

# Detection of HBV DNA and its existence status in liver tissues and peripheral blood lymphocytes from chronic hepatitis B patients \*

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**Subject headings** hepatitis B; hepatitis B virus; DNA, viral; lymphocytes

## INTRODUCTION

Cumulative clinical and experimental evidence indicates that host immune responses to hepatitis B virus (HBV) are responsible for liver damage in chronic hepatitis B, which leads to cirrhosis and hepatocarcinogenesis. The results of *in vitro* experiments by use of B cell lines expressing HBV antigens as effector cells have demonstrated the presence of cytotoxic T cells recognizing viral antigen epitopes in conjunction with MHC class I molecules<sup>[1]</sup>. Extrahepatic tropism of HBV has been increasingly recognized in recent years. Detection of HBV DNA in peripheral blood leukocytes (PBL) was reported recently<sup>[2]</sup>, but no further study was made on HBV DNA existence status in these cells. In this study, the positive PBL DNA specimens were selected by dot blot hybridization, and HBV DNA existence status was analyzed by Southern blot hybridization in order to provide experimental bases for the treatment of hepatitis B and some fundamental data for the research of hepatocarcinogenesis.

## MATERIALS AND METHODS

### Materials

**Subjects** Liver tissues of 37 cases were obtained from Shandong Provincial Hospital. Their diagnosis was confirmed by histopathological examination ( $n = 37$ , 26 males and 11 females, mean age 33.4 years). At the same time, PBL were separated and stored at  $-20^{\circ}\text{C}$  immediately for use.

**Reagents** Recombinant plasmid pBR<sup>322</sup>-2HBV was provided by Professor CB, and DIG High Prime Labeling and Detection Starter Kit was the product of Boehringer Mannheim Company, Germany. Restriction Endonucleases *EcoR* I, Leukocyte lysis buffer and Proteinase K were purchased from Huamei Company.

### Methods

**HBV DNA labeling** Isolation of recombinant plasmid pBR<sup>322</sup>-2HBV and preparation of HBV DNA probe were carried out as described by Sambrook<sup>[3]</sup>, HBV labeling procedures were described before<sup>[4]</sup>. Template DNA (1.0 $\mu\text{g}$ ) and sterile redist water were added into a reaction vial. The DNA was denatured and DIG high prime was added and incubated for 20 hours at  $37^{\circ}\text{C}$ . Reaction was stopped by adding EDTA. Quantification of labeling efficiency was performed according to the instructions of the manufacturer.

**DNA extraction** Genomic DNA from the liver tissues was isolated according to the standard methods<sup>[3]</sup>. Genomic DNA from PBL was extracted according to WANG *et al*<sup>[5]</sup>. The PBL aliquots were mixed with leukocyte lysis buffer, and proteinase K was added to a final concentration of 100  $\mu\text{g}/\text{L}$ . After being incubated at  $37^{\circ}\text{C}$  for 12h - 16h, the digested preparation was extracted twice with buffer-equilibrated phenol and once with a phenol-chloroform mixture. DNA was precipitated by adding sodium acetate and followed by ice-cold absolute ethanol and stored overnight at  $-20^{\circ}\text{C}$ . After being centrifuged and washed with ice-cold ethanol, the DNA was dried under vacuum at room temperature. It was redissolved in TE buffer and stored at  $-20^{\circ}\text{C}$ .

**HBV DNA dot blot hybridization** Genomic DNA 5 $\mu\text{L}$ ,  $\lambda\text{DNA}$  10ng, HBV DNA 10pg, and 1.0pg were heated in a boiling water bath for 5min - 10min, and cooled quickly in an ice/ethanol bath for 5min. They were mixed with  $20 \times \text{SSC}$  and tipped to nitrocellulose filters. The filters were prehybridized for 8h - 12h, hybridized for 24h - 36h at  $42^{\circ}\text{C}$ , washed and subjected to immunodetection according to the standard dot blot

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hybridization procedures.

**Southern blot hybridization** Positive DNA of liver tissues and PBL containing HBV DNA verified by dot blot hybridization were then digested completely with specific restriction endonucleases according to the proportion of 5U *EcoR* I to 1.0 $\mu$ g DNA electrophoresed in agarose gels, denatured, and transferred to nitrocellulose filters. The filters were performed with standard Southern blot procedures<sup>[6]</sup>.

**Statistical analysis**  $\mu$  test and  $\chi^2$  test of paired data were employed to determine the significance between groups. *P* values <0.05 were considered to be significant.

## RESULTS

### *Probes sensitivity and specificity*

The high sensitivity and specificity of HBV DNA probe were confirmed to be 1.0pg by dot blot hybridization which also confirmed its high specificity.

### *Results of HBV DNA detection in liver tissues and PBL*

The positive rates of liver tissues and PBL are shown in Table 1.

**Table 1 HBV detection in liver tissues and PBL**

| Specimens     | <i>n</i> | Positive rate |      |
|---------------|----------|---------------|------|
|               |          | <i>n</i>      | %    |
| PBL           | 31       | 27            | 87.1 |
| Liver tissues | 37       | 31            | 83.8 |

### *HBV DNA detection in liver tissues and PBL*

The results of HBV DNA in liver tissues and PBL are shown in Table 2.

**Table 2 HBV correlation between PBL and liver tissues**

| Specimens     | <i>n</i> | Positive rate |      |
|---------------|----------|---------------|------|
|               |          | <i>n</i>      | %    |
| PBL           | 31       | 27            | 87.1 |
| Liver tissues | 31       | 28            | 90.3 |

$r = 0.81, P < 0.05$ .

### *Analysis of HBV DNA existence status*

The existence status of HBV DNA is shown in Table 3.

**Table 3 HBV DNA existence status**

| Specimens     | <i>n</i> | Integrated type |      | Mixed type |      |
|---------------|----------|-----------------|------|------------|------|
|               |          | <i>n</i>        | %    | <i>n</i>   | %    |
| PBL           | 9        | 5               | 55.6 | 4          | 44.4 |
| Liver tissues | 9        | 5               | 55.6 | 4          | 44.4 |

## DISCUSSION

In traditional sense, hepatitis B virus (HBV) has

strict hepatic tropism, but in recent years, extrahepatic tropism of HBV has been increasingly recognized. HBV antigen or HBV DNA has been demonstrated in the spleen, kidney, skin, pancreatic tissues and bone marrow cells. Lymphocytes are immunoreactive cells. Cytotoxic lymphocyte recognizes viral antigen epitopes expressed in the surface of target cell<sup>[7]</sup>, and subsequently induced-cytotoxic function which may contribute to the elimination and clearance of HBV<sup>[8]</sup>. In the process of HBV clearance, virus may infect lymphocytes by way of infecting stem cells, phagocytic function of lymphocytes, or the cytotoxic effect mediated by hepatic surface-receptors. In our study, the positive rate of HBV DNA in PBL isolated from chronic hepatitis B (CHB) was 87.1%. The HBV existence in lymphocytes may affect the secretory function of tumor necrosis factor alpha (TNF $\alpha$ ) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), which will influence the prognosis of hepatitis<sup>[9]</sup>. So, attention should be paid to the regulation of the function of lymphocytes after HBV infection.

In our study, no statistical significance was found between the HBV DNA positive rates in Liver tissues and PBL. Six specimens of liver tissues which did not acquire PBL because of some reasons were removed from the study, and the data of the 31 paired cases were studied. To our astonishment, a statistical correlation of HBV DNA was found between liver tissues and PBL ( $r=0.81, P<0.05$ ), indicating that HBV DNA of PBL is almost equal to that of liver tissues and HBV DNA of PBL can reflect the viral status in liver tissues. Clinically, HBV DNA of PBL should be detected at first in order to reduce the occurrence of complications in liverbiopsy.

On the basis of HBV DNA integration into chromosome or free existing in plasma, HBV DNA positive specimens can be divided into three types: integrated type which has integrated HBV DNA only, free type which has free HBV DNA in plasma, and mixed type which has HBV DNA both in chromosome and in plasma. As a usual carcinogen, integrated HBV DNA can mediate secondary rearrangements of chromosome and lead to chromosome instability. Also, as a transactivator, integrated HBx DNA can cause cell overproliferation and contain p53-mediated apoptosis, which may be the possible reason of hepatocarcinogenesis. In order to detect HBV existence status in PBL, 9 PBL specimens were selected and analyzed by Southern blot hybridization. The results showed 55.6% was integrated type and 44.4% was mixed type. HBV DNA integration was found in each case of specimens. This result raised the question that can

not be answered by “how PBL function after HBV DNA integration has changed”. The further study on HBV DNA integration may answer it in the future.

The research of HBV status in PBL is of certain significance in the treatment of chronic hepatitis B (CHB). Among the medicines used to treat CHB, interferon  $\alpha$  (IFN $\alpha$ ) is the best one, which contains virus replication by influencing interferon stimulated gene (ISG) protein synthesis and regulating host immune response. HBV DNA in plasma of PBL may be integrated into chromosome with homologous sequence. This integration may lead to repression of ISG expression and make the cell lose its response to IFN $\alpha$ , indicating that HBV existence status should be considered before IFN $\alpha$  is used to treat CHB patients.

#### REFERENCES

- 1 Nancy WY, John ST, Gene TC, Thomas WT, Lau WY, Arthur KC. Hepatitis B virus DNA in peripheral blood leukocytes. *Cancer*, 1994; 73:1143-1148
- 2 Pollicino T, Campo S, Raimondo G. PreS and core gene heterogeneity in hepatitis B virus (HBV) genomes isolated from patients with long-lasting HBV chronic infection. *Virology*, 1995;208:672-677
- 3 Sambrook J. Molecular cloning. 2nd ed. *New York: Cold Spring Harbor Laboratory Press*, 1992
- 4 Gao FG, Sun WS, Cao YL, Zhang LN, Song J. HBx-DNA probe preparation and its application in study of hepatocarcinogenesis. *WJG*, 1998;4:320-322
- 5 Wang SW. Gene diagnosis techniques. *Beijing: Beijing Medical University and Xiehe Medical University Press*, 1993:24-34
- 6 Gao FG, Sun WS, Cao YL, Zhang LN, Song J. HBV DNA detection in liver tissues and peripheral blood leukocytes from chronic hepatitis B patients and its correlation research. *Shandong Yixueyuan Xuebao*, 1996;(2):99-102
- 7 Michalak TI, Lau JYN, Mcfarlane BM, Alexander GJM, Eddleston ALWF, Williams R. Antibody directed complement mediated cytotoxicity to hepatocytes from patients with chronic hepatitis B. *Clin Exp Immunol*, 1995;100:227-232
- 8 Brugger SA, Oesterreicher C, Hofmann H, Kalhs P, Greinix HT, Muller C. Hepatitis B virus clearance by transplantation of bone marrow from hepatitis B immunised donor. *Lancet*, 1997;349:996-997
- 9 Guang LX, Gu CH. The change of TNF $\alpha$  and PGE2 secreted by HBV-infected monocytes and its possible significance in hepatitis patients. *Zhonghua Chuanranbing Zazhi*, 1995;13:204-207

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