Detection of hepatitis B virus DNA at high frequency in liver neoplasias using a PCR technique

A. HALIASSOS[1], D. ARVANITIS[2], J. PARLIARAS[1] and D. A. SPANDIDOS[1,3]

[1] Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48, Vas. Constantinou Avenue, 116 35 Athens; [2] Department of Pathology, Children's Hospital Aghia Sophia, Athens; [3] Medical School, University of Crete, Heraklion, 711 10 Greece.

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Abstract. Hepatocellular carcinoma is the most frequent liver cancer and hepatitis B virus is included among the risk factors for the development of this type of neoplasia. Direct detection of this virus is difficult due to the lack of a simple tissue culture system for growing the virus. Amplification of HBV nucleic acid sequences with the Polymerase Chain Reaction technique leads to the direct detection of the virus, but involves several critical steps and it is prone to false positive results due to inter sample contaminations. We overcame these shortcomings by using a simple boiling method for extracting DNA from histological slides of tissues coupled with double ('nested') PCR amplification. In this study we evaluated the frequency of presence of HBV nucleic acid sequences in samples of neoplastic liver tissues from patients in Greece. We studied 20 DNA samples from hepatocellular carcinomas and we found 11 positive for HBV DNA and 8 DNA samples from hepatoblastomas and we found 3 positive for the viral DNA.

Introduction

Hepatocellular carcinoma (HCC) is the most frequent liver cancer. Hepatitis B virus (HBV) and aflatoxins are risk factors for HCC (1), but the molecular mechanism of human hepatocellular carcinogenesis is largely unknown. Hepatitis B virus infection causes either acute or chronic hepatitis and usually is diagnosed after finding the hepatitis B surface antigen (HBsAg) in serum. Normally infection is associated with the production of large amount of viral antigen that is readily detected in the serum. However, not all of the HBsAg in serum represents intact virions because the majority of this antigen consists of HBsAg particles produced in great excess of complete HBV particles. Direct detection of HBV is difficult due to the lack of a simple tissue culture system for growing the virus.

Correspondence to: Professor D.A. Spandidos, Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48 Vas. Constantinou Avenue, 11635 Athens, Greece

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Molecular techniques including dot-blot hybridization using recombinant radiolabeled DNA have been employed for the detection of HBV DNA in tissue and serum specimens (2,3).

The Polymerase Chain Reaction (PCR) is an *in-vitro* method for the primer directed enzymatic amplification of specific DNA sequences (4). Amplification of HBV nucleic acid sequences with this technique leads to a powerful method for the detection of the virus during acute or chronic HBV infection (5).

Although this method of HBV detection is extremely sensitive it involves several critical steps and is prone to false positive results due to inter sample contaminations. We overcame the shortcomings of the standard PCR method by using a simple boiling method for extracting DNA from histological slides of tissues (6) coupled with double PCR amplification, agarose gel electrophoresis and ethidium bromide (EB) staining for the detection of specific HBV DNA sequences (7).

This sensitive method, connected with the feasibility of polymerase chain reaction amplification of DNA in formalin-fixed and paraffin-embedded tissues, prompted us to evaluate the frequency of HBV nucleic acid sequences present in samples of liver tissues from patients in Greece with hepatocellular carcinomas and hepatoblastomas in order to establish a possible correlation.

Methods

DNA *samples.* DNA from histological slides of tissues from hepatocellular carcinomas and hepatoblastomas, fixed by formol and embedded in paraffin, was extracted by boiling in a lysis mixture after the dissolution of paraffin in chloroform (6).

Slices of paraffin with no embedded tissues, empty vials and normal lymphocytes were processed together with the DNA specimens and used as negative tests for PCR amplification and the presence of HBV nucleic acid sequences.

DNA extracted by the simplified guanidium method (8) from peripheral lymphocytes was used as a negative control.

Oligonucleotide primers. The oligodeoxynucleotides were synthesized by the solid phase triester method in an

Applied Biosystems DNA synthesizer. The primers were designed to amplify a specific region of the HBV genome.

Primer 1763 (5' GCT TGG GGG CAT GGA CAT TGA CCC GTA TAA 3') begins at map position 1763 of the HBV genome (9). Primer 2032R (5' CTG ACT ACT AAT TCC CTG GAT GCT GGG TCT 3') from the complementary or reverse strand begins at map position 2032. Primer 1778 (5' GAC GAA TTC CAT TGA CCC GTA TAA AGA ATT 3') begins at map position 1778 and primer 2017R (5' ATG GGA TCC CTG GAT GCT GGG TCT TCC AAA 3') begins at map position 2017. The position of these primers in the viral genome is depicted in Fig. 1.

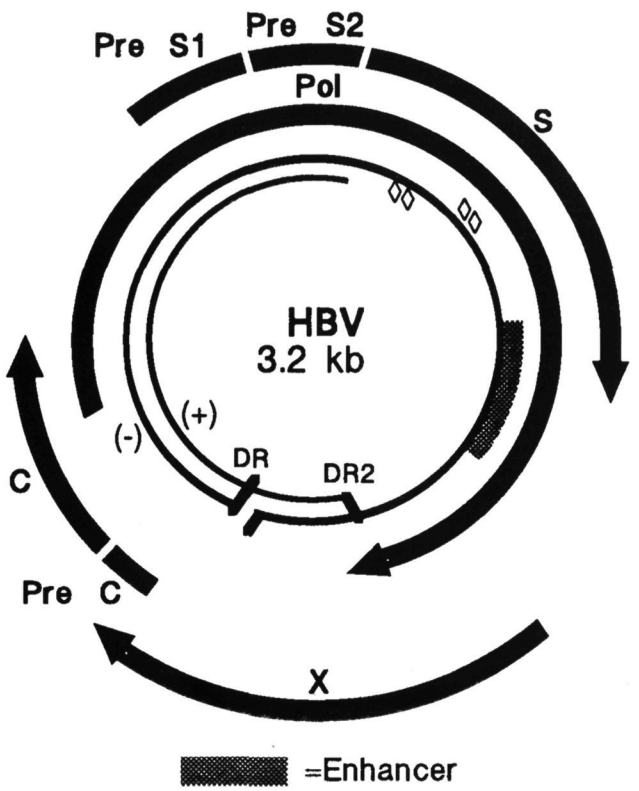

Figure 1. The organization of the HBV genome:
◊ denotes the positions of primers used for the PCR amplifications.
The diagram is adapted from Schröder *et al* (1).

Polymerase chain reaction. In-vitro enzymatic DNA amplification (PCR) was performed on an automated apparatus (DNA thermal Cycler from Perkin Elmer Cetus).

We performed 35 cycles of amplification using primers 1763 and 2032R. Each cycle includes 3 steps: (i) Denaturation of DNA at 94°C for 20 sec. (ii) Annealing of the primers at 42°C for 30 sec. (iii) Enzymatic extension at 72°C for 1 min. For reamplification of the samples a 10 µl aliquot of the PCR was amplified as described above using primers 1778 and 2017R.

The PCR products were analyzed by electrophoresis on LMP agarose gels and visualized by ultraviolet light

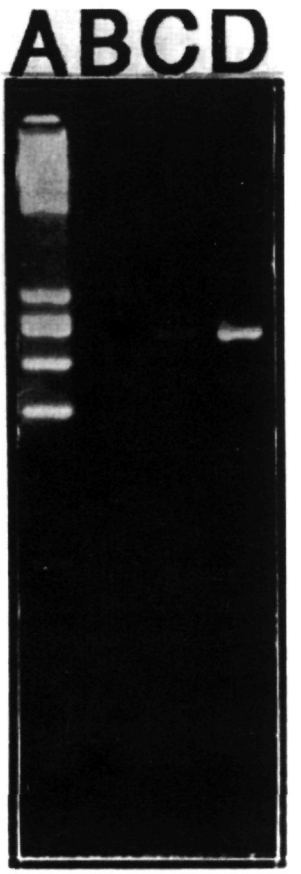

Figure 2. Electrophoresis of PCR products:
DNA from histological slides of tissues fromhepatocellularcarcinomas and hepatoblastomas were amplified by PCR (35 cycles) using the primers 1763 and 2032R. A 10 µl aliquot of the PCR was reamplified using primers 1778 and 2017R. 20 µl of the reamplified samples were electrophoresed on a 3% NuSieve agarose gel.
Lane A: 2µg of the ΦX 174/Hae III molecular weight marker.
Lane B: This sample is negative for HBV DNA (-).
Lane C: This sample is weakly positive for HBV DNA (+).
Lane D: This sample is strongly positive for HBV DNA (++).

fluorescence after staining with EB (Fig.2).

When a visible DNA band corresponding to the expected size (i.e. 270bp) was demonstrated in the product of the first PCR reaction the sample was classed as strongly positive for HBV DNA (++). When a visible DNA band corresponding to the expected size (i.e. 258bp) was found in the product of the reamplification the sample was classed as weakly positive for HBV DNA (+). Samples which did not demonstrate a visible band even after reamplification were classed as negative for HBV DNA (-).

HBsAg staining. Orcein staining for the hepatitis B surface antigen was performed as previously described (10).

Results and Discussion

We used the above technique to detect the presence of HBV nucleic acid sequences in cell populations from formalin-fixed and paraffin-embedded liver tissues from patients with hepatocellular carcinomas and hepatoblastomas.

Table I. Hepatitis B virus DNA in hepatocellular carcinomas.

Patient N°	Age (years)	Sex	Histological type[a]	Degree of differentiation	Liver cirrhosis	HBV DNA[b]	HBsAg staining
1	60	M	Trab.	High	No	+	-
2	55	M	Trab. + Sol.	Poor	Yes	++	++
3	55	M	Tub.	Moderate	Unknown	+	Unknown
4	69	M	Trab. + Tub.	Moderate	Yes	+	-
5		F	Trab.	High	Yes	-	-
6	47	M	Tub.	Moderate	No	-	-
7	75	F	Sol.	Moderate	No	+	-
8	56	M	Trab.	High	Yes	+	-
9	59	M	Trab.	Poor	Yes	-	Unknown
10	52	M	Trab.	High	No	-	-
11	74	M	Trab. + Dif.	Moderate	No	+	-
12	65	F	Trab. + Dif.	Poor	Unknown	-	-
13		M	Trab. + Sol.	Poor	No	-	-
14	93	M	Trab. + Tub.	High	No	-	-
15	65	M	Trab.	Moderate	Yes	+	+
16	75	F	Trab.	High	Yes	-	-
17	76	M	Trab. + Sol.	Poor	Yes	-	-
18	68	M	Trab. + Tub.	Poor	No	+	-
19	63	M	Trab. + Sol.	Moderate	No	+	-
20	9	M	Sol. + Tub.	Moderate	Yes	+	++

a. Trab.=Trabecular, Tub.=Tubular, Sol.=Solid, Dif.=Diffuse
b. The HBV DNA detected by PCR was judged as strongly positive (++) or weakly positive (+) after detection by a single or double PCR amplification respectively.

Table II. Hepatitis B virus DNA in hepatoblastomas.

Patient N°	Age (years)	Histological type	HBV DNA[a]	HBsAg staining
1		Mixed type: fetal epithelial component + mesenchyma with osteoid stroma	+	-
2	28 days	pure fetal type	++	-
3	3	pure fetal type	-	Unknown
4	2	Epithelial (fetal + embryonal component)	-	Unknown
5	3	Mixed type: fetal embryonal epithelial component + osteoid stroma	-	Unknown
6	11 months	Mixed type: embryonal + epithelial components and mesenchyma with osteoid	-	Unknown
7	4 months	pure fetal type	-	-
8		Mixed type: fetal embryonal epithelial component + osteoid	+	-

a. as b in Table I

We studied 20 DNA samples from patients with hepatocellular carcinomas, and we found 11 samples positive for HBV DNA and 8 DNA samples from patients with hepatoblastomas and we found 3 samples positive for this DNA. These results are presented in Tables I and II.

Analyzing further these experimental data we arrive at the following conclusions:

The PCR method for HBV DNA detection is significantly more sensitive compared to the orcein stain because it picks up positive cases which are negative for HBsAg staining.

Seven out of ten positive cases of hepatocellular carcinomas for HBV DNA appear negative for HBsAg with orcein stain. The case No 3 is marked unknown because no tissue was available for orcein stain since the biopsy block was small and the whole tissue was used for DNA extraction. In addition there is no case which is positive for HBsAg and negative for HBV DNA. The HBV DNA is incorporated in to the nuclei of tumor cells since cases 3,7,18 and 19 contained exclusively tumor tissues. It has to be noted that the HBsAg positive cells belonged to the normal liver cells from adjacent areas of the hepatic parenchyma and not to tumor cells which appear negative for HBsAg in all studied cases.

Hepatoblastomas are not associated with HBV infection and yet three out of eight cases gave positive results for HBV DNA. In addition these three positive cases appeared negative for HBsAg although the stained sections contained both tumor and adjacent areas of normal hepatic parenchyma.

These findings can be explained theoretically in two ways: these three cases are false positive cases which cannot be considered because in our study we used negative and positive controls during all the stages of samples manipulation, or these three patients are carriers of hepatitis B infected probably during birth from their mothers and the association of the presence of HBV DNA with hepatoblastomas is just accidental.

The fact that these three patients are negative for HBsAg with orcein stain can be explained. Positivity for HBsAg requires chronicity of infection and as we can see in case two which gave the strongest positivity the infant is only 28 days old. Unfortunately the age of the other two positive patients is not available although hepatoblastomas do not occur in children older than three years.

Definitive correlations between the presence of HBV DNA and these forms of liver neoplasias could be established only after a large study of patients using this sensitive method of detection in DNA samples extracted by a standard protocol.

Finally, this philosophy will permit to correlate clinical information about the cancer and stages of HBV infection with data from histology and molecular biology in these interesting cases of liver carcinogenesis.

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