

# Application of HBx-induced anti-URGs as early warning biomarker of cirrhosis and HCC

Wen Wang<sup>a,1</sup>, Lan-Juan Zhao<sup>a,1</sup>, Yan Wang<sup>a,1</sup>, Qing-Yuan Tao<sup>a</sup>, Mark A. Feitelson<sup>a,b</sup>, Ping Zhao<sup>a</sup>, Hao Ren<sup>a,\*</sup> and Zhong-Tian Qi<sup>a,\*</sup>

<sup>a</sup>Department of Microbiology, Shanghai Key Laboratory of Medical Biodefense, Second Military Medical University, Shanghai, China

<sup>b</sup>Department of Biology, College of Science and Technology, Temple University, Philadelphia, PA, USA

**Abstract.** *Background:* Hepatitis B virus (HBV) carriers are at high risk for the development of hepatocellular carcinoma (HCC), but there are no reliable markers that will identify such high-risk patients. HBV up-regulates the expression of selected genes (URGs) in the liver during chronic infection. These aberrantly expressed proteins trigger corresponding antibodies (anti-URGs) that appear prior to the detection of HCC. This study was undertaken to see if the anti-URGs could be used as early warning biomarker of HBV-induced liver cirrhosis and HCC.

*Methods:* A cross sectional study using a total of 625 serum samples from HBV infected and uninfected controls were tested for the anti-URGs using specific ELISAs.

*Results:* The number and specificity of anti-URGs correlated with the severity of liver disease. Anti-URGs were predominantly present among patients with HBV-associated HCC (55.2%) and cirrhosis (60.7%), and at a lower frequency among patients with chronic hepatitis (35.8%), and at still lower frequencies in most asymptomatic carriers (12.3%) with normal ALT, among patients with chronic hepatitis C (38.5%) and blood donors (0.9%). These anti-URGs were rarely detected in sera from those with tumors other than HCC, except among HBV infected patients with cholangiocarcinoma and in some patients with drug induced hepatitis. 3 or more anti-URGs could precede the diagnosis of cirrhosis or HCC 11.8 months on average, and HBV hepatitis patients with 3 or more anti-URGs have much higher risk (5/20 vs 0/30) to develop cirrhosis and HCC than those patients with less anti-URGs. As the early warning biomarker, 3 or more anti-URGs were served as the threshold to separate the cirrhosis and HCC from others with a moderate sensitivity (58.3%) and specificity (80.0%), which was better than other biomarkers (AFP, AFP-L3, GPC3 and GP73) and would improve up to 70.3% when combined with another biomarker.

*Conclusions:* The results of this clinical validation study suggest that the anti-URGs might have diagnostic/prognostic utility among patients at high risk for the development of cirrhosis and HCC.

**Keywords:** Anti-URGs, Hepatocellular carcinoma, hepatitis B virus x antigen, biomarker, early diagnosis

## 1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, with an estimated 250,000 new cases diagnosed each year [1]. The hepatitis B virus (HBV) carrier state and chronic liver disease are

the most important risk factors for cirrhosis and/or HCC [2,3]. Patients with early cirrhosis and/or HCC are often asymptomatic, and most of these tumors are undetectable by current biochemical and magnetic resonance imaging methods. Therefore, most clinical diagnosis of cirrhosis and/or HCC is made in patients with very late and advanced stage of disease. Hence, identifying carriers at high risk for tumor development and establishment of markers for the detection of patients with early stage cancers is very urgent for improving survival.

Hepatitis B x antigen (HBx) is a transcriptional-regulatory protein [4,5] that transactivates virus gene expression and replication [6,7] and may alter patterns

\*Corresponding author: Hao Ren, Department of Microbiology, Second Military Medical University, 800 Xiangyin Road, Shanghai 200433, China. Tel./Fax: +86 21 81870988; E-mail: renhm@yahoo.cn; Zhong-Tian Qi, Department of Microbiology, Second Military Medical University, 800 Xiangyin Road, Shanghai 200433, China. Tel./Fax: +86 21 81870988; E-mail: qizt@smmu.edu.cn.

<sup>1</sup>These three authors contributed equally to this work.

of cellular gene expression that contributes to tumor development [8,9]. Previous studies [10–13,15] have found that HBx contributes centrally to the development of HCC by altering patterns of host gene expression. Some of the HBx associated up-regulated genes (URGs) trigger corresponding antibodies in the sera of patients prior to tumor development and/or at early stage tumorigenesis (15 ~ 19) Given that China is also endemic for chronic HBV infection, and that this is also associated with a high frequency of cirrhosis and/or HCC, it became important to test whether the anti-URGs (antibodies to HBx up-regulated cell proteins: URG4, URG7, URG11, S15a and Sui1) were also present among patients at high risk of cirrhosis and HCC and/or among tumor bearing patients. The results of this study provide clinical validation of the earlier study, and suggest that the anti-URGs may have diagnostic/prognostic utility among patients at high risk for the development of cirrhosis and/or HCC.

## 2. Materials and methods

### 2.1. Ethics statement

These studies were reviewed and approved by the Medical Ethics Committees at the Second Military Medical University, Shanghai Changhai Hospital and Temple University. Informed written consent was obtained from each participant according to the Declaration of Helsinki.

### 2.2. Populations and sera

Serum samples used in this study were obtained from a variety of populations. Test samples were collected from HBV infected patients who are asymptomatic carriers (ASC), or with chronic hepatitis, cirrhosis or HCC (Table 1). In all cases, the diagnosis was determined by physical examination, blood chemistry, and magnetic resonance imaging. Samples were from patients visiting Shanghai Changhai Hospitals during June, 2008 to December, 2009. Among these, 278 were males and 138 were females ranging in age from 28 to 68 years old. All were tested for HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc and alanine aminotransferase (ALT) as part of a hepatitis B campaign to identify patients at high-risk for cirrhosis and HCC. Control samples collected from the same locations included 13 patients with hepatitis C virus (HCV) associated HCC cirrhosis or hepatitis who were sero-negative for HB-

sAg, HBeAg, anti-HBs, and negative for HBV DNA by PCR, but positive for anti-HCV by ELISA and HCV RNA by real time RT/PCR. Additional controls were obtained from 73 patients with several other types of cancers, and from 15 patients with drug-induced hepatitis. Additional samples were collected from 108 blood donors in Shanghai Changhai Hospital, in which standard markers for HBV or HCV infection tested negative (see above) (Table 1). In all of the populations, each serum sample was stored at  $-80^{\circ}\text{C}$  until use and then tested blindly.

### 2.3. Detection of anti-URGs by ELISA

ELISAs were performed as previously described with minor modifications [21]. Peptides sequences spanning hydrophilic regions of each HBx up-regulated gene were made by solid phase peptide synthesis (Shanghai JiEr Biotech Co), as previously described [21]. In brief, each ELISA was constructed and performed the same way with the exception of the peptides used for coating the wells. For example, to detect antibodies to up-regulated gene 4 (anti-URG4), a mixture containing 1  $\mu\text{g}$  of each URG4 synthetic peptide L4A and L4B in 100  $\mu\text{L}$  of phosphate buffered saline (PBS), was used to coat each well (Greiner Bio-One, Germany) in a 96-well plate. After overnight incubation at  $4^{\circ}\text{C}$ , the wells were washed six times with PBS and then blocked for 4 hrs at  $37^{\circ}\text{C}$  with PBS containing 5% bovine serum albumin (BSA). After washing, a test serum (100  $\mu\text{L}$ /well at a 1:10 dilution in PBS/ BSA) was added to each well, and the plates were incubated overnight at  $4^{\circ}\text{C}$ . After washing six times with PBS/0.05% Tween-20, affinity-purified horseradish peroxidase-conjugated anti-human immunoglobulin (100  $\mu\text{L}$ /well at a 1:1000 dilution in PBS/ BSA; KPL, Oklahoma City, USA) was added to each well. Plates were incubated for 45 min at  $37^{\circ}\text{C}$  and were washed six times with PBS; binding was determined by the addition of tetramethylbenzidine (TMB) (Sigma, USA) with an automated ELISA plate reader at 450 nm. Controls included performing the assays in wells coated with irrelevant peptides or PBS/ BSA only for 1 hour at  $37^{\circ}\text{C}$  before the assay. The cut-off value was calculated from the following equation:  $X + n \times \text{SD}$ , where  $X$  is the average absorbance value of serum samples from blood donors with no serologic evidence of HBV or HCV infection or biochemical evidence of liver disease, and  $n$  is a positive integer. The sensitivity and specificity of these ELISAs have been previously published [21].

Table 1  
Characteristics of patients used in this study

Population	Number of patients	Mean age and range (yrs)	M:F ratio	Patients with ALT >65u/L	HBs Ag/Ab	HBe Ag/Ab	HBe Ab	HBV DNA >10 <sup>3</sup> copies
HBV associated								
Msymptomatic carriers (ASC)	146	39.3 (28–45)	2.1	0	139/6	44/92	144	110
Chronic hepatitis	95	52.1 (38–63)	2.5	95	91/4	47/42	93	85
Liver cirrhosis	97	50.2 (38–68)	1.6	67	87/8	40/44	89	82
HCC	78	53.6 (46–66)	1.9	53	73/1	32/40	73	74
Cholangiocarcinoma	18	58.9 (51–68)	5.6	3	15/1	5/8	18	8
Chronic HCV	13	47.5 (29–58)	4.5	5	2/2	2/1	3	2
Drug induced hepatitis	15	59.1 (56–65)	1.5	15	0/3	0/0	2	0
Lung cancer	10	56.4 (52–66)	6.0	0	1/6	0/1	1	0
Colorectal cancer	30	58.6 (47–62)	3.1	2	12/12	10/1	14	0
Gastric cancer	15	56.6 (49–68)	4.2	1	3/8	1/3	8	0
Blood donors	108	49.7 (22–58)	1.4	0	0/0	0/0	0	0

#### 2.4. Detection of other biomarkers in serum

HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc, anti-HCV, alpha-fetoprotein, and ALT were determined using the corresponding commercially available kits (Shanghai Changzheng Bio, China). HBV DNA and HCV RNA were quantified by commercial real-time PCR kits (Shenzhen PG Biotech, China). AFP, AFP-L3, GPC3 were determined by commercially available kits (Wuhan Huamei Bio, China). GP73 was determined by commercially ELISA kit (Beijing Hotgene Bio, China).

#### 2.5. Statistical analysis

The Chi-square test was used to compare the difference in the number of anti-URGs in the various tests and control populations listed in Table 1. A significant value was indicated when  $P < 0.001$

### 3. Results

#### 3.1. Detection of anti-URGs by ELISA

To see whether the patients with antibodies against a panel of 5 different HBx induced URGs (anti-URGs) were at high risk for the development of cirrhosis and/or HCC, a cross-sectional study was performed with serum samples collected from HBV associated asymptomatic carriers (ASC), hepatitis, cirrhosis and HCC groups. (Fig. 1A) If use 3 or more anti-URGs as a threshold, the percent of patients with 3 or more anti-URGs in their serum is 61.7%, 55.2% 35.8% and only 12.3% in HBV associated cirrhosis, HCC, hepatitis and asymptomatic carriers (ASC) groups respective-

ly (Fig. 1B), with a significantly difference between the number of anti-URGs and HBV associated disease ( $P < 0.001$ ), which indicated a direct correlation between the number of anti-URGs and the severity of underlying liver disease among patients chronically infected with HBV. Moreover, there was no correlation between the presence of 3 or more anti-URGs and HBe/anti-HBe status and the levels of HBV DNA (data not shown).

Among patients with other types of cancers, 22% of patients with cholangiocarcinoma had 3 or more anti-URGs, which is not surprising in light of the findings that 15 of the 18 patients (83%) with this tumor type were HBsAg positive in serum, while the additional three patients had one or more HBV antibodies in their serum, suggesting all patients were infected with HBV. Among 55 serum samples collected from patients with lung, colorectal or gastric cancers, only 3 serum samples had three or more detectable antiURGs (Table 2). Samples collected from 108 blood donors only had one serum that tested positive for three or more anti-URGs (Table 2). In contrast, among patients with chronic HCV infection with elevated ALT, about 38% had 3 or more anti-URGs. While this may be due to underlying HBV infection in 3 patients who were anti-HBc positive, it is also possible that HCV up-regulates some of the same gene products as HBV. Surprisingly, 4 out of 15 patients with drug induced hepatitis also had 3 or more anti-URG (Table 2). Two of these patients had been previously infected with HBV, as indicated by the finding that they were anti-HBc positive, while in the other patients, the presence of antibodies remains to be elucidated. Hence, the anti-URGs were present in other populations, although most of the positive sera also had one or more markers of HBV infection.

Table 2  
Anti-URGs frequencies in different populations

Population	Number of patients	Average number of anti-URGs	Number(%) of patients with anti-URGs			
			0	1	2	3
HBV associated						
Asymptomatic carriers (ASC)	146	1.02	58 (39.7)	55 (37.7)	15 (10.3)	18 (12.3)
Chronic hepatitis	95	1.77	28 (29.5)	23 (24.2)	10 (10.5)	34 (35.8)
Liver cirrhosis	97	2.57	19 (19.6)	9 (9.3)	10 (10.3)	59 (60.7)
HCC	78	2.64	9 (11.5)	17 (21.8)	9 (11.5)	43 (55.2)
Cholangiocarcinoma	18	1.44	5 (28.0)	6 (33.3)	5 (28.0)	2 (11.0)
Chronic HCV	13	1.00	5 (38.5)	3 (23.0)	5 (38.5)	0 (0.0)
Drug induced hepatitis	15	0.80	10 (66.7)	4 (26.6)	0 (0.0)	1 (6.7)
Lung cancer	10	0.60	5 (50.0)	4 (40.0)	1 (10.0)	0 (0.0)
Colorectal cancer	30	0.93	17 (55.6)	5 (16.7)	6 (20.0)	2 (7.7)
Gastric cancer	15	0.33	12 (80.0)	1 (6.7)	2 (13.0)	0 (0.0)
Blood donors	108	0.42	73 (67.6)	26 (24.0)	8 (7.4)	1 (0.9)

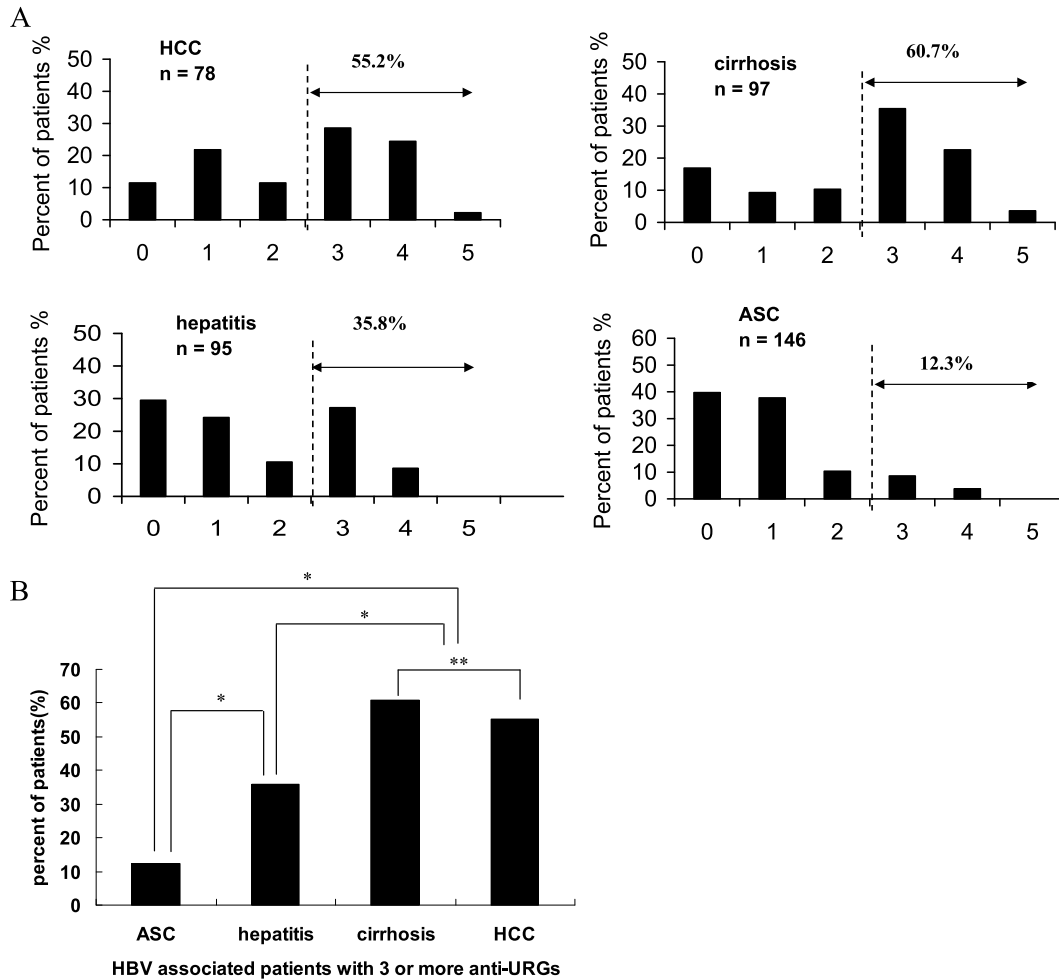


Fig. 1. Anti-URGs among groups with HBV associated disease. A, The percent of patients with 0, 1, 2, 3, 4 or 5 anti-URGs in HBV associated ASC, hepatitis, cirrhosis and HCC groups. B, The percent of patients with 3 or more anti-URGs in HBV associated ASC, hepatitis, cirrhosis and HCC groups. \*:  $P < 0.001$ ; \*\*:  $P > 0.05$ .

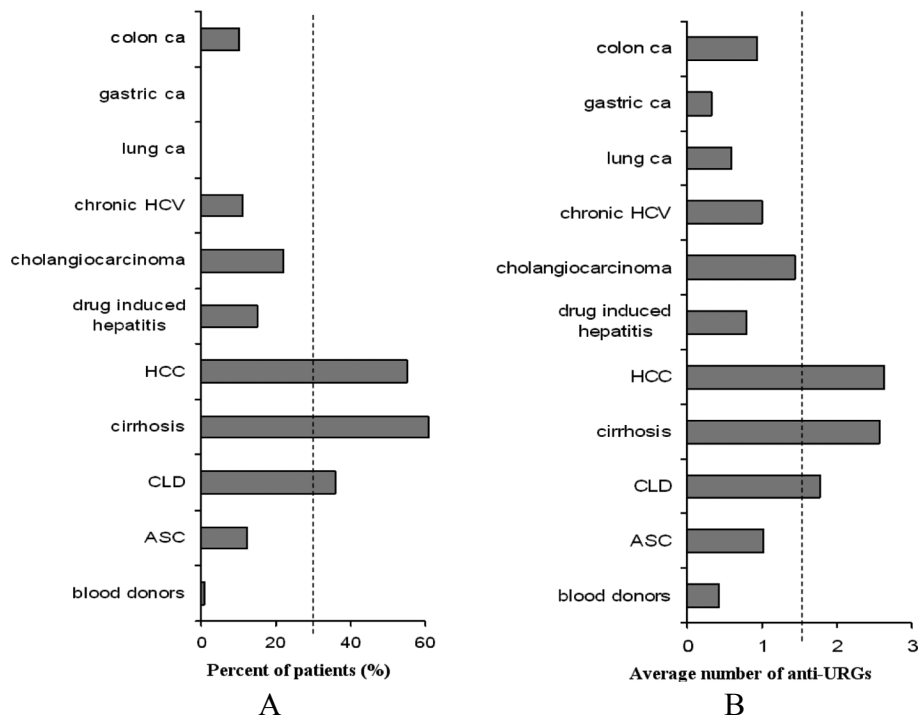


Fig. 2. Percent of patients with 3 or more anti-URGs (A) and average number of anti-URGs (B) in the indicated groups.

### 3.2. Relationship between number of anti-URGs and disease state

The relationship between the average number of anti-URGs and the various categories of patients is listed in Table 2 and graphically presented in Fig. 2A. In Table 2, the average number of antibodies varies greatly, from 0.42 among blood donors to 2.57 in patients with liver cirrhosis and 2.64 in patients with HCC. When these averages were plotted for each category of patients, it appears that groups with  $> 1$  antibody marker identify HBV carriers with chronic liver disease (CLD), cirrhosis, HCC and cholangiocarcinoma, but not other groups, above this threshold (Fig. 2A). The fact that 6 out of 13 HCV patients (46%), 22 out of 30 patients with colorectal cancer (73%), 7 out of 10 patients with lung cancer (70%), and 11 out of 15 patients with gastric cancer (73%) have one or more serological markers of HBV infection, suggests that the presence of anti-URGs in these categories of patients may reflect underlying HBV infection and not due to chronic HCV infection or the presence of tumor types unrelated to HCC. Further, the finding of at least one anti-URG in  $> 30\%$  of blood donors is not surprising in light of the observation that most people in China have been exposed to HBV, and that at least some of these expo-

sure would result in infection without the appearance of typical virus associated serological markers. Previous work showed that the frequency of anti-URGs is extremely low (0.6%) in blood donor serum samples tested from Iceland, which has a very low frequency of HBV infection [21]. Importantly, a fraction of patients with CLD have an average of  $> 1$  antibody marker, and have slightly elevated risk for the development of HCC. In cirrhotic patients, the risk for tumor appearance is very high, and the majority of these patients have  $> 1$  antibody marker. Among HCC patients, most had 3, 4 or 5 antibody markers, but upon closer examination, there was no correlation between the number of antibody markers, the grade of HCC differentiation, or the levels of alpha-fetoprotein (data not shown). Likewise, in cirrhotic patients, there was no correlation between the number of antibody markers and alpha-fetoprotein levels (data not shown).

When the relationship between the number of anti-URGs  $\leq 1$  were compared to the number of anti-URGs  $> 1$  for each group of HBV infected patients (Fig. 2B) by chi-square analysis, there was an inverse correlation between the asymptomatic carrier state with serum samples from this group testing positive for  $> 1$  antibody marker ( $P < 0.001$ ). For patients with chronic hepatitis, there was no significant correlation between

the number of antibody markers and disease. In contrast, among patients with cirrhosis or HCC, there was a strong, direct correlation between the number of antibody markers and disease state ( $P < 0.001$ ). This was not only true for patients with  $> 1$  antibody marker, but also for the same patients when calculations were done for  $> 2$  antibody markers. Interestingly, among other categories of patients similarly analyzed, there was a strong, direct correlation in HBV infected patients with cholangiocarcinoma having  $> 1$  or  $> 2$  antibody markers ( $P < 0.001$ ). In this context, hepadnavirus replication has been shown to occur in cholangiocytes [28] and strong HBx staining has been shown in clinical specimens of cholangiocarcinoma [29]. In contrast, these analyses demonstrated no correlation between these antibodies and disease in patients with lung, gastric, and colorectal cancers, suggesting the appearance of these antibodies is not a general characteristic of tumorigenesis ( $P > 0.5$ ). Moreover, there was no correlation between the appearance of these antibodies and chronic HCV infection ( $P > 0.7$ ). Among patients with drug induced hepatitis, however, there was a weak correlation between patients with  $> 1$  antibody and liver disease ( $P < 0.05$ ), but this correlation was lost when calculations were repeated for  $> 2$  antibodies. Finally, there was a strong inverse correlation between these antibodies and blood donation ( $P < 0.001$ ), verifying that individuals who are not HBV carriers and with no evidence of liver disease lack these antibodies. These statistical analyses further demonstrate a strong relationship between the presence of these antibodies in cirrhotic patients at high risk for the development of liver cancer, and in patients with HCC and cholangiocarcinoma. When these results are shown graphically for patients in each category having  $> 2$  antibodies, most of these antibodies are present in sera from HBV infected patients with cirrhosis and HCC, and to a lesser extent in serum samples from other groups of patients, some of which have evidence of underlying HBV infection (Fig. 2B), suggesting that in the context of chronic HBV infection, the appearance of these antibodies may reflect HBx mediated changes in the liver that are prevalent prior to tumor development and among early tumors.

### 3.3. Longitudinal studies

To test whether the anti-URGs were detectable in HBV patients with hepatitis or cirrhosis before the diagnosis of HCC and/or in newly diagnosed HCC patients, previously stored serial serum samples from each of 3

HBV patients who later developed HCC or cirrhosis were tested blindly for anti-URGs. On average the first anti-URGs appeared 22.7 months before diagnosis and 3 or more antiURGs appeared 11.8 months before diagnosis. The earliest appearance of 3 anti-URGs was detected in patient 6 ( $-36$  months). In patient 14 and patient 26, 3 anti-URGs were detected around 30 months before diagnosis, but there were no much earlier serum sample available (Fig. 3A) In HBV associated hepatitis group, patients were divided to two groups: patients with 3 or more anti-URGs (group A) and patients with 2 or less anti-URGs (group B). In the following 32 months observation, 4 patients in group A developed cirrhosis and 1 patient in this group developed HCC till Jun 2011, but no patients did in group B (Fig. 3B). These results further confirmed the anti-URGs's value for early warning of cirrhosis and HCC.

### 3.4. Anti-URGs and other biomarkers

To evaluate the potential of anti-URGs as a biomarker, the specificity and sensitivity of anti-URGs were analyzed by SSPS v16.0. As shown in Fig. 4A, when 3 anti-URGs were used to separate HBV associated cirrhosis and HCC groups from others, the sensitivity was 58.3% the specificity was 80.0%, and the area under the curve (AUC) was 0.721. Besides anti-URGs, the serum samples from patients in HBV associated cirrhosis (97 isolates) or HCC (78 isolates) were also tested for AFP, AFP-L3, GPC3 and GP73 alone. For cirrhosis, the positivity is 38.8%, 42.3%, 45.2% and 41.1%, this rate increased to 65.7%, 64.6%, 70.3% and 69.1% when combining these biomarkers with the anti-URGs (Fig. 4B, upper panel). For HCC, the positivity is 32.8%, 46.3%, 36.1% and 33.2%, this rate increased to 6.7%, 60.5%, 71.2% and 64.1% when combining these biomarkers with the anti-URGs (Fig. 4B, lower panel).

## 4. Discussion

HBV is a leading cause of CLD and HCC. Given that the liver is capable of extensive regeneration following bouts of hepatitis, and that homeostasis could be maintained by a minority of hepatocytes in the liver, the development of HCC often remains asymptomatic until it becomes quite advanced. This has severely limited the therapeutic options available for HCC patients, and underscores the importance of identifying early markers associated with the pathogenesis of this tumor

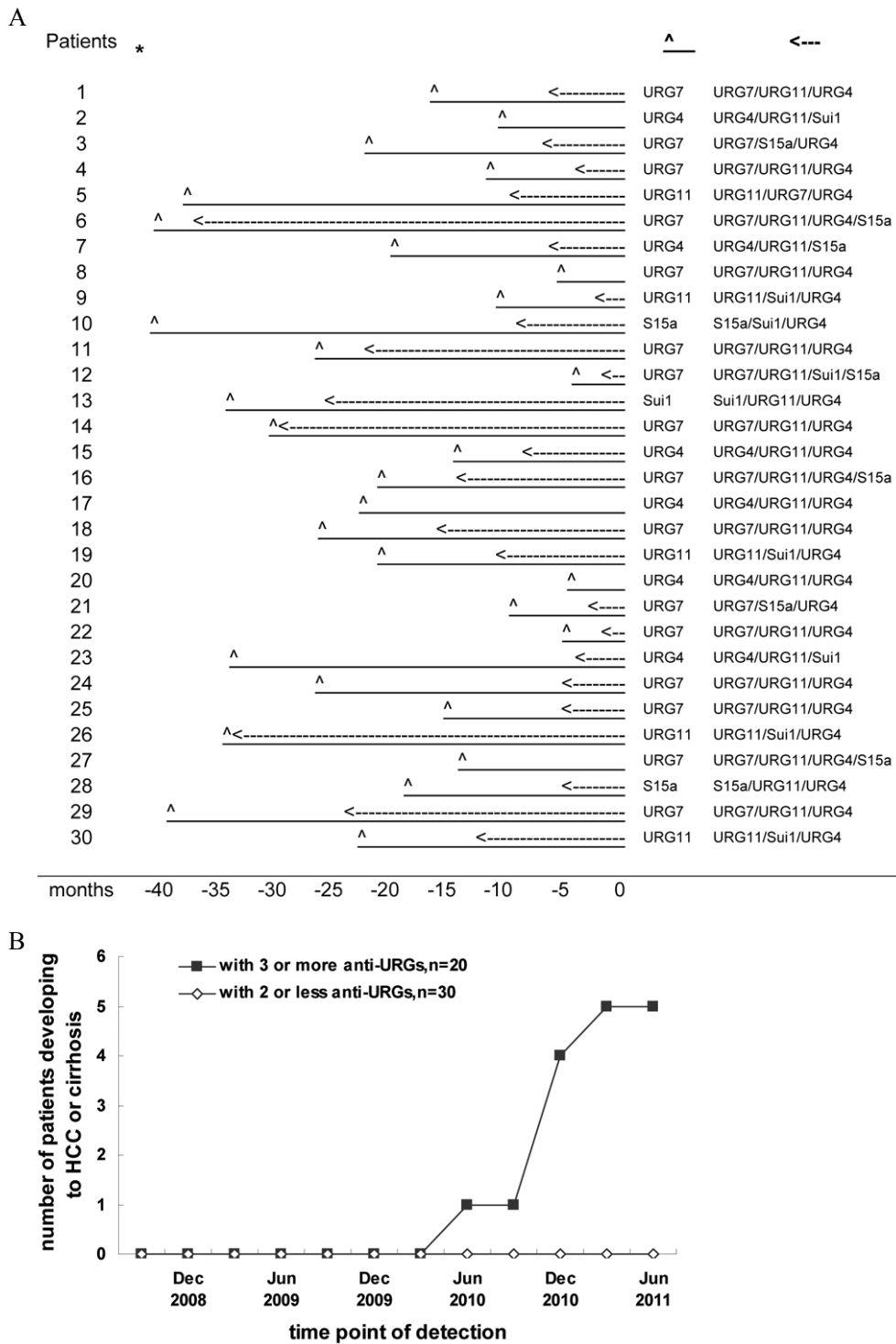


Fig. 3. A, The first or 3 anti-URGs appeared before clinical diagnosis as cirrhosis or HCC in 30 patients. ^: the first anti-URGs appeared; <---: the 3 anti-URGs appeared. \*: clinical diagnosis. B, Comparison of the number of patients who developed to cirrhosis or HCC in observation period. HBV associated hepatitis patients were divided to 2 groups: patients with 3 or more anti-URGs (■) and patients with 2 or less anti-URGs (◇).

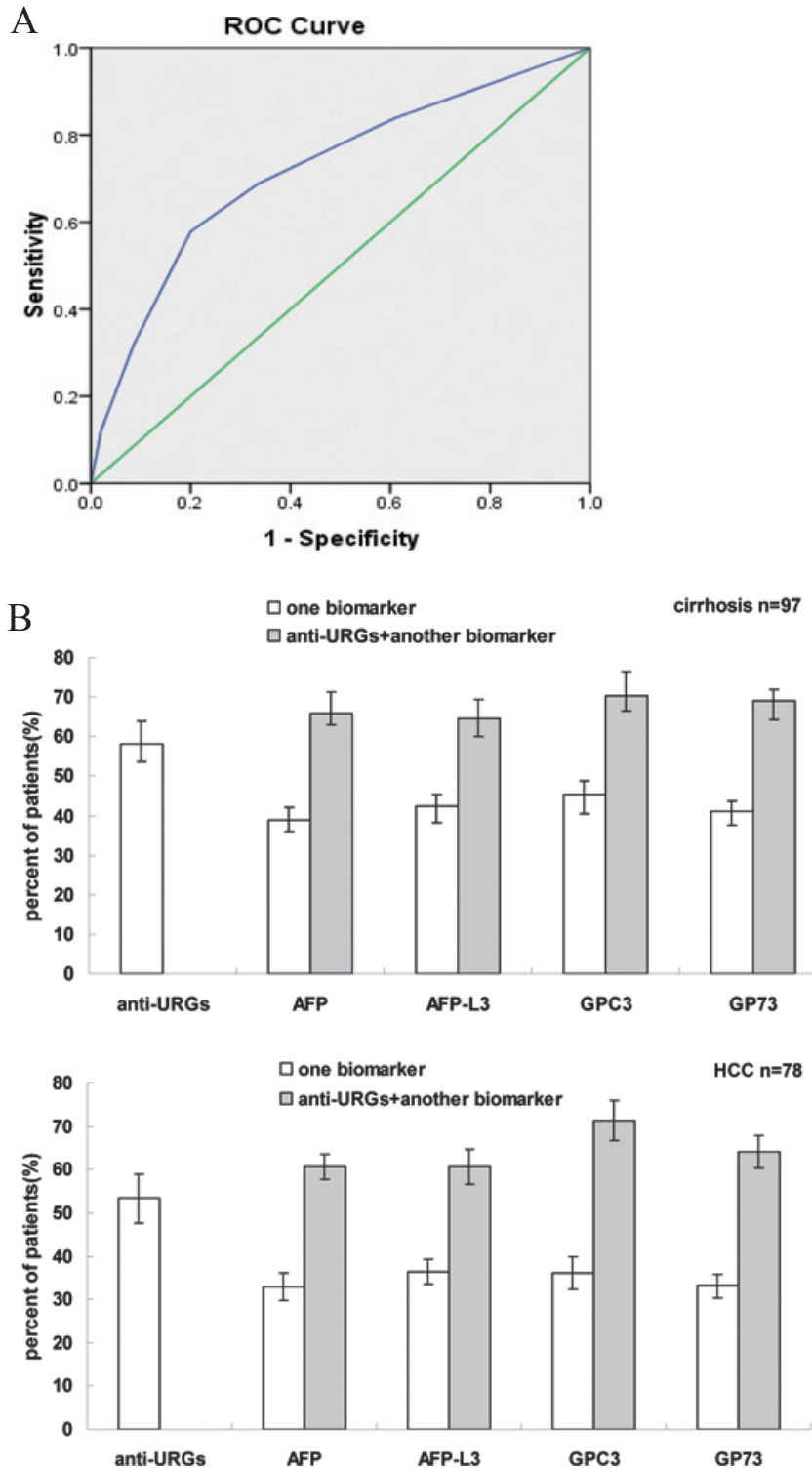


Fig. 4. A, ROC curve of anti-URGs ( $\geq 3$ ) between HBV associated HCC/cirrhosis groups and others. AUC = 0.721, sensitivity is 0.578, specificity is 0.800, asymptotic 95% confidence interval is 0.673 ~ 0.768. B, Sensitivity of anti-URGs and AFP, AFP-L3, GPC3, GP73 alone (□) or combination of anti-URGs and another biomarker (■) for the detection of HCC or cirrhosis.



Table 3  
Characteristics of individual antibodies in different patient populations

Population	Number of patients	Frequency of anti-URGs: number (%)				
		URG4	URG7	URG11	S15a	Sui1
HBV associated						
Asymptomatic carriers (ASC)	146	27 (18)	23 (15.8)	18 (12)	28 (19)	53 (36)
Chronic hepatitis	95	42 (44)	20 (21)	37 (38.9)	27 (28)	42 (44)
Liver cirrhosis	97	41 (42)	47 (48)	53 (54.6)	42 (43)	66 (68)
HCC	78	39 (50)	38 (48.7)	41 (52.5)	34 (43.6)	50 (64)
Cholangiocarcinoma	18	5 (27.7)	3 (16.7)	5 (27.7)	3 (16.7)	9 (50)
Chronic HCV	13	5 (38)	4 (30.7)	5 (38)	0 (0)	6 (46)
Drug induced hepatitis	15	5 (33)	1 (6.6)	3 (20)	0 (0)	4 (26.7)
Lung cancer	10	2 (20)	1 (10)	0 (0)	0 (0)	3 (30)
Colorectal cancer	30	3 (10)	6 (20.7)	5 (17)	4 (13.7)	7 (24)
Gastric cancer	15	1 (6.6)	2 (13)	1 (6.6)	0 (0)	1 (6.6)
Blood donors	108	12 (11)	7 (6.5)	9 (8)	10 (9)	6 (5.5)

The strong epidemiologic relationship between chronic HBV infection and HCC [2,3], and the important contribution of HBx to HCC [22–26], suggest that the molecular pathways altered by HBx are important for tumor development [26]. Previously, HBx has been shown to alter the expression of genes that promote hepatocellular growth, survival, and tumorigenesis [16–20]. These up-regulated proteins appear to trigger corresponding antibody responses that may accompany the onset of critical transforming events. The strong correlation between the anti-URGs in patients with cirrhosis and HCC suggests that their presence may mirror changes that contribute to tumor development [21,27].

This work was conducted with the intent of answer the question whether the anti-URGs were early warning biomarker in populations of Chinese HBV patients at high risk for the development of cirrhosis and/or HCC. This work was conducted with the intent of asking whether these markers are also present in populations of Chinese HBV patients at high risk for the development of chronic liver disease and HCC. The results confirmed the presence of all five antibody markers that are predominantly distributed among HBV infected patients who have developed cirrhosis and/or HCC (Tables 2 and 3). The frequency of each antibody increased with disease intensity, and the patients with the greatest number of anti-URGs (3, 4 or 5) were also those with cirrhosis and/or HCC (Fig. 1, Table 2). The average number of anti-URGs in the panel is also highest among patients with cirrhosis and/or HCC (Fig. 2, Table 3). These findings are consistent with the multistep nature of carcinogenesis and suggest that the up-regulated expression of the corresponding cellular proteins reflects an increased risk for the appearance of HCC. Thus, the evidence provided here further supports the hypothesis that this panel of anti-URGs may

be molecular based risk factors and, therefore, preneoplastic markers for HCC. While the role of the anti-URGs in the pathogenesis of HCC is suggested by the study here, the prognostic value of anti-URGs will need to be assessed in future, longitudinal studies.

Although the number of anti-URGs has been reported to have an impact on survival [21], the more concern is focused on how early the anti-URGs could imply the cirrhosis and HCC accurately. In the 30 patients whose serial serum samples were available, 3 or more anti-URGs appeared 11.8 months (range, –36 months; Fig. 3A) before diagnosis of HCC, and preceded the diagnosis of HCC at least 5 months in 70.3% of patients (Fig. 3A). To verify whether the patients with 3 or more anti-URGs will develop cirrhosis or HCC, longitudinal studies were performed in two groups of HBV hepatitis patients, it was showed that the patients with 3 or more anti-URGs have much higher risk (5/20 vs 0/30, till June 2011) to develop cirrhosis or HCC (Fig. 3B).

Presently, a-fetoprotein (AFP) is the generally accepted serological marker for HCC diagnosis, but sensitivity of the AFP assay is only 47% (The specificity is 96%) and cut-off value useful in tumor diagnosis is high (50 mg/L) [31]. Combined determination of AFP with AFP-L3 (Lens culinaris agglutinin reactive AFP), des- $\gamma$ -carboxy prothrombin (DPC) could improve the sensitivity to 85% while the specificity would decrease to 59% [32,33]. Given these limitations, AFP has been clinically used in combination with hepatic echography in the screening of cirrhotic patients for HCC, but clearly, much improvement is needed. Additional markers have been developed that may be useful in the early diagnosis of HCC. These include a-1-fucosidase (AFU), g-glutamyl transferase (GGT), glypican-3 (GPC3), DCP, squamous cell carcinoma antigen (SCCA), golgi protein-73, hepatocyte growth factor (HGF), nerve growth factor (NGF) and

transforming growth factor b1 (TGF-b1) [33]. None of these have thus far been shown to identify patients with early HCC, although most of these remain to be studied in combination.

In this study, anti-URGs and AFP, AFL-L3, GPC3, GP73 were measured in serum of patients. As the ROC curve showed in Fig. 4A, when the anti-URGs was used to separate the cirrhosis and HCC groups from others, the AUC was 0.721 (sensitivity: 58.3%; specificity: 80%). The sensitivities of the other biomarkers were all lower than 50%. The anti-URGs showed significantly better in identifying patients who then develop HCC and/or cirrhosis than AFP, AFL-L3, GPC3 and GP73. Moreover, combination of anti-URGs with above biomarker could improve the sensitivity to 75% (Fig. 4B) as well as the specificity was kept around 80% (data not shown). The anti-URGs presented herein, either alone or in combination with existing markers, may increase the chances of identifying patients at high risk for cirrhosis and/or HCC or with early cirrhosis and/or HCC. This would permit the identification and close monitoring of patients most likely to develop cirrhosis and/or HCC, and timely treatment of those with early stage tumor.

Given that the anti-URGs presented herein reflect HBxAg mediated changes in the corresponding gene expression profile that promotes tumor development, and that the pathogenesis of HCC is multi-step, future studies will be designed to evaluate different combination therapies that will be useful in identifying patients who will benefit from aggressive intervention.

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