

Immunopathology on Hepatocyte Expression of HBV Surface, Core, and x Antigens in Chronic Hepatitis B: Clinical and Virological Correlation

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Abstract

Purpose Hepatocyte expression of HBV surface, core, and x antigens (HBsAg, HBcAg and HBxAg), semi-quantitated by immunopathology, were correlated with clinical and virological data in 80 patients with chronic hepatitis B.

Results Seventy patients were HBsAg positive in cytoplasm, 61 were HBcAg positive, including 45 in both nucleus and cytoplasm and 16 in cytoplasm only, and 47 were HBxAg positive in cytoplasm. The detection rates for HBcAg increased while those for HBsAg and HBxAg decreased with HBV DNA levels. Positive HBcAg staining usually suggested the presence of HBV DNA levels $>10^6$ copies/ml. HBcAg, HBsAg, and HBxAg expressions showed no significant differences between patients with genotype B and C. Serum HBeAg and HBV DNA levels correlated positively with nuclear or cytoplasmic HBcAg expression but inversely with HBsAg expression. By multiple regression analysis, HBV DNA levels correlated significantly only with nuclear HBcAg expression. ALT levels and inflammatory grades correlated with cytoplasmic HBcAg expression. There was an inverse quantitative relationship between HBcAg and HBsAg expression. Furthermore, HBxAg expression correlated significantly with HBsAg expression as well as male gender.

Conclusions With diminishing HBV DNA levels following HBeAg seroconversion, HBcAg expression decreased but HBsAg expression increased with a concomitant increase in HBxAg expression. Whether the finding that a

significantly higher expression of HBxAg observed in males than females may account for the gender difference in long-term sequelae of chronic HBV infection needs further investigation.

Keywords Chronic hepatitis B · HBcAg · HBsAg · HBxAg · Hepatitis B virus · HBV DNA · Immunopathology

Introduction

Hepatitis B virus (HBV) is the smallest known DNA virus with a partially double-stranded DNA genome of only 3.2 kDa, which encodes four known overlapping open reading frames, called S (surface), C (core), P (polymerase), and X (HBx protein). By immunopathology, HBcAg is detectable in the hepatocyte nucleus or cytoplasm, while HBsAg is localized in the cytoplasm and occasionally on the cell membrane but not in the nucleus [1, 2]. The demonstration of HBcAg in tissues is almost invariably correlated with circulating Dane particles. Hepatocyte expression of HBcAg thus can be recognized as a marker of active viral replication. On the contrary, the tissue localization of HBsAg is linked to circulating 20-nm spherical and tubular HBsAg particles, but not necessarily to circulating Dane particles [1].

The clinical implication of intrahepatic expression of HBsAg and HBcAg in chronic HBV infection has long been studied [3–9]. Recently, there has been much progress in HBV virological testing. For example, HBV genotyping is of increasing clinical significance and the recent availability of polymerase chain reaction (PCR)-based assays can detect serum HBV DNA levels as low as 10^2 copies/ml. Further studies to correlate the expression of viral antigens

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in tissues with clinical and virological characteristics are needed. On the other hand, data on the clinical implication of intrahepatic HBxAg expression in chronic HBV infection are relatively limited and results remain inconclusive.

In this study, we performed immunopathology to study the expression of HBcAg, HBsAg and HBxAg in tissues from patients with chronic B hepatitis. The degrees of viral antigen expression were semi-quantitated and correlated with the clinical and virological characteristics as well as with the expression of other viral antigens.

Materials and Methods

Patients

Eighty patients with clinico-pathologically verified chronic hepatitis B were studied. None had ever received antiviral or immunomodulatory therapy. This investigation was carried out in accordance with the Helsinki declaration and signed consent was obtained from all patients. Each patient was recorded for age, sex, aspartate aminotransferase (AST), alanine aminotransferase (ALT), hepatitis B e antigen (HBeAg), HBV DNA levels, HBV genotype, inflammatory grades and fibrosis stages. Clinical and laboratory data of the 80 patients are summarized in Table 1.

Laboratory Methods

HBsAg and HBeAg were tested by radioimmunoassays (Abbott Diagnostics, Chicago, Illinois). Serum levels of HBV DNA were tested using the COBAS Amplicor HBV Monitor test (Roche diagnostics, Branchburg, NJ). The detection sensitivity was 200 copies/ml. HBV genotypes were determined by using the PCR-restriction fragment length polymorphism of the surface gene of HBV, as previously described [10]. Percutaneous needle biopsies were

performed with a Menghini needle. A 1- to 3-cm biopsy core was available from each patient for study. The liver histological findings including inflammatory grades and fibrosis stages were interpreted in accordance with the scoring system proposed by Ishak et al. [11]. Immunostaining of viral antigens in hepatocytes was studied by the avidin-biotin immunoperoxidase methods with paraffin sections of liver specimens, as described in detail before [5, 6]. The primary antibodies used in this study included rabbit polyclonal antibody against HBcAg, mouse monoclonal antibody against HBsAg (Dakopatts, Copenhagen, Denmark) and rabbit polyclonal antibody against HBxAg (Acris Antibodies GmbH, Germany). Analysis of staining patterns was made by microscopic examination. The degrees of viral antigen expression were expressed as a proportion of the immunolabeled cells without consideration for the staining intensity. The degrees of HBcAg and HBsAg expression were semi-quantitated using a scale of 0–4, corresponding to positivity in 0, 1–10, 11–25, 26–50, and >50% of hepatocytes examined, respectively, as reported previously [4, 6]. The proportion of the HBxAg positive hepatocytes was usually less than that of HBcAg or HBsAg positive cells, so the degree of HBxAg expression was semi-quantitated using a scale of 0–4, corresponding to positivity in 0, 1–5, 6–10, 11–20, and >20% of hepatocytes examined, respectively.

Statistical Analysis

The degrees of hepatocyte expression of HBcAg, HBsAg and HBxAg were correlated with clinical and virological data and with the expression of other viral antigens by using logistic regression for nominal data or Spearman's rank correlation for continuous data. Multiple regression analysis was performed for independent variables with colinearity. Statistical procedures were performed with statistical software (3rd edition, Stat View; SAS Institute Inc., Cary, NC). *P* values of <0.05 were considered significant.

Table 1 Clinical and laboratory data of the study patients

Data	Patients (<i>n</i> = 80)
Age (years)	39.2 ± 10.7
Male:female	59:21
HBeAg (positive:negative)	61:19
HBV genotype (B:C)	47:33
HBV DNA (log copies/ml)	7.56 ± 1.47
AST (IU/l)	67 ± 40
ALT (IU/l)	124 ± 87
Inflammatory grades	5.1 ± 2.7
Fibrosis stages	3.1 ± 1.3

Data are given as number value or mean ± standard deviation

Results

Seventy patients (88%) were HBsAg positive in cytoplasm, 61 patients (76%) were HBcAg, including 45 in both nucleus and cytoplasm and 16 in cytoplasm only, and 47 patients (59%) were HBxAg positive in cytoplasm. The detection rates for HBcAg increased while the detection rates for HBsAg and HBxAg decreased with increasing serum levels of HBV DNA (see Table 2). Nuclear HBcAg was rarely detected if serum levels of HBV DNA were less than 10⁷ copies/ml, as was cytoplasmic HBcAg if serum levels of HBV DNA were less than 10⁶ copies/ml. The

Table 2 Relationship between hepatocyte staining of HBcAg, HBsAg and HBxAg and serum levels of HBV DNA

HBV DNA (log copies/ml)	Number (%) with positive staining			
	Nuclear HBcAg	Cytoplasmic HBcAg	HBsAg	HBxAg
<5 (<i>n</i> = 4)	0	0	4 (100)	3 (75)
5–5.99 (<i>n</i> = 11)	0	1 (9)	11 (100)	11 (100)
6–6.99 (<i>n</i> = 7)	0	5 (71)	6 (86)	4 (57)
7–7.99 (<i>n</i> = 20)	12 (60)	18 (90)	16 (80)	12 (60)
8–8.99 (<i>n</i> = 25)	20 (80)	25 (100)	22 (88)	11 (44)
≥9 (<i>n</i> = 13)	13 (100)	13 (100)	11 (85)	6 (46)

Difference in positive rates between patients with HBV DNA $\geq 10^6$ copies/ml and $< 10^6$ copies/ml was significant for nuclear HBcAg ($P < 0.0001$), cytoplasmic HBcAg ($P < 0.0001$) and HBxAg ($P = 0.0025$), and marginally significant ($P = 0.1$) for HBsAg

Table 3 Semi-quantitative expression of HBcAg, HBsAg and HBxAg on hepatocytes in 80 patients with chronic hepatitis B

Degrees	Nuclear HBcAg	Cytoplasmic HBcAg	HBsAg	HBxAg
0	35	19	10	33
1	24	11	29	19
2	10	10	17	11
3	7	13	21	11
4	4	27	3	6

results of semi-quantitative expression of HBcAg, HBsAg and HBxAg are summarized in Table 3.

The degrees of hepatocyte expression of nuclear HBcAg, cytoplasmic HBcAg, cytoplasmic HBsAg and HBxAg were correlated with clinical (age, gender, ALT levels, inflammatory grades and fibrosis stages) and virological (HBeAg, HBV DNA levels and HBV genotypes) characteristics, as well as with the expression of other viral antigens, as summarized in Table 4.

Serum HBeAg and HBV DNA levels correlated significantly with the degrees of nuclear or cytoplasmic HBcAg expression but inversely with the degrees of HBsAg expression (Table 4; Fig. 1). Because nuclear HBcAg, cytoplasmic HBcAg and HBsAg expression correlated closely with each other (Table 4), further multiple regression analyses were performed. The results revealed that serum levels of HBV DNA correlated significantly with nuclear HBcAg ($P = 0.014$), but not with cytoplasmic HBcAg ($P = 0.06$) or HBsAg expression ($P = 0.19$).

Serum ALT levels correlated significantly with the degrees of nuclear and cytoplasmic HBcAg expression but inversely with the degree of HBsAg expression (Table 4). Multiple regression analysis revealed that ALT levels correlated significantly with cytoplasmic HBcAg expression ($P = 0.0005$) but not with nuclear HBcAg ($P = 0.80$) or HBsAg expression ($P = 0.86$). The inflammatory grades also correlated significantly with the degree of cytoplasmic HBcAg expression but not with nuclear HBcAg, HBsAg or HBxAg expression.

Table 4 Correlation of hepatocyte expression of nuclear and cytoplasmic HBcAg, HBsAg and HBxAg with clinical and virological characteristics

Clinical and virological characteristics	Nuclear HBcAg		Cytoplasmic HBcAg		HBsAg		HBxAg	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Age (years)	-0.025	0.82	-0.135	0.23	0.102	0.37	0.011	0.92
Sex (male = 1, female = 0)	0.012	0.48	0.144	0.36	0.066	0.78	0.59	0.019
HBV DNA (copies/ml)	0.746	<0.0001	0.713	<0.0001	-0.399	0.0004	-0.095	0.40
HBeAg (positive = 1, negative = 0)	0.503	<0.0001	1.469	<0.0001	-4.28	<0.0001	-0.333	0.09
Genotype (B = 1, C = 2)	0.138	0.48	0.147	0.31	-0.154	0.47	-0.092	0.63
ALT (U/l)	0.395	0.0004	0.55	<0.0001	-0.272	0.016	0.100	0.37
Inflammatory grades	0.129	0.25	0.400	0.0004	-0.083	0.46	0.159	0.16
Fibrosis stages	0.083	0.46	0.044	0.69	-0.109	0.34	-0.089	0.43
Nuclear HBcAg expression	–	–	0.649	<0.0001	-0.254	0.024	-0.231	0.07
Cytoplasmic HBcAg expression	0.649	<0.0001	–	–	0.479	<0.0001	-0.107	0.34
HBsAg expression	-0.254	0.024	-0.367	0.0011	–	–	0.479	<0.0001
HBxAg expression	-0.231	0.07	-0.107	0.34	0.479	<0.0001	–	–

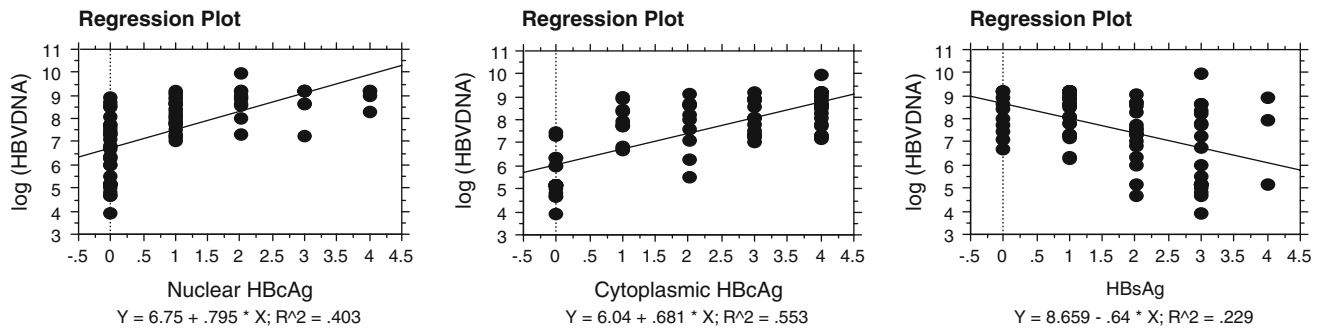
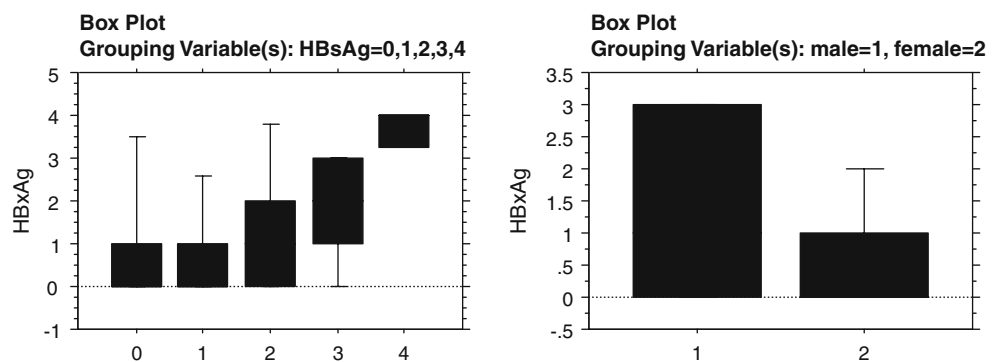


Fig. 1 Correlation between serum levels of HBV DNA (log copies/ml) and degrees of hepatocyte expression of nuclear and cytoplasmic HBcAg and HBsAg

Fig. 2 Box plot of the degrees of hepatocyte HBxAg expression, stratified according to the degrees of hepatocyte HBsAg expression (*left*) and gender (*right*), in 80 patients with chronic hepatitis B



There was no significant correlation between HBxAg expression and serum HBeAg, HBV DNA levels, HBV genotypes, biochemical and histological activities. However, HBxAg expression correlated significantly with HBsAg expression (Table 4). The median values (interquartile range) of HBxAg expression were 0 (0–1), 0 (0–1), 1 (0–2), 2 (1–3), and 3 (3–4) for patients with degrees of HBsAg expression of 0, 1, 2, 3, and 4, respectively (Fig. 2). In addition, the degrees of HBxAg expression were significantly higher in the males than the females (Table 4). The median values (interquartile range) of HBxAg expression were 1 (0–3) and 0 (0–1) for males and females, respectively (Fig. 2).

Discussion

It has long been recognized that positive staining of HBcAg in liver indicates active viral replication [1, 2]. In this investigation, the detection rates of HBcAg in liver increased with increasing serum levels of HBV DNA. As shown in Table 2, HBcAg can rarely be detected in tissues if serum levels of HBV DNA were less than 10^6 copies/ml. The absence of HBcAg in tissues therefore cannot exclude the presence of modest levels ($<10^6$ copies/ml) of HBV DNA in serum. This range of HBV DNA levels indeed is not uncommon in patients with chronic hepatitis B [12].

This investigation showed that serum HBeAg and HBV DNA correlated significantly with the degrees of nuclear or cytoplasmic HBcAg expression but inversely with the degrees of HBsAg expression. However, nuclear HBcAg, cytoplasmic HBcAg and HBsAg expression correlated closely with each other (Table 4). Multiple regression analysis revealed that serum HBV DNA correlated significantly with nuclear HBcAg expression but not with cytoplasmic HBcAg or HBsAg expression. This finding seems to be in keeping with the observations that HBcAg was predominantly localized in the nucleus during the immune tolerance phase of chronic HBV infection when there are high HBV DNA levels in serum [3, 4, 7]. Nuclear HBcAg expression thus can be recognized as a marker of high levels of HBV replication. The presence of nuclear HBcAg usually indicates relatively high serum HBV DNA levels ($>10^7$ copies/ml), as shown in Table 2.

Furthermore, there is a positive correlation between the degrees of cytoplasmic expression of HBcAg and biochemical (ALT levels) and histological (inflammatory grades) activities. Previous studies have shown that in the course of chronic HBV infection HBcAg shifted from the nucleus during the immune tolerance phase with normal ALT to cytoplasm during the immune clearance phase with elevated ALT [3, 5, 7]. Our data further suggest that the degrees of cytoplasmic expression of HBcAg can be recognized as a marker of histological activity in liver.

This investigation did not measure serum levels of HBsAg in our patients, but previous studies have shown that there was a highly significant and positive correlation between serum levels of HBV DNA and HBsAg [13]. As shown in Table 4, serum levels of HBV DNA correlated negatively with expression of HBsAg in liver, so there was an inverse relationship between levels of HBsAg in serum and in liver. In addition, there was also an inverse relationship between hepatocyte expression of HBsAg and HBcAg (Table 4). Taken together, these findings suggest that during active viral replication, intracellular HBsAg, packed with viral DNA and nucleocapsid proteins as a complete virion, is efficiently exported, resulting in high levels of HBsAg in serum and low levels of HBsAg in liver. Conversely, when viral replication becomes inactive, synthesis of HBcAg is diminished and the export of HBsAg is reduced; as a result, there is increased accumulation of intracellular HBsAg with decreased levels of HBsAg in serum. Accordingly, hepatocytes with a wide-spread cytoplasmic expression of HBsAg (so-called “ground glass” hepatocytes) are usually observed in inactive HBsAg carriers who carry a low level of viral replication.

The reported frequency of hepatocyte expression of HBxAg by immunopathology in patients with chronic type B hepatitis varies considerably from 30 to 95% [14–21]. HBxAg was distributed exclusively in cytoplasm [14, 15, 18, 21] or in cytoplasm and nucleus [16, 17]. The expression of HBxAg correlated active viral replication in some studies [14, 18], but not in others [15, 20, 21]. Most studies showed the expression of HBxAg did not correlate with histological activity [15, 17, 19, 21]. In our study, 59% of patients with chronic type B hepatitis were positive for HBxAg in the cytoplasm, and the degrees of hepatocyte expression of HBxAg did not correlate with age, serum HBeAg, HBV DNA levels, HBV genotypes, AST and ALT levels, inflammatory grades and fibrosis stages. However, there was a significant positive correlation between the degrees of HBxAg and HBsAg expression (Table 4; Fig. 2). Given that hepatocyte expression of HBsAg increased with decreasing levels of viral replication, these data may suggest that during the course of chronic HBV infection hepatocyte expression of HBxAg tends to increase along with increasing accumulation of intracellular HBsAg when the viral replication decreases. The clinical implication of these observations is unclear, but several studies have shown that there is an over-expression of HBxAg in HBV infected patients with hepatocellular carcinoma [16, 21–23]. Perhaps another interesting finding of our study is that the degrees of HBxAg expression, but not of HBcAg or HBsAg expression, were significantly higher in the male patients than the female patients (Fig. 2). Further studies on a larger number of subjects are indicated to clarify whether this finding may contribute to

the gender-related difference in the development of hepatocellular carcinoma in chronic HBV infection.

In conclusion, hepatocyte expression of HBcAg and HBsAg correlates with serum HBV DNA levels and histological activity. Nuclear HBcAg expression correlates with serum HBV DNA levels while cytoplasmic HBcAg expression correlates with inflammatory activities. With diminishing serum HBV DNA levels, hepatocyte HBcAg expression decreases but HBsAg expression increases with a concomitant increase in HBxAg expression. Additionally, HBxAg expression is higher in males than females.

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References

- Bianchi L, Gudat F. Histo- and immunopathology of viral hepatitis. In: Deinhardt F, Deinhardt J, eds. *Viral hepatitis: Laboratory and clinical science*. New York: Marcel Dekker; 1983:335–382.
- Goodman Z. Histopathology of hepatitis B virus infection. In: Lai CL, Locarnini S, eds. *Hepatitis B virus*. London: International Medical Press; 2002:131–143.
- Chu CM, Liaw YF. Intrahepatic distribution of hepatitis B surface and core antigens in chronic hepatitis B virus infection. *Gastroenterology*. 1987;92:220–225.
- Chu CM, Liaw YF. Intrahepatic expression of HBcAg in chronic HBV hepatitis: Lessons from molecular biology. *Hepatology*. 1990;12:1443–1445.
- Chu CM, Yeh CT, Sheen IS, Liaw YF. Subcellular localization of hepatitis B core antigen in relation to hepatocyte regeneration in chronic hepatitis B. *Gastroenterology*. 1995;109:1926–1932.
- Chu CM, Yeh CT, Chien RN, Sheen IS, Liaw YF. The degrees of hepatocyte nuclear but not cytoplasmic expression of hepatitis B core antigen reflect the level of viral replication in chronic hepatitis B virus infection. *J Clin Microbiol*. 1997;35:102–105.
- Hsu HC, Su IJ, Lai MY, Chen DS, Chang MH, Sung JL. Biologic and prognostic significance of hepatocyte hepatitis B core antigen expression in the course of chronic hepatitis B virus infection. *J Hepatol*. 1987;5:45–50.
- Kim CW, Yoon SK, Jung ES, et al. Correlation of hepatitis B core antigen and beta-catenin expression on hepatocytes in chronic hepatitis B virus infection: Relevance to the severity of liver damage and viral replication. *J Gastroenterol Hepatol*. 2007;22:1534–1542.
- Serinoz E, Varli M, Erden E, et al. Nuclear localization of hepatitis B core antigen and its relations to liver injury, hepatocyte proliferation, and viral load. *J Clin Gastroenterol*. 2003;36:269–272.
- Chu CM, Liaw YF. Genotype C hepatitis B virus infection is associated with a higher risk of reactivation of hepatitis B and progression to cirrhosis than genotype B: A longitudinal study of hepatitis B e antigen-positive patients with normal aminotransferase levels at baseline. *J Hepatol*. 2005;43:411–417.
- Ishak K, Baptista A, Bianchi L, et al. Histological grading and staging of chronic hepatitis. *J Hepatol*. 1995;22:696–699.
- Chu CJ, Hussain M, Lok AS. Quantitative serum HBV DNA levels during different stages of chronic hepatitis B infection. *Hepatology*. 2002;36:1408–1415.
- Ozars R, Tabak F, Tahan V, et al. Correlation of quantitative assay of HBsAg and HBV DNA levels during chronic HBV treatment. *Dig Dis Sci*. 2008;53:2995–2998.

14. Haruna Y, Hayashi N, Katayama K, et al. Expression of X protein and hepatitis B virus replication in chronic hepatitis. *Hepatology*. 1991;13:417–421.
15. Hoare J, Henkler F, Dowling JJ, et al. Subcellular localisation of the X protein in HBV infected hepatocytes. *J Med Virol*. 2001; 64:419–426.
16. Pal J, Somogyi C, Szmolenszky AA, et al. Immunohistochemical assessment and prognostic value of hepatitis B virus X protein in chronic hepatitis and primary hepatocellular carcinomas using anti-HBxAg monoclonal antibody. *Pathol Oncol Res*. 2001;7: 178–184.
17. Seo JH, Kim KM, Murakami S, Park BC. Lack of colocalization of HBxAg, insulin like growth factor II in the livers of patients with chronic hepatitis B, cirrhosis and hepatocellular carcinoma. *J Korean Med Sci*. 1997;12:523–531.
18. Suzuki K, Uchida T, Shikata T, et al. Expression of pre-S1, pre-S2, S and X peptides in relation to viral replication in livers with chronic hepatitis B. *Liver*. 1990;10:355–364.
19. Vitvitski-Trepo L, Kay A, Pichoud C, et al. Early and frequent detection of HBxAg and/or anti-HBx in hepatitis B virus infection. *Hepatology*. 1990;12:1278–1283.
20. Wang WL, London WT, Lega L, Feitelson MA. HBxAg in the liver from carrier patients with chronic hepatitis and cirrhosis. *Hepatology*. 1991;14:29–37.
21. Zentgraf H, Herrmann G, Klein R, et al. Mouse monoclonal antibody directed against hepatitis B virus X protein synthesized in *Escherichia coli*: Detection of reactive antigen in liver cell carcinoma and chronic hepatitis. *Oncology*. 1990;47:143–148.
22. Hwang GY, Lin CY, Huang LM, et al. Detection of the hepatitis B virus X protein (HBx) antigen and anti-HBx antibodies in cases of human hepatocellular carcinoma. *J Clin Microbiol*. 2003;41: 5598–5603.
23. Wang XZ, Chen XC, Chen YX, et al. Overexpression of HBxAg in hepatocellular carcinoma and its relationship with Fas/FasL system. *World J Gastroenterol*. 2003;9:2671–2675.