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USING 3 PRIMER PAIRS TO DETECT HBV DNA IN LIVER TISSUES FROM HEPATOCELLULAR CARCINOMAS WITH PCR TECHNIQUE

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PCR technique was used to detect HBV DNA in liver tissue samples for study of the prevalence of HBV DNA in tumorous and nearby nontumorous liver tissues from 16 hepatocellular carcinoma (HCC) patients. Three primer pairs, S1/S2, C1/C2 and X1/X2, used in this study were selected from S region, pre C and C region, and X region of HBV DNA, respectively. The detecting with agarose gel electrophoresis and ethidium bromide staining (PCR-EB) was 10^{-2} pg, and that with Southern blot hybridization was 10^{-6} pg. The positive rates in amplification of HBV DNA by S, C and X region primer pairs in liver samples were 43.8% (14/32), 71.9% (23/32) and 71.9% (23/32), respectively. There was significant difference between the positive rates in amplification with S primer and with C primer ($P<0.05$), but no significant difference between the C primer and the X primer ($P>0.05$), and between the S primer and the X primer ($0.10>P>0.05$). HBV DNA fragments were detected in the livers from all 16 cases. The results indicated that X gene integration inducing hepatocellular carcinogenesis and arrest of C gene expression causing escape from host immune surveillance are the possible mechanisms of HCC development in patients with perisistent HBV infection.

Key words: Hepatocellular carcinoma, DNA, PCR, Hepatitis B virus.

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Hepatitis B virus (HBV) infection is closely associated with hepatocellular carcinoma (HCC). According to this serological study, Beasley[1] noted that HBV infection is probably the leading cause of HCC throughout the world, accounting for 75%–90% of the world's cases. Recently reports care been made on the HBV antigen prevalence in HCC tissue by using immunohistochemistry and on HBV DNA integrated in HCC tissue by in situ hybridization.[2,3] But the process how HBV cause HCC remains to be elucidated. As little is known about the prevalence of HBV DNA open reading frames in HCC liver, we tackled the problem by selecting 3 primer pairs, S, C and X gene of HBV DNA, for the sensitive PCR technique to amplify and detect HBV DNA in HCC liver tissue.

MATERIALS AND METHODS

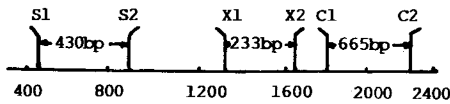
HCC Tissue Specimens

Thirty-two formalin-fixed, paraffin-embedded surgical specimens were taken from 16 patients with pathologically diagnosed HCC in the Affiliated Tumor Hospital of Sun Yat-sen University of Medical Sciences. Fifteen 10 μm microsections of each specimen, with parafin melted, were treated with phenol

/chloroform to extract DNA stored at -40 ℃. The recevered DNA was for use.

Oligonucleotide Primers

The primers specific for HBV C, S and X genes are diagrammed in Figure 1. Primers specific for the Duchenne's muscular dystrophy (DMD) gene xP21 were used as internal control. The sequences of them were P1 F-GAAGATCTAGACAGTGGATACATA-ACAAATGCATG and P2 R-TTCTCCGAAGGTA-ATTGCCTCCCAGATCTGAGTCC. The amplified product was 536 bp long (provided by Shanghai Biochemical Institute of CAS).



S1 413 5' GTGCTGCTATGCCTCATCTT 3';
S2 842R 5' CCCATATGTAAATTTGGGAT 3';
X1 1392 5' TGCCAACTGGATCCTGCGCG 3';
X2 1624R 5' TTCACGGTGGTCTCCATGCG 3';
C1 1730 5' CTGGGAGGAGTTGGGGGAGGAGATT 3';
C2 2394R 5' GGCGAGGGACTTCTTCTTCTAGGGG 3'

Fig. 1. The primer pairs of HBV DNA and the products by PCR with them

Conditions for PCR

The target sequences were amplified in a 50 μl reaction volume with each containing 50 pmol of the primer, 0.2 pmol of dNTP, 1.5 units of FD.DNA polymerase, and 5μl of DNA extract equivalent to 3 microsections. The reaction was continued for 35 cycles in a programmable DNA thermal cycler (Perkin Elmer Cetus). For each cycle, the reaction mixture was heated to 93 ℃ for 0.5 min., cooled to 55 ℃ for 1 min. and incubated at 72 ℃ for 2 min. In the last cycle, the 72 ℃ incubation was extended to 7 min.

Analysis of Products

The DNA was fractionated by 1% agarose gel with ethidium bromide electrophoresis (PCR-EB). The products were transferred to Zeta probe membranes and hybridized with digoxigenin-labelled full-long HBV DNA probe. Besides, the product of PCR with C primer was hybridized with ^{32}P-labelled C gene fragment probe by nick translation.

Special cares were taken to avoid contamination in the procedures as noted in reference.[4]

RESULTS

Specificity and Sensitivity

The bands of products amplified by C, S and X primer pairs corresponded to molecular weight markers (Figure 2, 3). The S and X products were developed by hybridization with digoxigenin labelled HBV DNA probe and the C product was observed after hybridization with ^{32}P-labelled C gene fragment probe. In contract, the product amplified by P1/P2 primers was not visualized by hybridization with the two probes. The detecting with PCR-EB was 10^{-2} pg, and with PCR-SBH was 10^{-6} pg, as revealed by the amplification of pAM6 plasmid with C and S primers.

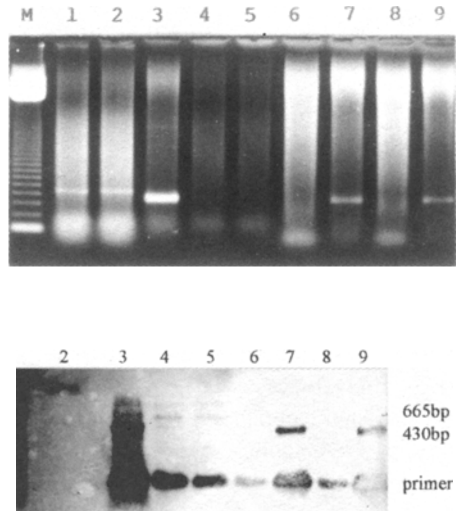


Fig. 2. The result of PCR amplification in liver specimens. M: Molecular weight standard, 123 bp (BRL). 2a: PCR-EB: Internal control (Lane 1, 2); negative control (Lane 6); S product (Lane 3, 7, 9). 2b: PCR-SBH: Hybridized with digoxigenin-labelled full-long HBV DNA probe; Not developed (Lane 1, 2); C product developed (Lane 3–5); S product developed (Lane 3, 7, 9).

The Detection of HBV Markers in Sera and HBV DNA in Liver Tissues

As shown in Table 1, thirteen of the HCC patients were tested for serum HBV markers. Nine of them were HBsAg positive and 4 were HBsAg negative, including 2 cases of simple anti-HBs positivity, 1 of anti-HBs and anti-HBe positivity and 1 without any HBV markers. All of the 13 patients were HBeAg negative.

Table 1. Results of serum HBV DNA marker and HBV DNA in liver of HCC

Number	Sera HBV markers					HBV DNA in cancer tissue			HBV DNA in adjacent tissue		
	HBsAg	HBeAg	AntiHBs	AntiHBe	AntiHBc	C1/C2	S1/S2	X1/X2	C1/C2	S1/S2	X1/X2
1			+	–	+	+	+	–	+	–	+
2	+	–	–	–	–	–	–	–	–	+	–
3	–	–	–	–	–	–	–	–	–	+	+
4	+	–	–	+	+	+	+	–	–	–	+
5	+	–	–	+	+	+	+	–	+	+	+
6	+	–	–	+	+	+	+	+	+	+	–
7	–	–	+	–	–	–	–	+	+	–	+
8	+	–	–	+	+	+	–	+	–	–	+
9	+	–	–	–	–	+	+	+	+	+	+
10	nd	nd	nd	nd	nd	–	–	+	+	+	+
11	+	–	–	–	+	+	–	+	+	–	+
12	–	–	+	+	–	+	+	+	+	–	+
13	+	–	–	+	+	+	–	+	+	+	+
14	–	–	+	–	–	+	–	+	–	–	+
15	nd	nd	nd	nd	nd	+	–	–	+	–	+
16	nd	nd	nd	nd	nd	+	–	–	+	+	+

nd=Not don e

HBV DNA fragments in liver tissues were detected in all of the 16 cases. The positive rates of HBV DNA in the cancerous and the adjacent tissues were 100% (16/16) and 87.5% (14/16) respectively, with the difference of no significance ($P>0.05$). The positive rates in amplification of HBV DNA by S, C and Xgene primer pairs in 32 liver samples were 43.8% (14/32), 71.9% (23/32) and 91.9% (23/32) respectively. The difference between the positive rate in amplification with S primer and that with C primer was significant ($P<0.05$), but the difference between the positive rates in amplification with C and X primers was no significant ($P>0.05$), nor was that between these with S and X primers ($0.10>P>0.05$).

DISCUSSION

Persistent HBV infection has been recognized as the probable leading cause of HCC. Once the clinical diagnosis of HCC is established, HBV DNA replication in the patients is usually not active or has ceased.[1] The present study showed serum HBV markers were positive in 12/14 cases, but the HBeAg, which indicates active replication of the virus, was negative in all of the cases. In amplification of serum HBV DNA by C primer (PCR-EB), only 1 case was found to be HBV DNA positive. These results also support that HBV DNA replication in HCC is not active. Our study also showed HBV DNA fragments were detected in all of the HCC liver specimens, demonstrating that the absence of HBeAg and HBV DNA in sera does not mean that HBV have been completely cleared from the body. It is worthy to note that our positive rate of HBV DNA in HCC liver

was higher than that reported by foreign scholars.[5,6] This result provides evidence on a DNA molecular level for the process that HBV causes HCC. It also suggests that HBV is especially closely correlative with HCC in China, and hence, the prevention and treatment of HBV infection here should be most important measure for grade A prevention of HCC.

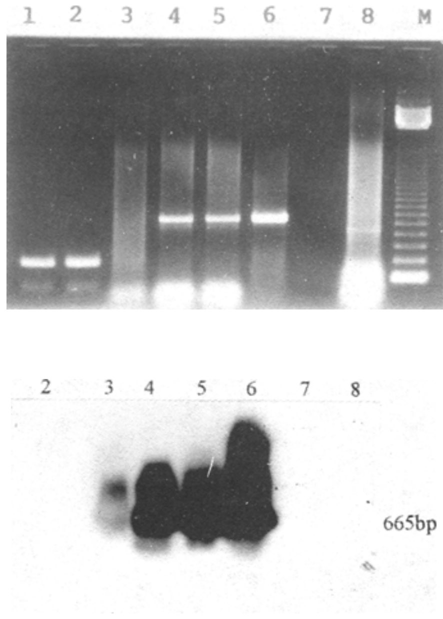


Fig. 3. Specificity of PCR amplified products. M: Molecular weight standard, 123 bp (BRL). 3a: X product (Lane 1, 2); C prodect (Lane 3–6); negative control (Lane 7); internal control (Lane 8). 3b: Hybridization with ^{32}P labelled C gene probe, Lane 3–6 developed.

It was documented that the HBV DNA integrated in HCC liver tissue are incomplete virus genoma.[6] In our study when S, C and X primer pairs were used to amplify HBV DNA separately, only 8 cases were all positive for the 3 primers and 2 all negative for them, giving a consistent rate of 31.3% (10/32). The results testify the above mentioned view. Therefore, in order to make the results more reliable, several pairs of primer should be selected when PCR is use to detect HBV DNA in liver tissue.

DNA integration is an important link in the oncogenesis of HCC due to persistent HBV infection. HBV DNA fragment integration may result in activation of pro-oncogene and loss of antioncogene, and consequently the reinless hepatocellular growth becomes malignant. Our study showed there was no significant difference between the positive rates of HBV DNA in tumorous and nearby non tumorous liver tissues, suggesting that HBV DNA integration occurs prior to the development of hepatocellular malignancy.

Blum[7] reported that HBV DNA in HCC liver have multi-locus mutations, and the stop mutation at procore region 1896 site may lead to the stop of HBV translation and failure of HBeAg expression. The present study showed HBeAg was negative for all of the sera, but the positive rate of C gene fragment detected in liver tissue after amplification with C primer pair was as high as 71.9%, indicating that there might be infection with HBV strains that mutated at procore region.

The action of X gene in the pathogenesis of HCC has been considered very important recently. A close correlation between HBxAg and human HCC has been established and the X protein has been shown to be a trans-activator. Takada[8] considered that the X protein has the characteristics of Kunitz-type serine protease inhibitor and it may bring about trans-activation by activating certain transcriptional factors through proteolytic cleavage alteration. Kim[9] farther demonstrated in transgenic mice the direct involvement of the regulatory HBx gene aione in development of HCC. In our investigation the detection rates of C and X genes were high, these results may imply the posibility that X gene integration may lead to activation of proto-oncogene and induce hepatocellular carcinoma genesis; meanwhile the stop mutation in procore of HBV DNA will arrest the expression HBeAg and allow the cancerous liver cells to evade the host immune surveillance and than proceed to the unchecked selective cloning proliferation developing into a clinical HCC.

REFERENCES

1. Beasley RB. Hpatitis B virus: The major etiology of hepatocellular carcinoma. Cancer 1988; 61 (10): 1942.
2. 胡锡琪, Millward S GH, Robertson D. 肝癌及癌旁肝组织内 HBsAg 和 HBV DNA 定位研究. 肿瘤 1990; 10(4):148.
3. Paterlini P, Brechot C. The detection of hepatitis B virus (HBV) in HBsAg negative individuals with

primary liver cancer. Dig Dis Sci 1991; 36(8):1122.

4. Kwoks, Higuchi R. Avoiding false positives with PCR. Nature 1989; 339(18):237.
5. Lai MY, Chen DS, Chen PJ, et al. Status of hepatitis B virus DNA in hepatocellular carcinoma: a study based on paired tumor and notumor liver tissues. J Med Virol 1988; 25(3):249.
6. Lee HS, Kim ST, Kim CY. Identification of integrated hepatitis B virus DNA sequences in human hepatocellular carcinoma in Korea. J Korean Med Sci 1990; 15(3):145.
7. Blum HE, Liang TJ, Galun E, et al. Persistence of hepatitis B virus DNA after serological recovery from hepatitis B virus infection. Hepatology 1991; 14:56.
8. Takada S, Koike K. X protein of hepatitis B virus resembles a serine protease inhibitor. Jpn J Cancer Res 1990; 81(12):1191.
9. Kim CM, Kazahiko K, Saito I, et al. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. Nature 1991; 351(6324):317.