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Localization of Hepatitis B Virus DNA in Hepatocellular Carcinoma by Polymerase Chain Reaction In Situ Hybridization

The polymerase chain reaction in situ hybridization (PCR-ISH) is a new technique that combines the sensitivity of PCR with the localizing ability of ISH. To investigate the expression pattern of hepatitis B virus (HBV) in the tissue of hepatocellular carcinoma (HCC), we detected HBV-DNA with PCR-ISH in paraffin-embedded tumor and corresponding non-tumor tissues from 11 HCC patients. HBV-DNA was detected in 4 of 11 tumor tissues and in 7 of 10 non-tumor tissues. In tumor tissues, positive signals were scattered in the tissue with occasional clustering, and were found mainly in the cytoplasm of HCC cells rather than in the nucleus. In non-tumor tissues, the number of positive signals was higher than in tumor tissues and they were found in regenerating nodules with differing patterns and intensities. When we compared the detection rate of PCR-ISH with nested PCR among 10 tissue samples, HBV-DNA was detected in 5 tissue samples by PCR-ISH, but the S gene was detected in 10, precore gene in 9 and X gene in 8 by nested PCR. The findings suggest that PCR-ISH is a sensitive technique for localizing HBV in tissue sections and that the low level of HBV replication persists in HCC cells.

Key Words : *Hepatitis B virus; Carcinoma, Hepatocellular; Polymerase chain reaction; In situ hybridization*

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INTRODUCTION

Chronic infection with hepatitis B virus (HBV) continues to be the major etiology of hepatocellular carcinoma (HCC), and geographic distribution of this infection correlates with the geographic distribution of HCC (1-3). However, it is still unknown whether and how HBV contributes directly to the oncogenic transformation of hepatocytes (4). As a free replicating virus, HBV is rarely found in HBV-related HCC and its viral DNA is usually found integrated in majority of HCC (5-8), resulting in low expression of HBV genome and protein. HBsAg was detected in 11.6-32% and HBcAg was detected in 4.2-14.7% of HCC (9, 10). By using reverse transcription polymerase chain reaction, HBs-mRNA was detected in 2, HBc-mRNA in 7, and HBx-mRNA in 40 among 48 HCC patients (11).

Polymerase chain reaction (PCR) in situ hybridization is a new molecular technique that combines the extreme sensitivity of PCR with the cellular localization provided by in situ hybridization, through the amplification of specific gene sequences within intact cells or tissue sections and increasing copy numbers to levels detectable by in situ hybridization or immunohistochemistry (12-16). Though, at first, multiple overlapping primers were used to prevent the diffusion of the amplified product from its site of synthesis (12), Noubo et al. (14) reported PCR in situ hybridization technique, employing a single primer pair by "hot-start" method.

In situ amplification of target sequences and visualization of PCR products can be done either by direct detection of labeled nucleotides, which have been incorporated during PCR (in situ PCR) or by subsequent in situ hybridization (PCR in situ hybridization) (17). Long et al. (18) compared different approaches to in situ PCR in the detection of viral DNA in cell suspensions, cytospins and tissue sections and reported that the specificity of the results was greater with indirect in situ PCR than with direct in situ PCR, where false positive results were frequent.

Now, in addition to the detection of viral DNA, in situ PCR is used for the study of DNA rearrangements, chromosomal translocations and viral RNA in cell suspension, cytocentrifuge preparations or archival tissue sections (17). Thus, we investigated the expression pattern of HBV in tumor and non-tumor liver tissue of

HCCs with indirect in situ PCR.

MATERIALS AND METHODS

Patients

Liver specimens were obtained from 11 patients who underwent hepatic resection due to HCC. All 11 patients were seropositive for HBsAg determined by commercially available assays according to the manufacturer's instructions (Abbott Laboratories, North Chicago, IL).

Tissue preparations for PCR in situ

Five micrometer-thick formalin-fixed paraffin-embedded liver specimens were prepared from two different tumor and non-tumor portions of 11 HBsAg positive patients with HCC. Each block containing tumor and nontumor portions was cut with a new disposable blade to prevent cross-contamination of HBV-DNA. Liver sections from patients with chronic hepatitis C infection seronegative for HBV markers served as controls. The sections were affixed to poly-l-lysine coated glass slides using 1% Elmer's glue (Borden Inc. Columbus, OH) and deparaffinized with fresh xylene twice for 5 minutes each. After rehydration through an alcohol series (70-100%), the tissues were washed with phosphate buffered saline (PBS) for 5 minutes and digested with proteinase K (5-40 μg/ml, 37℃, Sigma, St. Louis, MO) for 5-30 minutes and washed with Tris-EDTA buffer [TE, (pH7.4)].

"Hot-start" PCR in situ

Tissue sections were covered with 20 μl of the DNA amplifying solution. The composition of 20 μl of PCR reaction mixture was as follows: 10×PCR buffer, 4.5 mM $MgCl_2$, 200 μM each dNTP, 2 U Taq polymerase (Promega, USA), internal sense and antisense oligonucleotide primers (20 pmol each) (Table 1). The slide was placed on the premade aluminum boat (with mineral oil on top) and placed on the heating block of a PCR machine (Perkin Elmer Cetus, Emeryville, CA) cushioned with mineral oil. In the "hot-start" PCR modification, Taq DNA polymerase and primers were withheld from the initial amplifying solution. When the temperature was brought to 82℃, the cover slip was lifted, the enzyme and primers were added, the plastic cover was replaced immediately and overlaid with about 1 ml of mineral oil preheated to 82℃ to avoid evaporation. After the initial denaturation step at 95℃ for 5 minutes, 25-30 cycles were accomplished with the following parameters: annealing at 55℃ for 1 minute, extension at 72℃ for 2 minutes, denaturation at 94℃ for 1 minute, and final extension at 55℃ for 10 minutes. After amplification, the mineral oil was removed with xylene and the slides were treated with 100% ethanol.

In situ hybridization

A labeled HBV-DNA probe was prepared, and in situ hybridization was performed as previously described with some modifications (19). A probe representing the complete 3.2-kb genome of HBV was labeled with digoxigenin-dUTP by random priming with DNA labeling and detection kit (Boehringer Mannheim Biochemical Co., Indianapolis, IN). Control DNA probes from pBR 322 were prepared in an identical manner. The sections were prehybridized for 1 hr at room temperature, washed and then overlaid with 30 μl hybridization cocktail (Am-

Table 1. PCR primers

Primers		Nucleotide position	Polarity	Sequence (5'-3')
HBs*	1	41-58	(+)	ATG GAG AGC ACA ACA TCA
	2	721-704	(−)	TCA AAT GTA TAC CCA AAG
	3	329-348	(+)	CAA GGT ATG TTG CCC GTT TG
	4	587-568	(−)	AAA GCC CTA CGA ACC ACT GA
preC/C†	1	1730-1755	(+)	CTG GGA GGA GTT GGG GGA GGA GAT T
	2	2394-2369	(−)	GGC GAG GGA GTT CTT CTT CTA GGG G
	3	1796-1814	(+)	GGT CTG TTC ACC AGC ACC
	4	2009-1985	(−)	AGC TGA GGC GGT GTC GAG GAG ATC
HBx†	1	1376-1389	(+)	ATG GCT GCT CGG GT
	2	1841-1827	(−)	TTA GGC AGA GGT GAA
	3	1400-1423	(+)	CTG GAT CCT GCG CGG GAC GTC CTT
	4	1627-1610	(−)	GTT CAC GGT GGT CTC CAT

*Hepatitis B virus S-gene; †Hepatitis B virus precore/Core-gene; †Hepatitis B virus X-gene.
Nucleotide numbering is taken from Ono et al. (27) or Kim et al. (28).

resco; Solon, Ohio) containing 1 μl of the appropriate digoxigenin-labeled probe. The probe and tissue DNAs were denatured in situ by heating at 95℃ for 4 minutes. After 12 to 16 hrs of incubation at 42℃, the digoxigenin-labeled hybrids were detected with a digoxigenin antibody/alkaline phosphatase conjugate and an enzyme substrate/chromogen (NBT/BCIP) according to the manufacturer's instructions (Boehringer Mannheim). The color reaction was stopped with Tris-EDTA buffer (pH 8.0). All samples were repeated at least twice.

Control study

To confirm the specificity of the PCR in situ hybridization, several controls were carried out in the serial sections. In situ amplification was done without HBV specific primers or Taq DNA polymerase, and Helicobacter pylori or HCV specific primers were used instead of HBV specific primers. During in situ hybridization procedure, control DNA probes from pBR322 were used instead of HBV-DNA specific probe. Liver specimens seronegative for HBV markers were included in every experiment as negative controls. All samples were repeated at least twice.

Nested PCR of hepatitis B virus S, preC/C, X gene from liver tissue

DNA extraction

Out of 11 HBsAg positive patients with HCC, liver DNA extraction for nested PCR was performed in 5 patients as described before by Kam et al. (20). Briefly, frozen liver tissue was mechanically shattered and incubated for protein digestion in lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 150 mM NaCl, 2% SDS) containing proteinase K at 2 mg/ml for 18 hr at 37℃. After incubation, cellular DNA was extracted twice with phenol chloroform/isoamylic alcohol. The DNA was precipitated with ethanol overnight at -20℃, and resuspended in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA.

Nested PCR of hepatitis B virus S, preC/C and X gene

HBV-DNA was amplified by a nested polymerase chain reaction (PCR) assay with sense and antisense oligonucleotide primers located in the S, precore and X gene. The primers are listed in Table 1. PCR was carried out as previously described (21). Five units of Taq polymerase (Promega, USA) were used for each reaction. Each cycle of PCR using the primers was performed in a PCR machine (Perkin Elmer Cetus, Emeryville, CA) with thermal denaturation at 94℃ for 2 minutes, annealing at 55℃ for 2 minutes, extension at 72℃ for 3 minutes and final extension at 55℃ for 7 minutes. Thirty cycles were performed. One-tenth of the PCR products from the first amplification was added to reaction mixture for the second round of PCR using a nested set of primers to increase the sensitivity and specificity of the amplification. For each sample, PCR reactions were performed in duplicate. Negative samples were included in each run to control for contamination. 10 μl aliquots of PCR products were fractionated by electrophoresis on 1.3% agarose gel. DNA was visualized by ultraviolet fluorescence after staining with ethidium bromide.

RESULTS

Control study

The specificity of the PCR in situ hybridization was examined in a number of control experiments. Staining was not detected when HBV specific primers or Taq DNA polymerase were omitted. Use of H. pylori or HCV specific primers during in situ amplification also abrogated HBV staining signals. During in situ hybridization, we could not detect any staining signals with biotin-labeled pBR322 DNA probe instead of HBV probe. HBsAg negative liver specimens did not show hybridization signals.

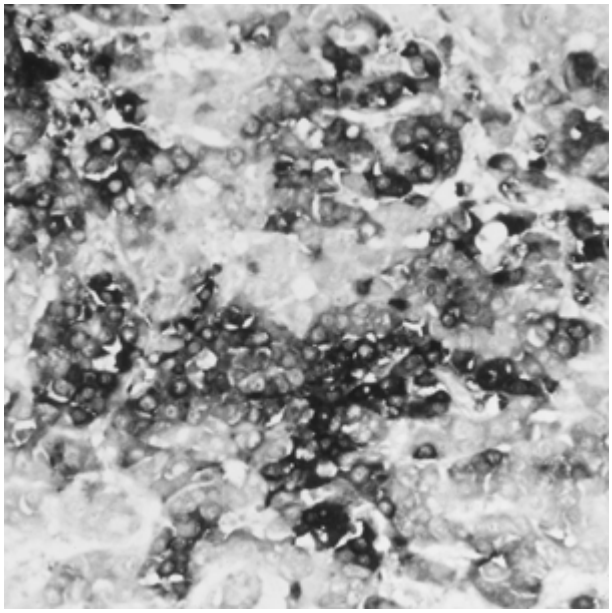

Fig. 1. Cellular localization of HBV-DNA in the tumor tissue of primary hepatocellular carcinoma by in situ PCR: Strong positive signals were mainly found in the cytoplasm, and also found in the nucleus of HCC cells (×200).

Results of in situ PCR in tumor and non-tumor tissue of HCC

HCC cells with positive staining were scattered in tumor tissues with occasional clustering, and the signals were obtained mainly in the cytoplasm of HCC cells and sometimes in the nucleus as well (Fig. 1). The staining patterns were divided into three types: diffuse, cluster or focal (Fig. 2). In the diffuse pattern, HBV-DNA was stained throughout the liver sections. In the cluster pattern, groups of hepatocytes in some parts of the section were stained strongly. In the focal pattern, only a few isolated hepatocytes were stained. Out of 11 tumor tissues, HBV-DNA was detected in 4. Three cases showed cluster pattern and 1 focal pattern. The positive signals were found in the pericapsular area of tumor tissue and in some hepatocytes around fibrous bands. In non- tumor tissue, a number of positive signals were found in regenerating nodules with different patterns and intensities. The number of positive cells was higher than in tumor tissue. Among 10 surrounding cirrhotic liver tissues, HBV-DNA was detected in 7. Two cases showed diffuse pattern, 3 cluster pattern and 2 focal pattern (Table 2).

Comparison of the detection of hepatitis B virus by in situ PCR and conventional PCR with specific HBV primers

The sensitivity of in situ PCR and nested PCR was

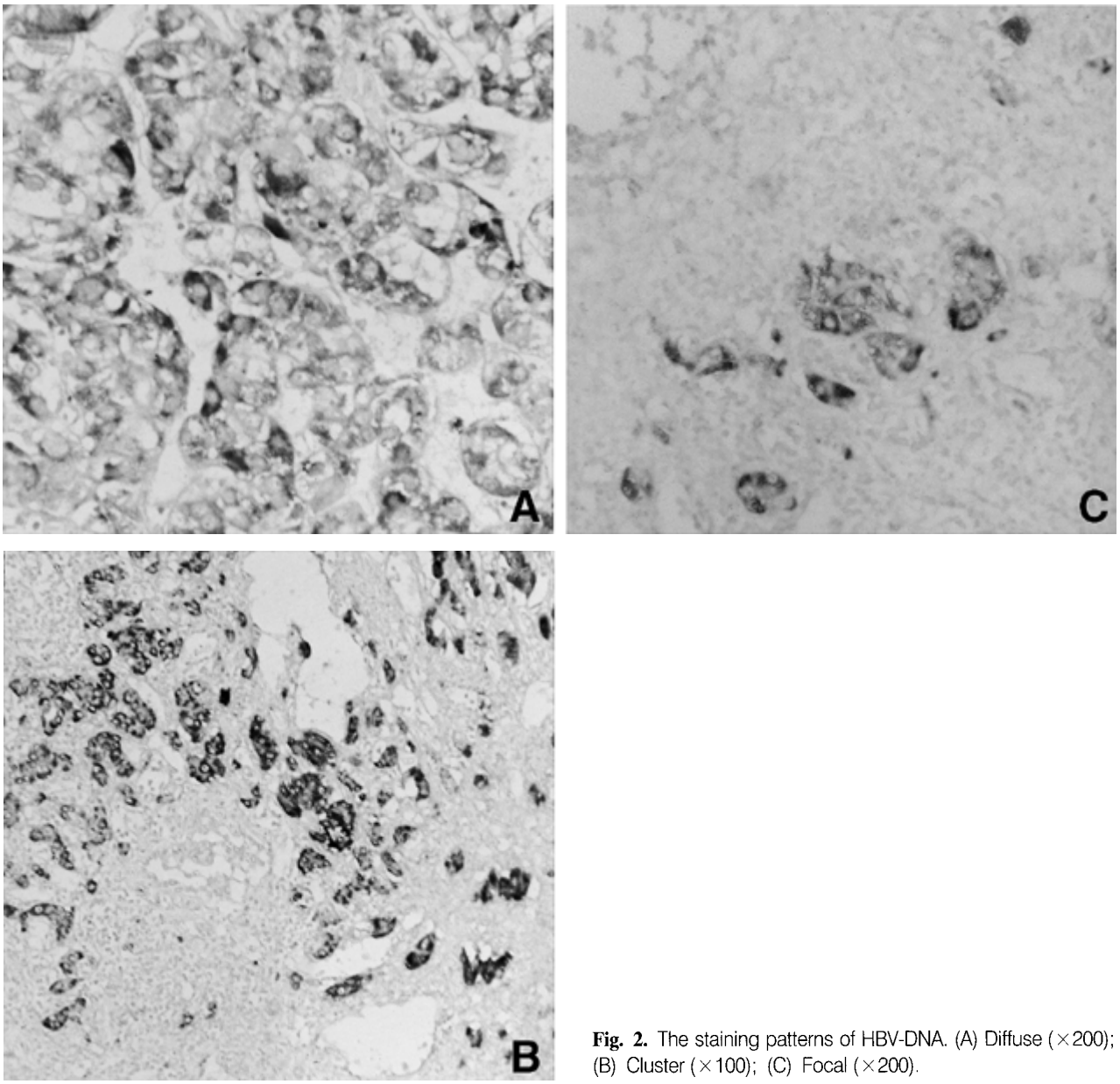

Fig. 2. The staining patterns of HBV-DNA. (A) Diffuse (×200); (B) Cluster (×100); (C) Focal (×200).

Table 2. Distribution of HBV-DNA in patients with HCC* by PCR in situ hybridization

Tissue	No. of specimen	Distribution of HBV-DNA			
		Diffuse	Cluster	Focal	Negative
Tumor	11	0	3	1	7
Non-tumor	10†	2†	3	2	3

*Hepatocellular carcinoma; †All but one specimen were from cirrhotic liver; †One of two specimens was from fatty liver.

Table 3. Detection of HBV-DNA in patients with HCC

Tissue	No. of specimen	PCR in situ hybridization	PCR		
			S	Pre-C/C	X
Tumor	5	2	5	5	4
Non-tumor	5	3	5	4	4

compared in the detection of HBV-DNA in 10 tissuses available for nested PCR among 21 tissues tested for in situ PCR (Table 3). Among 10 tissues (5 tumor and 5 non-tumor tissues), HBV-DNA was detected in 5 by PCR in situ hybridization, but HBV-S gene was detected in 10, HBV-preC/C gene was detected in 9, and HBV-X gene was detected in 8 tissues by nested PCR.

DISCUSSION

Viral DNA in HCC has been shown integrated in the great majority of HCC (5-8). Hsu et al. (10) detected HBcAg in 22% of the cases, suggesting that free, episomal forms of HBV-DNA may occur in HCC of HBsAg carriers. Fowler et al. (22) detected free HBV-DNA in needle biopsies of HCC by using southern blot hybridization. These observations suggest that HCC cells can support HBV replication. In situ detection of HBV-DNA at the single-cell level should be helpful in gaining insights into HBV-induced oncogenesis. But conventional in situ hybridization has limitations in detecting HBV-DNA in HCC because of low level gene expression.

Since amplification and detection of lentiviral DNA inside cells was done by Hasse et al. (12) using in situ PCR with multiple primers, this useful technique which combines the sensitivity of soluble-phase PCR with the localizing ability of in situ hybridization was further improved with the introduction of "hot-start" PCR method using single primer pair but until now, detection of HBV in HCC with this method was not reported in English literature. We detected HBV specific signals in HCC in 4 cases (36.2%) by using in situ PCR. The positive signals were found in the nucleus and cytoplasm of HCC cells. The intensity of signals was stronger in the cytoplasm than in the nucleus of HCC cells. The detection of HBV-specific sequences in the cytoplasm of the HCC cells by using in situ PCR suggests that replicative forms of HBV-DNA may exist in HCC and the HCC cells can support HBV replication. The presence of HBV-specific sequences in the nucleus of the HCC cells may indicate that HBV-DNA is integrated into host genome. But both integrated and free forms of HBV-DNA can exist in the nucleus of the hepatocytes. Further investigations using southern blot hybridization are necessary to evaluate whether or not integration of HBV-DNA in the carcinoma cells is present in these samples.

HBV-DNA was found in 4 tumor tissues with 3 as cluster and 1 as a focal pattern. In the non-tumor part, HBV-DNA was detected more often (7/10) than in the tumor part (4/11) and a number of positive signals were found in regenerating nodules with different patterns and intensities. Inflammatory cell infiltration is not uncommon in HCC (23). Hsu et al. (10) reported that a local host immune response was generated in HBV antigen-positive HCCs, particularly in those expressing HBcAg. It was not uncommon for the antigen-bearing tumor portion to be replaced and compressed by the less-differentiated, antigen-negative tumor. These findings are in accord with the observation that HBV antigen-expressing hepatocytes are preferentially eliminated by host immune response and are gradually replaced by antigen negative hepatocytes (24). This negative selection may lead to the cluster or focal localization of HCC cells supporting HBV replication.

When we compared the HBV-DNA detection rate of PCR in situ hybridization with nested PCR, the detection rate of PCR in situ hybridization was 50% (2 of 5 in tumor and 3 of 5 in non-tumor tissue), while the detection rate of nested PCR was 100% (S), 90% (pre-C/C), and 80% (X), respectively. Even though Nuobo et al. (13, 14, 25) reported that one copy of a target sequence per cell can be detected using PCR in situ hybridization, low amplification and detection efficiency of PCR in situ hybridization was noted when it is performed on tissue sections (26). The reason for this low detection rate may be due to mechanical factors such as poor thermal conduction and uneven convection patterns in the PCR reaction mixture under coverslip, possible adsorption of Taq DNA polymerase to glass, or tissue-related factors including inhibitors of the Taq polymerase and the poor quality of the target DNA (18). In conclusion, even though the sensitivity of PCR in situ hybridization was inferior to nested PCR, this technique allowed for sensitive detection of HBV-DNA with simultaneous localization of HBV in tissue sections with low copy number. We also found that low level HBV replication persisted in HCC cells.

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