

# Survivin is not a promising serological maker for the diagnosis of hepatocellular carcinoma

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**Abstract.** Survivin expression in the serum of patients with hepatocellular carcinoma (HCC) and nonmalignant chronic liver diseases remain to be elucidated. The aims of the present study were to evaluate the diagnostic role of survivin in the serum of patients with HCC and identify which ELISA kit performed best in detecting the levels of serum survivin. In total, 80 patients were included in the present study, including 20 patients with HCC, 20 patients with liver cirrhosis, 20 patients with chronic hepatitis B virus infection and 20 healthy volunteers. The levels of survivin protein in the serum were detected using two different ELISA kits (R&D and Abnova). The positive ratios of serum survivin detected by the R&D ELISA kit in all the cases were 8.75% (7/80; median, 0 pg/ml; range, 0-39.8 pg/ml) and in HCC patients were 5% (1/20; median, 0 pg/ml; range, 0-39.8 pg/ml). For the same samples analyzed using the Abnova ELISA kit, the positive ratios of serum survivin in all the cases were 22.5% (18/80; median, 0 pg/ml; range, 0-553.5 pg/ml) and in HCC patients were 25% (5/20; median, 0 pg/ml; range, 0-93.5 pg/ml). The results obtained by the different ELISA kits demonstrated no statistically significant differences in the level of survivin between HCC patients and healthy controls. The correlation coefficient was 0.0064 ( $P=0.481$ ) when analyzing the same serum samples with the different ELISA kits. In addition, the highest positive ratio of serum survivin was observed using the Abnova kit. A statistically significant difference in the results was observed between the R&D and Abnova kits. In general, the levels and positive ratios of serum survivin in the patients with HCC were significantly low. Furthermore, no difference was observed between HCC patients and controls in regard to the levels of serum survivin detected by the R&D and Abnova ELISA kits. In conclusion, survivin is unlikely to be a promising serological maker for the diagnosis of HCC.

## Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignant neoplasm and the third most common cause of cancer-associated mortalities worldwide (1,2). At present, the combination of  $\alpha$ -fetoprotein (AFP) with ultrasonography or computed tomography (CT) is widely used in clinical practice for the early diagnosis of HCC. However, a previous study identified that the sensitivity of AFP was only 25-65%. In addition, a number of patients with chronic hepatitis, liver cirrhosis and other liver diseases or gastrointestinal cancers have been found to exhibit elevated levels of AFP (3). Therefore, identifying novel and promising makers that may improve the diagnostic accuracy of HCC is required. Survivin, also known as baculoviral inhibitor of apoptosis repeat containing 5 (BIRC 5), is an inhibition of apoptosis protein. It was initially separated by researchers from Yale University, and has since been identified to be involved in the inhibition of apoptosis (4) and the regulation of mitosis (5).

Previous studies have revealed that survivin is highly expressed in the majority of human tumors and fetal tissues, but is normally undetectable in normal human tissues (6). This suggests that survivin is associated with the occurrence and progression of carcinomas. A study by Ito *et al* (7) was the first to report the expression of survivin in HCC tissues. Those authors demonstrated that survivin mRNA expression was upregulated in HCC tissues and in four HCC cell lines using reverse transcription-polymerase chain reaction (RT-PCR) analysis. In addition, survivin protein expression was also found to be elevated in HCC tissues following immunohistochemical staining. By contrast, survivin mRNA and protein were undetectable in adjacent paraneoplastic tissues (7). Since then, a number of studies have also identified an elevation in the level of survivin in HCC tissues using immunohistochemical staining (8-10) and RT-PCR analysis (11,12). These findings indicate the potential value of survivin in the diagnosis of HCC.

To the best of our knowledge, only two studies have investigated the expression of survivin in the serum of patients with HCC. El-Attar *et al* (13) revealed that the concentrations of survivin in the serum of HCC patients infected with the hepatitis C virus were not significantly different to those in the serum of healthy humans. A study by Matteucci *et al* (14) failed to detect the expression of serum survivin in 262 patients using ELISA. However, the two studies had limitations concerning

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the selection of patients and controls. Therefore, the expression of survivin in the serum of HCC patients remains to be fully elucidated. The present study recruited healthy controls and controls with nonmalignant chronic liver disease, and used two different commercially-available ELISA kits in order to detect the levels of serum survivin and assess the diagnostic role of survivin in HCC. In addition, the study aimed to identify which ELISA kit performed best in detecting the levels of serum survivin.

## Materials and methods

**Patients and samples.** The present study included 60 patients with liver diseases who had been admitted to the Tianjin Third Center Hospital, Tianjin Medical University (Tianjin, China) between January 2010 and August 2013. In addition, 20 healthy individuals were included, who had a normal liver biochemical function, no history of liver diseases, no viral hepatitis and no history of any other tumors or malignant disease. The participants were classified into four groups (n=20 each) as follows: i) patients with HCC; ii) cirrhotic patients without HCC; iii) patients with chronic hepatitis B (HBV) infection; and iv) control individuals. The clinical characteristics of the patients are shown in Table I.

The diagnosis of HCC was established by the clinical characteristics in combination with imaging evidence (ultrasound, CT or magnetic resonance imaging findings) and biochemistry (including the AFP level and liver function). Following resection, histopathological analysis was used to confirm the final diagnosis according to guidelines provided by the American Association for the Study of Liver Diseases (15). All the HCC patients were initially admitted to hospital without receiving any surgery or intervention therapy. The tumor stage was defined according to the Barcelona Clinic Liver Cancer (BCLC) staging system (16). Early-stage HCC was defined as BCLC stage 0+A. The patients with hepatitis infection were diagnosed on the basis of a biochemical function examination, a positive hepatitis B surface antigen result that had been apparent for the previous 6 months, and HBV DNA concentrations  $>10^3$  IU/ml. Patients with cirrhosis were diagnosed using histopathological analysis of liver biopsy samples. Evaluation of liver reserve function used the Child-Pugh score system, and liver function were divided into grade A, B and C. Written informed consent was obtained from all the patients at the time of recruitment.

All the serum samples were obtained using serum separator tubes at the initial presentation prior to any treatment. The samples were left to clot for 30 min at room temperature, followed by centrifugation for 15 min at 1000 x g, and then stored at -80°C until use.

**Detection of serum survivin level.** Serum survivin levels were detected by two independent researchers who had no access to the patients' clinical information. The assays were performed using two different ELISA kits: The Quantikine® ELISA Human Survivin Immunoassay (cat. no. DSV00; R&D Systems, Inc., Minneapolis, MN, USA) and the BIRC5 Human ELISA (cat. no. KA0441; Abnova Corporation, Taipei City, Taiwan) kits, according to the manufacturer's instructions.

The levels of serum survivin were detected using the R&D kit (recombinant human survivin; range, 31.2-2000 pg/ml).

Briefly, 100  $\mu$ l RD1-9 assay diluent and 100  $\mu$ l of standards were added to each well. The specimen serum to be detected and the appropriate controls were added to the appropriate wells, which were precoated with a mouse anti-human monoclonal antibody specific for survivin (catalog no. MAB886; R&D Systems, Inc., Minneapolis, MN, USA). The plates were then incubated for 2 h at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 1,000 x g. Next, 200  $\mu$ l survivin conjugate was added to each well and incubated for 2 h at room temperature on the shaker. Subsequently, 200  $\mu$ l substrate solution was added to each well and incubated for 30 min at room temperature, whilst protected from light. Finally, the stop solution was added and the optical density was determined at 450 nm and referenced to 540 nm on a microplate reader (Multiskan 3, Thermo Fisher Labsystems, Waltham, MA USA). All the measurements were performed in duplicate.

The levels of serum survivin were also detected using the Abnova kit (recombinant human survivin; range, 62.5-4000 pg/ml). Briefly, 100  $\mu$ l of standards, the specimen serum to be detected and the controls were added into the appropriate wells, which were precoated with a rabbit anti-human polyclonal antibody specific for survivin (catalog no. PAB0272; Abnova Corporation, Taipei, Taiwan). The plates were then incubated at 37°C for 90 min. Next, 100  $\mu$ l biotinylated mouse anti-human polyclonal survivin antibody (catalog no. H00000332-B01P; Abnova Corporation) was added to each well and incubated for 1 h at 37°C, followed by addition of 100  $\mu$ l ABC working solution to each well and incubation at 37°C for 90 min. Subsequently, 90  $\mu$ l TMB color developing agent was added to each well and incubated at 37°C for 20 min. Finally, the stop solution was added and the optical density was determined. All the measurements were performed in duplicate.

**Statistical analysis.** All the statistical analyses were performed using SPSS version 19.0 software (IBM Corporation, Armonk, NY, USA). The data are expressed as the mean  $\pm$  standard deviation or median for nonparametric data. Differences between two independent groups were compared using the Mann-Whitney U test (continuous variables and nonparametric analyses). The Spearman correlation coefficient was used to analyze any associations between the results detected by the R&D and Abnova kits. A value of  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Serum survivin levels detected by two different kits for the diagnosis of HCC.** In order to detect the serum survivin levels with the R&D kit, a standard curve was constructed using serially diluted recombinant human survivin (Fig. 1A). The kit was able to detect survivin with a linear range from 0-2000 pg/ml. The regression coefficients ( $r^2$ ) were 0.9962 and 0.9995. Survivin was undetectable in 73 out of 80 serum samples (91.25%). The survivin-positive samples (n=7; median level, 11.0 pg/ml; range, 0-39.8 pg/ml) corresponded to 1 HCC patient, 1 cirrhosis patient, 1 chronic HBV patient and 4 healthy controls (Table II). The levels of serum survivin were not significantly higher in the patients with HCC and

Table I. Clinical characteristics of the patients.

Group	n	Median age, years	Gender, M/F	HBsAg, +/-	Child-Pugh score, A:B:C	AFP, ng/ml	
						<20	≥20
HCC	20	58.6±8.1	16/4	12/8	14:3:3	4	16
LC	20	60.0±11.3	12/8	0/20	10:7:3	15	5
CHB	20	44.6±13.8	11/9	20/0	-	20	0
NC	20	51.2±9.6	7/13	0/20	-	20	0

A:B:C, ratio of patients who had A, B or C score. M, male; F, female; HCC, hepatocellular carcinoma; LC, liver cirrhosis; CHB, chronic hepatitis B; NC, normal control; HBsAg, hepatitis B surface antigen; AFP, α-fetoprotein.

Table II. Comparative analysis of serum survivin levels in 80 patients, as detected by R&amp;D and Abnova ELISA kits.

Group	n	R&D kit				Abnova kit			
		Range, pg/ml	Positive, n (%)	Z	P-value <sup>a</sup>	Range, pg/ml	Positive, n (%)	Z	P-value <sup>a</sup>
HCC	20	0.0-39.8	1 (5)	-1.459	0.144	0.0-93.5	5 (25)	-0.756	0.449
LC	20	0.0-11.0	1 (5)	-2.078	0.038	0.0-107.0	4 (20)	-0.818	0.413
CHB	20	0.0-13.5	1 (5)	-1.023	0.306	0.0-553.5	3 (20)	-0.888	0.375
NC	20	0.0-26.6	4 (20)	-	-	0.0-437.0	6 (30)	-	-

<sup>a</sup>Compared with the NC group. Z, Mann-Whitney U test statistic; HCC, hepatocellular carcinoma; LC, liver cirrhosis; CHB, chronic hepatitis B; NC, normal control.

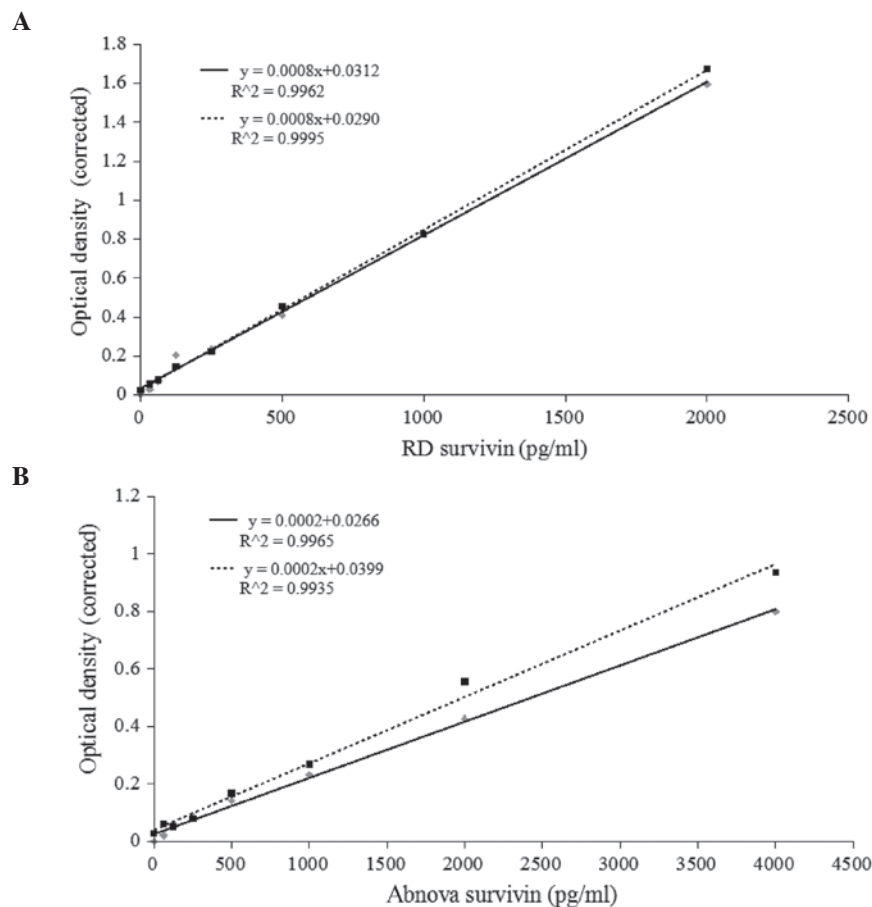


Figure 1. Standard curves of serum survivin ELISA obtained using the (A) R&amp;D and (B) Abnova kits.

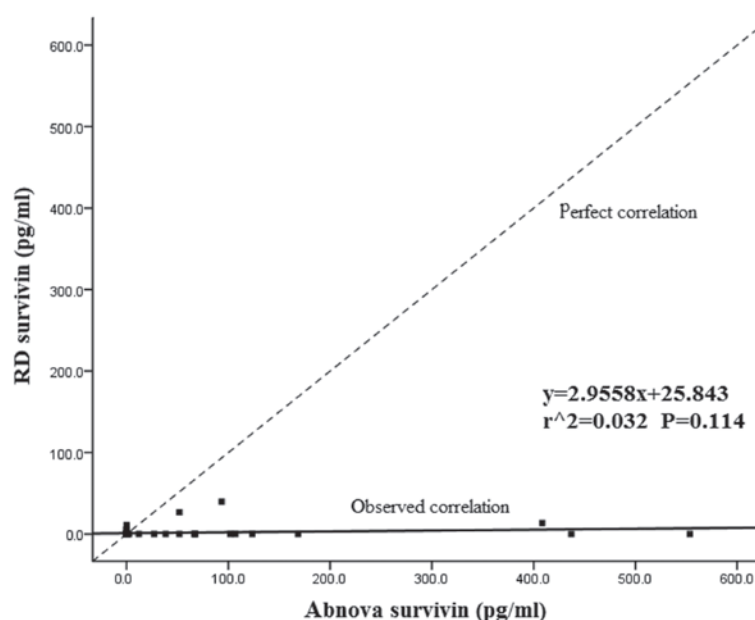


Figure 2. Association of the serum survivin levels detected using the R&D and Abnova ELISA kits.

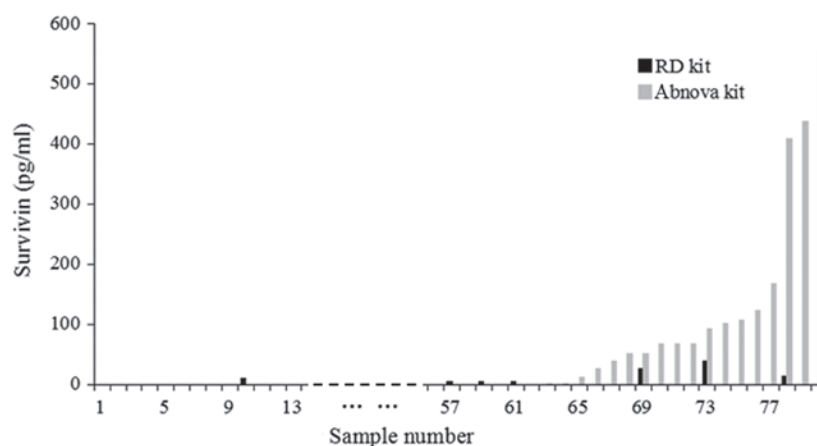


Figure 3. Comparative analysis of the serum survivin levels in each sample detected using the R&D and Abnova kits.

chronic HBV compared with the normal controls ( $P < 0.05$ ). Only patients with cirrhosis exhibited an elevation in serum survivin compared with the healthy controls ( $P = 0.038$ ).

The standard curves for the assays performed using the Abnova kit are shown in Fig. 1B. The kit was able to detect survivin with a linear range from 0–4000 pg/ml, using the regression coefficients ( $r^2$ ) 0.9964 and 0.9931. Survivin was detectable in 22.5% (18/80) of the serum samples (median level, 67.0 pg/ml; range, 2.0–553.5 pg/ml), which corresponded to 5 HCC patients, 4 cirrhosis patients, 3 chronic HBV patients and 6 healthy controls (Table II). No statistically significant differences were observed between the patients with HCC, cirrhosis and chronic HBV and the healthy controls ( $P > 0.05$ ).

*Association between R&D and Abnova kits in detecting survivin in the same serum samples.* As shown in Fig. 2, the Spearman correlation coefficient was 0.178 ( $P = 0.114$ ), which indicated that there were no associations between the results

of the R&D and Abnova kits in detecting the level of serum survivin. A histogram was constructed in order to compare the serum survivin levels of each sample analyzed by the R&D and Abnova kits (Fig. 3). A statistically significant difference was identified between the results of the two kits ( $P = 0.002$ ), which suggested that the sensitivity of the Abnova kit was higher compared to that of the R&D kit.

## Discussion

Survivin, which is a member of the inhibition of apoptosis protein family, is important in the inhibition of apoptosis and the regulation of mitosis (4,5). Previous studies reported that survivin was highly-expressed in the majority of human tumors and fetal tissues, but was undetectable in normal human tissue (6). This suggests that survivin is associated with the occurrence and progression of carcinomas. Ito *et al* (7) first evaluated the expression of survivin in HCC tissues using immunohistochemical and RT-PCR analyses. It was revealed



that the level of survivin protein was elevated in 70% (14/20) of the HCC tissues and that survivin mRNA was present in all eight types of HCC tissues, as well as the HepG2, Huh7, SK-Hep1 and HLE cell lines. By contrast, survivin mRNA and protein were undetectable in adjacent paraneoplastic tissues (7). Studies by Yang *et al* (8), Zhu *et al* (9) and Hui *et al* (10) that used immunohistochemical staining, as well as studies by Chau *et al* (11) and Augello *et al* (12) that used RT-PCR analysis, confirmed the elevated levels of survivin in HCC tissues. These studies indicated that survivin protein was a promising histological tumor marker. However, whether survivin can be used as a serological marker for HCC remains to be elucidated. To the best of our knowledge, only two studies have investigated the serum expression of survivin protein (7,14). However, the conclusions of these studies were unclear and limited by the absence of healthy controls or controls with nonmalignant chronic liver diseases. The identification of novel serum biomarkers is extremely important for the diagnosis of HCC, particularly noninvasive and inexpensive protein markers that require the collection of >100  $\mu$ l serum. Therefore, the present study recruited healthy controls and patients with HCC, cirrhosis and chronic HBV infection in order to evaluate the role of serum survivin protein in the diagnosis of HCC.

In the current study, the levels of serum survivin were detected using two different commercially-available ELISA kits (R&D and Abnova kits). However, the expression of survivin in the serum was undetectable in the majority of the patients and normal controls. The positive ratios were only 8.75% (7/80) for the R&D kit and 21.25% (18/80) for the Abnova kit. In HCC patients, the patients with a positive expression of survivin were only 5% (1/20) using the R&D kit and 25% (5/20) using the Abnova kit. Furthermore, no statistically significant differences were observed in the results of the two kits ( $r=0.178$ ;  $P=0.114$ ), which indicated that survivin was not a promising serum marker for the diagnosis of HCC. It may be reasonable to suggest that the levels of survivin protein in the serum of patients with HCC were low and close to the detection limits of the two different commercially-available ELISA kits. By contrast, survivin protein may not have a secreting type, although there has been conflicting evidence concerning the significance of the nuclear or cytoplasmic expression of survivin in HCC (17-20). El-Attar *et al* (13) reported that the median level of serum survivin in HCC patients was 13.9 pg/ml, which was close to the detection limits of the R&D and Abnova kits. In addition, Matteucci *et al* (14) demonstrated that serum survivin was undetectable in all 62 cases of HCC serum samples that were analyzed. Therefore, the present study may have failed to detect the expression of serum survivin with the use of these two kits.

Several previous studies have investigated the levels of the autoantibody of survivin in the serum of patients with HCC. Zhang *et al* (21) reported that the positive ratio of the survivin antibody was 11.3% (16/142) in patients with HCC and 2.4% (2/82) in healthy controls (capture antibody, goat anti-human immunoglobulin G (IgG); Caltag Laboratories, Burlingame, CA, USA). However, the study failed to identify the autoantibody in the serum of the patients with cirrhosis and chronic hepatitis using the ELISA assays (21). Using the same type of assay, Megliorino *et al* (22) established that the positive ratio was only 9.4% (15/160) in HCC patients (capture

antibody, goat anti-human IgG; Caltag Laboratories, San Francisco, CA, USA), whilst Yagihashi *et al* (23) reported that the positive ratio was 24.1% (7/29). These studies revealed that the positive ratios and the levels of survivin autoantibody were low in the serum of patients with HCC, which may be in accordance with the results of survivin protein in the serum of HCC patients in the present study.

The present study identified significant differences between the results of the two kits used. This may be due to the difference in the capture antibody used by each kit. The capture antibody for the R&D kit was a rat anti-human survivin monoclonal antibody (Met1-Asp142; accession number (AN), O15392), whereas that for the Abnova kit was a rabbit anti-human survivin polyclonal antibody (M1-D142; AN, O15392), which may have a stronger affinity. Therefore, the sensitivity of the Abnova kit may be higher compared with that of R&D kit.

In conclusion, the levels of serum survivin protein were close to the detection limits of the two ELISA kits used in the present study. In addition, the positive ratios of the serum survivin protein in the patients with HCC were significantly low and no statistically significant difference was observed between the levels of serum survivin in the HCC patients and controls, as detected by the R&D and Abnova ELISA kits. Therefore, survivin protein was not found to be a promising serological marker for the diagnosis of HCC.

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