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Over-expression of SERPINB3 in hepatoblastoma: A possible insight into the genesis of this tumour?

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ABSTRACT

Background: The serpin SERPINB3 (SB3) found over-expressed in human hepatocellular carcinoma and in regenerating liver in mice has been shown to induce apoptosis resistance, epithelial-to-mesenchymal transition and increasing cellular invasion. It has also been hypothesised that SB3 may provide a pro-proliferative stimulus for liver cells *in vivo*. No information is available on SB3 in hepatoblastoma (HB). Aims of the study were to analyse SB3 expression in HB specimens and to investigate its possible correlation with Myc expression and tumour extension at diagnosis as evaluated by the pre-treatment extent of disease evaluation system (PRETEXT).

Methods: Frozen tumour specimens from 42 children with HB were analysed for SB3 and Myc expression by real-time PCR. SB3 localisation in tumour specimens was assessed by immunohistochemistry.

Results: At transcription level SB3 was positive in 79% of the cases. By immunohistochemistry, SB3 expression was found mainly in the embryonic, blastemal, small cell undifferentiated (SCUD) components of HB, while it was not detectable in normal hepatocytes. High SB3 reactivity was also detected in neoplastic cell clusters of portal vein tumour thrombosis. A direct correlation was observed between SB3 gene expression, the up-regulation of Myc ($r = 0.598$, $p < 0.0001$) and tumour extension (PRETEXT III/IV versus I/II, $p = 0.013$).

Conclusions: SB3 is over-expressed in HB and its expression is positively correlated with Myc expression and high tumour stage. The role of SB3 in the genesis of HB and in defining the risk profile of children affected by this tumour is hypothesised.

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1. Introduction

SERPINB3 (SB3), a serine protease inhibitor also known as Squamous Cell Carcinoma Antigen 1 (SCCA1), is a member of the ovalbumin-serin protease inhibitor family (ov-serpins)[1] frequently upregulated in several malignancies of epithelial origin.[2,3] SERPINB3, undetectable in normal hepatocytes, is expressed in hepatocellular carcinoma (HCC), dysplastic nodules and surrounding cirrhotic tissue, suggesting that SB3 expression may represent a relatively early event in hepatocarcinogenesis.[4,5] Furthermore, considering that it has been documented that transgenic mice expressing SB3 showed higher liver regenerative potential compared to wild-type mice, a role of this protein in promoting cell growth and proliferation of those cell sustaining hepatic regeneration has been proposed.[6] The biological role of this serpin in carcinogenesis has not been yet completely defined. *In vitro* studies have shown that SB3 protects neoplastic cells from apoptotic death induced by several kinds of stimuli, being their suggested molecular target location upstream caspase 3.[7] Recent data have revealed that SB3 induces cell proliferation and deregulation of adhesion processes, leading to epithelial-to-mesenchymal transition (EMT) with increased invasiveness potential.[8] Nothing instead is known regarding the potential role of SB3 in hepatic organogenesis and the intimate genetic mechanism potentially linking its expression with hepatic regeneration.

Hepatoblastoma (HB), the most common liver malignancy in early childhood, is considered an embryonal tumour of the liver. The term 'embryonal' implies that its genesis is related to a derangement in the normal mechanisms regulating normal hepatic organogenesis; thus its cell of origin most likely is an embryonal cell which instead of completing its maturation fate undergoes a neoplastic transformation. Actually solid hypotheses have already linked the different histologic subtypes of HB to specific stages of the arrest of normal hepatic organogenesis.[9] Which developmental, signalling and transcriptional pathways are mainly affected, at which level, according to which mechanisms and, if more than one is involved, how they interplay amongst themselves it is all a matter of further investigation. To date, no information is available on the expression of SB3 in HB. Histopathological classification of this primary liver tumour has led to define a limited number of patterns ranging from differentiated to undifferentiated epithelial types and including various mesenchymal components.[9,10] This tumour is frequently characterised by morphological heterogeneity: the foetal type consists of clusters of hepatocytes with irregular two cell thick trabeculae, recapitulating those of the foetal liver, while the embryonal type presents a more immature appearance, with patterns of solid type exhibiting ribbons, rosettes and papillary formations. It has been reported previously that HBs frequently carry activating mutations in the ß-catenin gene associated with cytoplasmic and nuclear accumulation of ß-catenin.[11] Increased levels of Myc and cyclin D1 have been reported in proliferative and poorly differentiated HBs,[12,13] and activation of Myc signalling in the most aggressive subtypes has been associated with poor prognosis.[14]

Aims of the study were to analyse SB3 expression in HB specimens and to investigate its possible correlation with Myc expression and tumour extension at diagnosis as evaluated by the pre-treatment extent of disease evaluation system (PRETEXT) proposed by the International Childhood Liver Tumour Strategy Group (SIOPEL).[15]

2. Materials and methods

2.1. Patients and tissue samples

The study was carried out in tumour specimens of 42 consecutive patients with HB, collected in France and in the SIOPEL Tissue Bank, as part of clinical trials promoted by the SIOPEL group, where histologic classifications were reviewed by a central pathologist (Zimmermann).[16] All studied cases were post-therapy resected specimens and characteristics of the patients included in the study are reported in Table 1.

The study was approved by the SIOPEL Tissue Bank Scientific Committee. Informed consent was obtained at each medical centre in accordance with European Union guidelines for biomedical research.

Part of tumour samples was formalin-fixed and paraffin-embedded, while the remaining part was either snap frozen in liquid nitrogen or conserved in RNAlater (Ambion) and stored at −80 °C for further analysis.[17]

2.2. Immunohistochemistry

Immunohistochemistry was carried out, as reported previously,[14] in six human liver tumours (four were consecutive cases from 2007 and two were selected for rare compounds: macrotrabecular component for one, and crowded foetal and small cell undifferentiated for the other). In addition, six different controls were analysed, including two normal livers (both were donor livers, one adult liver on surgical specimen and one paediatric liver on surgical biopsy, obtained at the beginning of liver transplantation) and other tissues as skin, heart, intestine and lung. For each HB case, one block was chosen for containing both non-tumoural liver and tumour, allowing us to compare the expression difference between the normal liver and the tumour.

For the detection of SB3, a rabbit polyclonal and affinity purified antibody was used at 1:200 dilution (Hepa-Ab, Xeptagen Life Biotechnology). Antigen retrieval was achieved by heating sections in 1 M citrate buffer at pH 6.0 in a water bath heating for 30 min. The sections were incubated with primary antibody for 30 min. Reactions were visualised using the ChemMate Dako EnVision Detection kit (Dako). Skin tissue samples served as positive controls. Expression of SB3 in the normal bile ducts of the large portal tracts, in hepatic arteries and sometimes in the endothelial cells of the portal veins served as internal control (Supplementary Figs. 1–3). Evaluation of immunostaining was assessed by two investigators examining at least ten random high-power fields.

2.3. SERPINB3 mRNA expression

Expression of SB3 and Myc mRNA was assessed by real-time PCR in frozen tumour and liver specimens. Total RNA was extracted using RNasy Trizol (Invitrogen, Carlsbad, CA)

Table 1 – Baseline epidemiologic and clinical characteristics of the patients with hepatoblastoma included in the study.

No. patients	42
Age	
Months, mean ± SEM	39.85 ± 7.32
Gender (%)	
Male	27 (64.3)
Female	15 (35.7)
Metastasis (%)	
Yes	13 (31.0)
No	29 (69.0)
Vascular invasion (%)	
Yes	16 (38.1)
No	26 (61.9)
PRETEXT stage (%)	
I	5 (11.9)
II	16 (38.1)
III	10 (23.8)
IV	11 (26.2)
Histology (%)	
Epithelial	5 (11.9)
Epithelial/foetal	21 (50.0)
Mixed	16 (38.1)

according to the manufacturer's instructions and quantified by spectrophotometry at 260 nm. Total RNA (up to 1 μg) was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) in a reaction mix consisting of: 4 μl of 5× buffer, 2 μl DTT (0.1 M), 2 μl dNTPs (5 mM), 1 μl of primers oligo dT (500 μg/ml), 1 μl (200 U) Superscript II and 1 μl (40 U) RNase inhibitor (Invitrogen, Carlsbad, CA). Expression of SB3 and cMyc genes was determined using SYBR green master mix (Roche Diagnostics GmbH, Indianapolis, USA) as previously described[18] using the following primers: sense SB3, 5′-GCAAATGCTCCAGAAGAAAG-3′; reverse SB3, 5′-CGAGGCAAAATGAAAAGATG-3′; sense cMyc, 5′-AAGACA GCGGCAGCCCGAAC-3′; reverse cMyc, 5′- TGGGCGAGCTGCT GTCGTTG-3′.

The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analysed in all amplification sets to assess the integrity of total RNA extracts with the primers: sense, 5′-TGGTATCGTGGAAGGACTCATGAC-3′ reverse, 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′.

Single-tube RT-PCR assays were performed using the LightCycler instrument (Roche Diagnostics GmbH, Indianapolis, USA). Relative levels of GAPDH, SB3 and Myc genes were calculated according to the threshold cycle (C_T). Samples were run in triplicate and fold change, compared to normal liver, was generated for each sample by calculating $2^{-\Delta\Delta CT}$,[19] where values >2 were considered arbitrarily as positive. Specificity of the amplified PCR products was determined by melting curve analysis and confirmed by agarose gel electrophoresis and ethidium bromide staining.

2.4. Statistical analysis

Statistical significance was determined by non-parametric procedures using the Unpaired *t*-test, Welch corrected, Mann Whitney test. Normality of distribution for quantitative variables was assessed by Kolmogorov and Smirnov test. In order to evaluate simple linear relationships between quantitative variables, Spearman's correlation coefficient was applied, when indicated. All tests were two-sided. The calculations were carried out with Graph Pad InStat Software (San Diego, CA). The null hypothesis was rejected at $p < 0.05$.

3. Results

3.1. SERPINB3 mRNA levels in HB

SERPINB3 mRNA was quantified in each tumour sample by real-time RT-PCR using primers specific for the human SB3 gene and the results were normalised to GAPDH housekeeping gene. Fold change was generated for each sample by calculating $2^{-\Delta\Delta CT}$,[19] and a cut-off value >2, compared to normal liver, was considered arbitrarily as positive. Amongst the 42 HB samples analysed, SB3 mRNA was detectable in 33 samples (79%), with different relative levels in individual cases, as reported in Fig. 1.

3.2. SERPINB3 protein expression

To confirm SB3 expression in HB samples, immunohistochemistry analysis was carried out in paraffin-embedded liver specimens. As positive control, beside skin tissue, also sweat glands in the dermis of the skin (Supplementary Fig. 4) and endothelial cells of the veins and arteries walls in the intestine (Supplementary Fig. 5) were positive. Within the normal liver, SB3 protein expression was seen in portal interlobular ducts, in the walls (myocytes of the media) of the large and medium sized hepatic arteries and sometimes in the endothelial cells of the portal veins (Supplementary Figs. 1–3). Normal hepatocytes, sinusoidal cells and Kupffer cells did not exhibit any reactivity, except some hepatocytes in the limiting plate that showed focal faint positivity. A tumoural compound was considered positive if the expression in the tumoural cells was higher than in normal hepatocytes (often a slight labelling of normal hepatocytes was present, Fig. 2A) and if at least 30% of neoplastic cells were labelled. No single HB cell positivity was observed in this series. Expression of the SB3 protein was observed as diffuse cytoplasmic staining in all HB cases examined, but the intensity of the signal was higher in less differentiated components (macrotrabecular, small undifferentiated cells, embryonal) of the HB than in well differentiated foetal HB (Fig. 2C), especially at the invasion front of the tumours (Fig. 2D). In some cases, primitive mesenchyme and cells arranged as immature ductal structures were also labelled (Fig. 3). Furthermore, high SERPINB3 reactivity was detected in clusters of small, ovoid cells with a high nucleocytoplasmic ratio arranged in sheets or as islands interspersed with more mature elements, representing the undifferentiated variant, named small undifferentiated cell (SCUD) pattern, considered as primitive uncommitted progenitor cells[9] (Fig. 4A). In the same patient, invasive cell clusters determining portal vein thrombosis, were highly positive for SB3 (Fig. 4B), supporting the role of the serpin in the invasive potential of the most undifferentiated forms of this rare liver tumour.

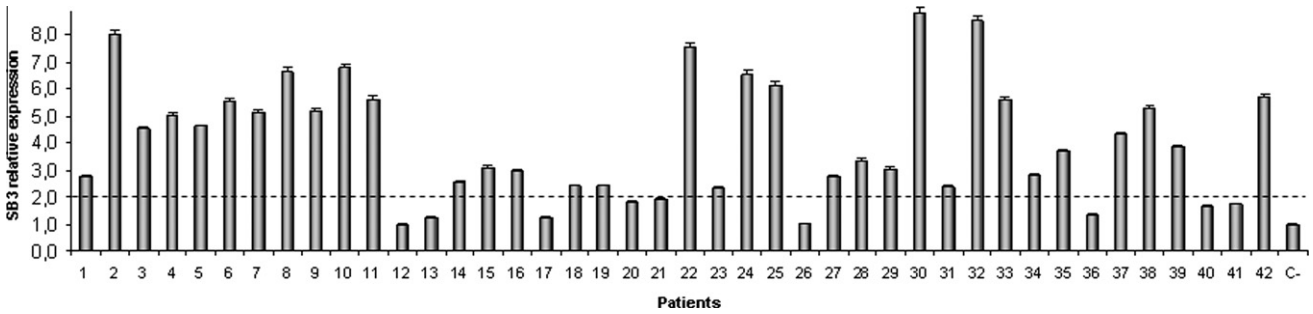

Fig. 1 – Distribution of SB3 mRNA expression in HB samples. Distribution of SB3 mRNA expression in HB samples from individual patients (Nos. 1–42). Data were normalised to gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene and expressed as fold differences, compared to normal liver control in SB3 mRNA. The *y*-axis represents the relative mRNA level of the SB3 gene calculated by $2^{-\Delta\Delta Ct}$ method. Dotted line indicates the cut off. C = control normal liver.

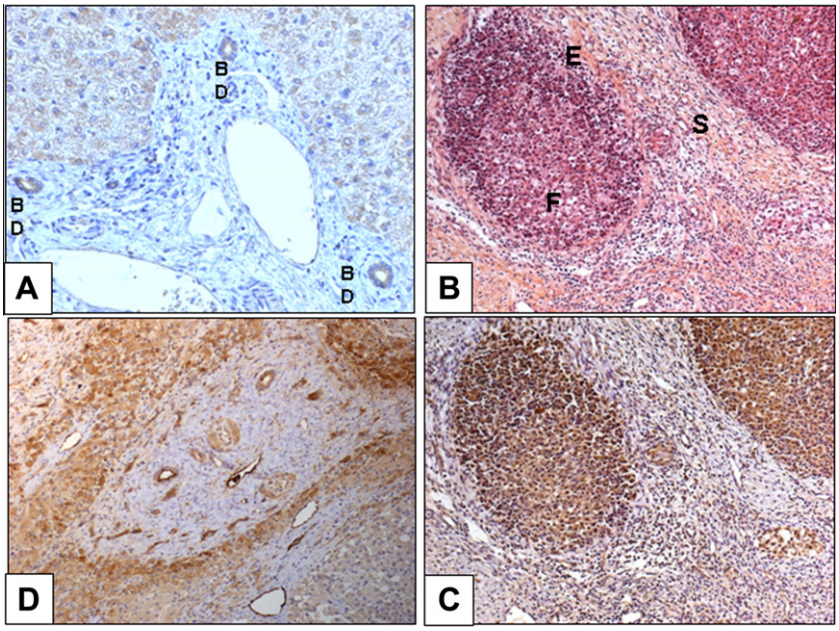

Fig. 2 – Immunohistochemical study of SB3 in HB samples. (A). Normal liver: SB3 expression faintly present in some periportal hepatocytes and expressed in the cytoplasm of biliary ducts. (B) Mixed HB stained with haematoxylin and eosin showing foetal (F), embryonal (E) and stromal (S) components. (C) Sequential sections of the same tumour stained for SB3, showing intense SB3 reactivity in embryonal and cholangioblastic cells, moderate in foetal and stromal cells. (D) Strong positivity for SB3 detected in embryonal cell clusters at the invasion front of the same tumour.

3.3. *Correlation between SERPINB3 and Myc gene expression*

To better define the potential correlation of SB3 with the expression of Myc gene, a parallel real-time PCR analysis was performed in frozen tissue samples. In HB cases a significant positive correlation was observed between the up-regulation of Myc and SB3 gene expressions ($r = 0.598$, $p < 0.0001$), as reported in Fig. 5A. The relationship between SB3 and Myc expressions was further explored in HepG2 cells stably transfected with the wild type human SB3 gene or with its reactive loop-deleted form, described in a previous study.[18] Quantitative PCR analysis showed higher Myc mRNA levels in cells expressing SB3, independently of the presence of the reactive loop, compared to control cells transfected with the vector alone (Fig. 5B). These results suggest the involvement of SB3 in up-regulation of Myc transcription and this effect does not require the antiprotease activity of the protein.

3.4. *Correlation of SERPINB3 expression with clinical prognostic parameters*

To better define the correlation of SB3 expression with clinical prognostic parameters, expression of the serpin in HB tissue samples was analysed in relation to vascular invasion, metastasis and PRETEXT system. This staging system is based on imaging at presentation, categorising the primary tumour on the basis of the extent of liver involvement at diagnosis

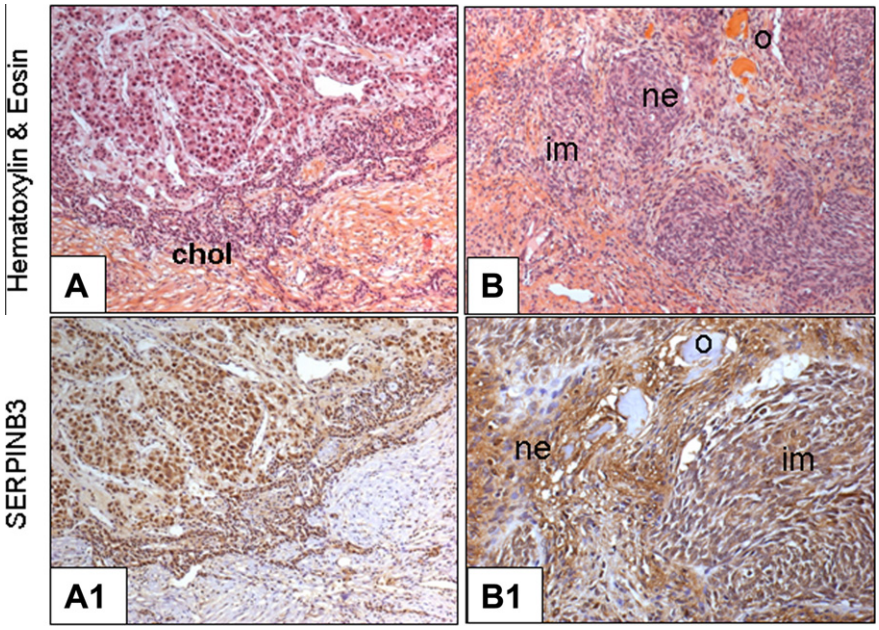

Fig. 3 – Histology and immunohistochemistry in mixed hepatoplastoma. Haematoxylin and eosin (HE) staining (upper panels A, B) and immunohistochemistry (A1, B1) for SB3 (lower panel) in another mixed HB with foetal, crowded foetal, cholangioblastic (chol), neuroectodermal (ne), osteoid (o) and immature mesenchymal (im) components. Positive immunostaining is observed in foetal and cholangioblastic areas (A1). Immature mesenchymal cells are labelled and some of them are arranged as immature neural structures (B1).

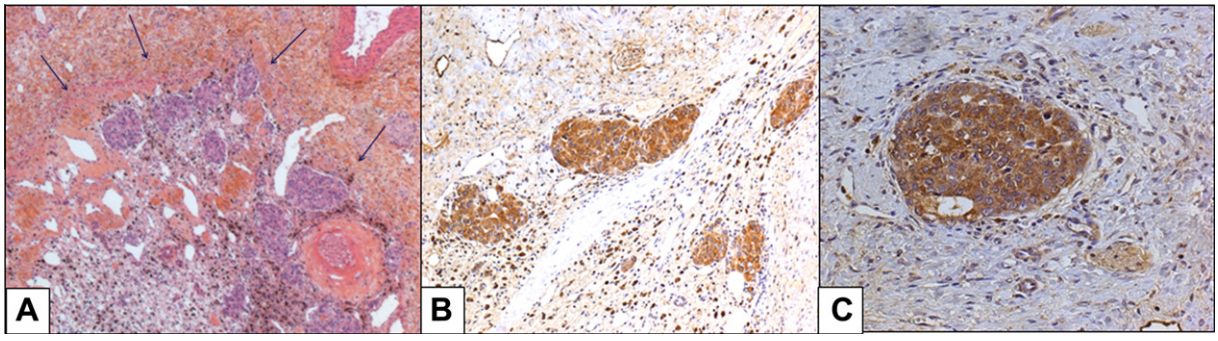

Fig. 4 – Histology and immunohistochemistry in a case of epithelial HB. (A) HE staining of large portal tract containing a portal vein thrombosis with several small cell undifferentiated (SCUD) clusters (arrow). (B, C) SB3 immunolabeling showing intense reactivity of SCUD clusters, not detectable in the surrounding fibrosis of the thrombosis. Siderophages are detectable in the surrounding fibrosis of the thrombosis (B).

and it has been used previously in the first SIOPEL study trial.[20] As shown in Fig. 6, SB3 mRNA levels were significantly higher in tumours involving 3 or 4 liver sections (PRETEXT stages III and IV) than in tumours involving only one or two liver sections (PRETEXT stages I and II) ($p = 0.013$). SB3 mRNA levels were slightly higher in tumour specimens showing vascular invasion, while the extrahepatic metastatic spread was not a discriminative parameter.

4. Discussion

To date little is known about the involvement of SB3 in HB. The results obtained in the present study indicate that SB3 is expressed in the majority of this neoplasm. SB3 has been found frequently over-expressed in epithelial tumours[3] and recently in primary liver cancer.[4,5] The biological role of this serpin in carcinogenesis has not been fully defined. Previous studies have described that SB3 can inhibit apoptosis and decrease NK cell tumour invasion.[7,21,22] More recently, a role for this serpin in tumour invasiveness has been proposed, since SB3 is able to promote the epithelial-to-mesenchymal transition programme and to increase cell invasion capacity.[8] In the present study the majority of HB cases were positive for SB3, although with different extent of expression in individual cases. Immunohistochemistry has revealed that SB3 was detectable also in the more immature embryonal cell compartment and in the SCUD pattern, recently identified as aggressive HB subtypes with a worse clinical prognosis.[9,14]

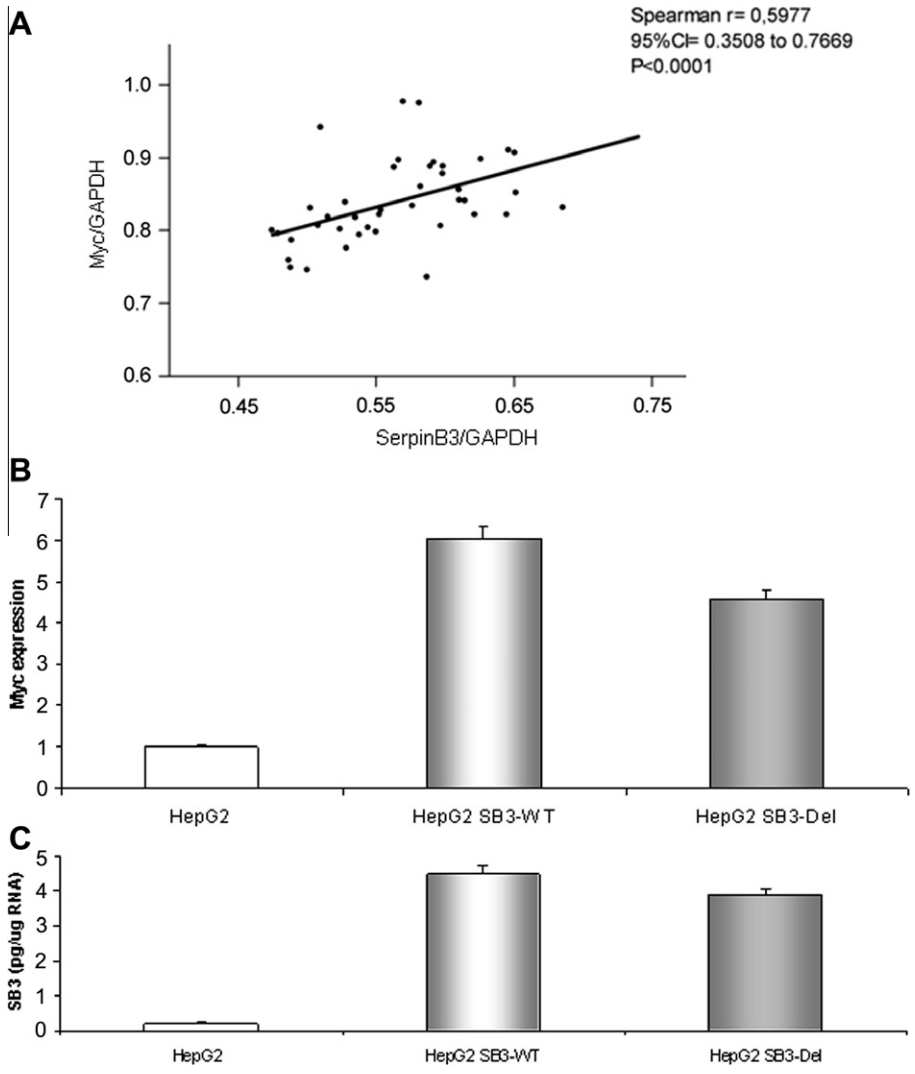

Fig. 5 – Relationship between SB3 and Myc transcriptional activity. (A) Comparative analysis in HB samples. (B) Analysis of Myc and SB3 mRNA levels in HepG2 cells stably transfected with the entire SB3 cDNA (HepG2 SB3-WT) or with the SB3 sequence lacking the reactive site loop (HepG2 SB3-Del).

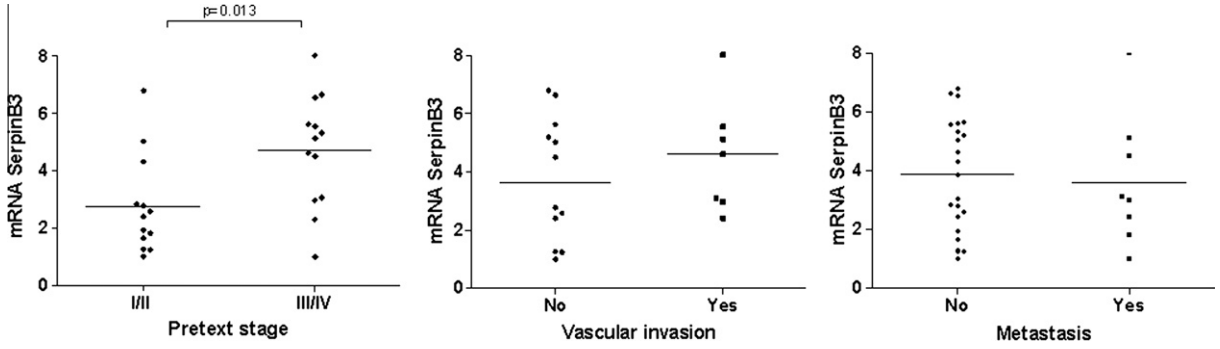

Fig. 6 – Correlation between SB3 and clinical staging. SB3 expression in 42 HB samples was analysed in relation to pre-treatment extent of disease evaluation system (PRETEXT) stage, vascular invasion and metastasis. Upregulation of SB3 was associated with the highest grades of PRETEXT stages (III/IV), compared to the lower stages (I/II) ($p = 0.013$).

So far no other biological markers have been so consistently reported associated with HB, thus a role of SB3 in the genesis or in promoting HB cell growth can be hypothesised. More precisely, considering that transgenic mice expressing SB3 showed higher liver regenerative potential compared to wild-type mice[6] and SB3 expression in HB, the embryonal

tumour of the liver, the hypothesis that SB3 may have something to do in regulating and/or promoting the growth of immature hepatic tissue and thus in the genesis of HB can be formulated. The fact that SB3 is highly expressed in the SCUD component of HB, considered the more immature form of this tumour, could be used to support this hypothesis. More should be investigated to validate this assumption and, in particular, to reveal which genetic and biological mechanisms intervene in regulating SB3 expression. The extent of expression of SB3 in HB samples was correlated directly to Myc gene expression, already identified as an indicator of aggressive phenotype and poor clinical prognosis.[23]

The overexpression of Myc is currently considered as a negative clinical prognostic factor that predicts poor outcome, irrespective of therapeutic treatment, often characterised by tumour propagation and disease progression. The close relationship between SB3 and Myc expressions, with progressive intra-hepatic tumour extension and also with the SCUD variant of hepatoblastoma, allows to hypothesise that SB3 may intervene in defining the risk profile of children affected by HB.

In conclusion, the present study indicates for the first time that SB3 is over-expressed in HBs. This finding allows to generate important hypotheses on the role of this protein in the genesis of this rare childhood tumour. If this assumption will be confirmed, and the genetic mechanism regulating SB3 expression revealed, possible innovative therapeutic targets for the treatment of HB could be identified. Finally, the association of SB3 expression with Myc expression, a high PRETEXT system and the SCUD variant of HB allow to assume that SB3 might help in defining the risk profile of children affected by this neoplasm.

Tumour cell immaturity in many tumour models is correlated with a more aggressive clinical behaviour. The maintenance by these immature cells of some stem cell characteristics like resistance to apoptosis, unlimited growth potential and invasiveness may explain why the tumour is partially or totally composed of highly immature cells usually having a more aggressive clinical behaviour. Following this hypothesis, it would sound not surprising that the presence of SB3, which confers to the cells the above mentioned characteristics, could be involved in the genesis and risk profile of HB.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.06.004.

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