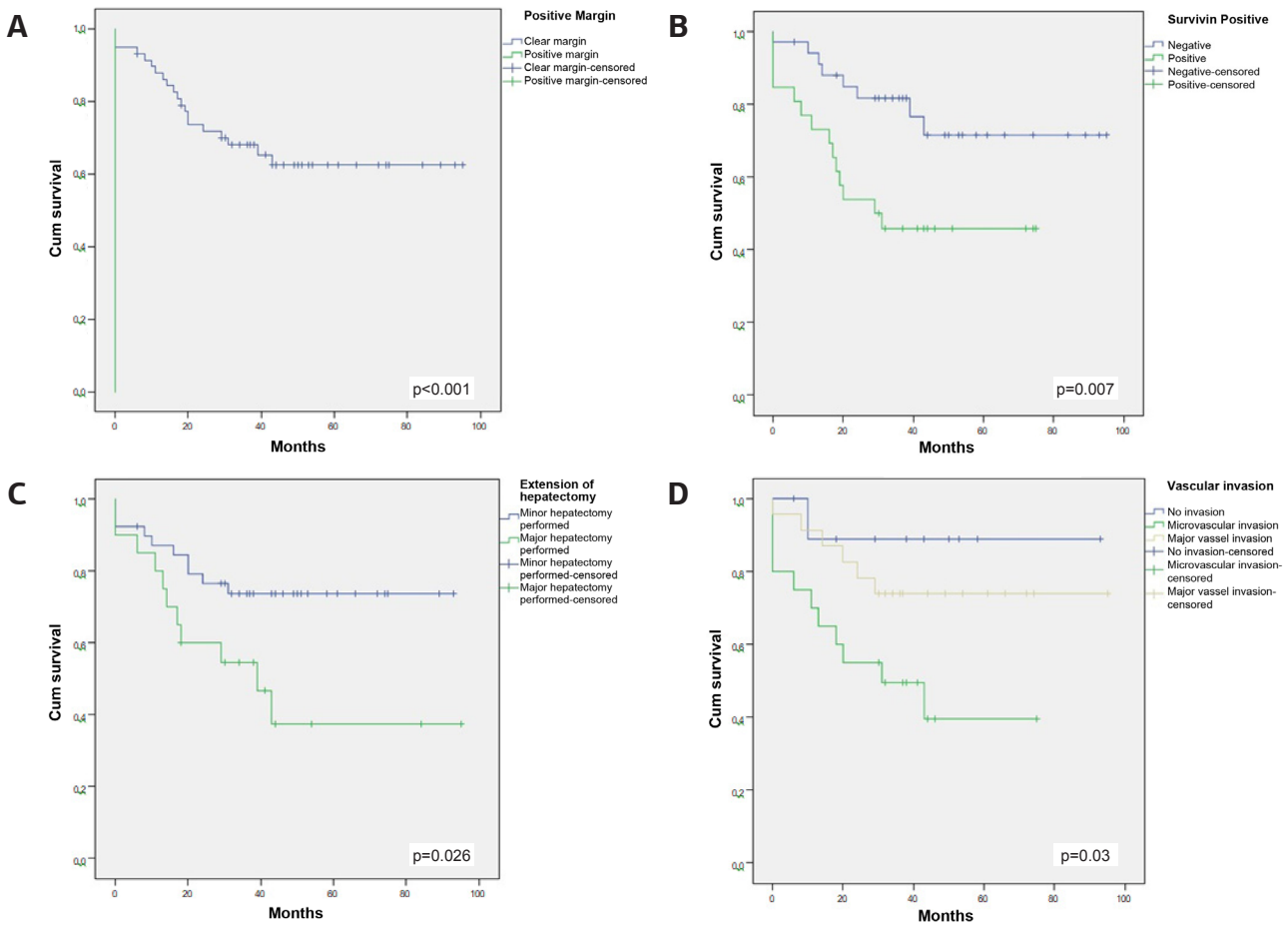

Figure 4. Kaplan-Meier overall survival according to margin status **(A)**, Survivin expression **(B)**, extent of hepatectomy **(C)** and vascular invasion **(D)**, demonstrating significant correlation.

curve for BCL-2 expression and survival (p=0.325) are also demonstrated.

Multivariate analysis

In multivariate analysis of survival (Cox regression), using variables with differences in survival in univariate analysis (margin status, Survivin expression, vascular invasion, extent of surgery), no variable was found to be an independent predictor of survival. Data are shown in Table 3.

Discussion

Apoptosis is a process of programmed cell death under certain physiological and pathological stimuli. Several factors, such as multiple genes and enzymes, define and control this process. Apoptosis plays an essential role in physiological processes such as tissue shaping, fetus developing, in the modulation of hematopoiesis, growing and aging, but also closely related to cancer occurrence and progression [18].

Survivin is a member of the inhibitor of apoptosis proteins (IAP) family, shown to bind and inhibit the cell-death terminal effectors caspase-3 and caspase-7, which induce cell apoptosis. The antiapoptotic mechanisms that involve in the process include: (1) The direct inhibition of the activity of the terminal responsive enzymes caspase-3 and caspase-7 which act during the apoptosis process and block apoptosis and (2) the starting of a combination process with the cell cycle modulator CDK4 to form Survivin-CDK4 complex. This complex releases p21 which further combines with caspase-3 inside the mitochondria. This results in inhibition of caspase-3 activity and blocks apoptosis [19]. Survivin is expressed at the G2/M phase of the cell cycle and is undetectable in terminally differentiated adult tissues.

Survivin is overexpressed in most human cancers, including esophageal, gastric, colorectal, breast, lung and pancreatic carcinomas [10-12,14,15]. Elevated Survivin mRNA expression is commonly associated with enhanced proliferative index and activation of p53 [20], which induces expression of the pro-apoptotic BCL-2 family proteins. Consequently, the reduced levels of apoptosis may result in more aggressive features of HCC,

Table 3. Multivariate analysis of factors predicting survival in 60 HCC patients

	SE	*Sig.*	*Exp(B)*	*95.0% CI for Exp(B)*	
	Upper	*Lower*	*Upper*	*Lower*	*Upper*
Positive margin	0.555	0.939	0.959	0.323	2.845
Survivin positive	0.548	0.200	2.018	0.690	5.902
Vascular invasion		0.502			
Microvascular invasion	1.143	0.307	3.210	0.342	30.133
Major vascular invasion	1.094	0.559	1.894	0.222	16.179
Major hepatectomy> 4 Segments	0.515	0.157	2.074	0.755	5.696

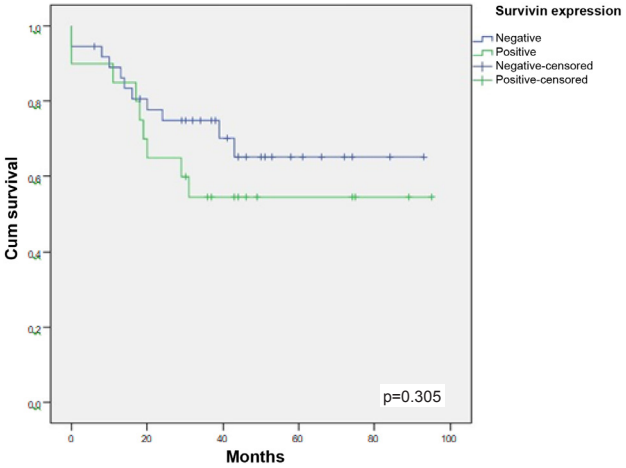

Figure 5. Kaplan-Meier overall survival according to Survivin expression on BCL-2 positive specimens demonstrating nonsignificant correlation.

resistance to chemotherapy and increased rate of tumor recurrence [21,22]. For HCC, Survivin has been reported as a useful marker for recurrence [23,24].

Studies have shown that the cytoplasmic expression of Survivin was closely correlated with poor prognosis of HCC patients and low overall survival (OS). On the other hand, nuclear expression of Survivin by immunofluorescence and immunohistochemistry strongly correlated with proliferation index (proliferation nuclear antigen) but not with the apoptotic index (Tunel method) [19,25]. Due to lack of studies reporting nuclear expression of Survivin in meta-analysis groups, further work is necessary to establish whether the nuclear expression of Survivin is associated with prognosis [26].

In the present study, the expression of Survivin in the cytoplasm of HCC cells was examined. Expression of Survivin was detected in 43.3% of patients with HCC. A large range (30-90%) of Survivin positivity has been noted in previous studies [18,20,21,23]. This heterogeneity of the results has been explained by the fact that detection of Survivin is variable, as a consequence of using different criteria for positive expression [24-26]. In the same studies it has also been noticed that the expression of the protein has been evaluated in different phases of the cell cycle where the expression level is extremely low (G1 phase), higher (S phase) or extremely high (G2/M phase). Therefore, the tumor cells in the G1/S phase may represent the negative expression, which would lead to different expression rate of Survivin in different studies [27].

In this study, Survivin-positive expression was significantly higher in poorly differentiated HCC with vascular infiltration. The results indicated that these factors might interact with each other in neoplastic invasion, neo-angiogenesis and metastasis and explain the high risk of recurrence in patients after hepatectomy with curative intent. Therefore, Survivin may play an important role not only in the development of HCC but also in the recurrence and disease progression process. These results are following previous reports that suggest risk factors for early disease recurrence, such as tumor volume, histopathologic differentiation, microscopic invasion and multifocal lesions [28].

Furthermore, this study revealed that Survivin-positive HCC patients had poorer OS (p=0.007), compared to Survivin-negative HCC patients. In multivariate analysis using the Cox model, Survivin was confirmed as a statistically significant independent prognostic factor for OS, along with positive surgical margins.

BCL-2 stain was found in the cytoplasm of 33.3% of the patients with HCC. A negative statistically significant correlation between Survivin and BCL-2 positive staining was noticed. It has been reported that BCL-2 is significantly expressed in HCC tissues, suggesting that this protein plays an important role in the formation of HCC [18]. On the contrary, lower expression in poorly differentiated HCC has also been noticed in other studies, suggesting that BCL-2 overexpression is associated with low grade HCC and, possibly, with a significant improvement in OS [29].

Conclusion

The present study confirms that Survivin expression reflects more aggressive histological and clinical behavior of HCC independently of other clinicopathological factors and affects OS significantly. Vascular infiltration and positive margins were, as expected, reliable predictors of recurrence. We suggest that Survivin could be used as a biomarker to evaluate the prognosis of HCC. Development of Survivin inhibitors or other molecules conferring pro-apoptotic action on HCC cells could provide useful targeted treatment options.

Conflict of interests

The authors declare no conflict of interests.

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