

Detection of hepatitis B virus DNA and mutations in *K-ras* and *p53* genes in human hepatocellular carcinomas

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Abstract. Hepatitis B virus (HBV) infection is considered as one of the major risk factors in the development of human hepatocellular carcinoma (HCC). Recent studies have also suggested the implication of oncogene and onco-suppressor genes in liver carcinogenesis. We studied 41 cases of HCC for the presence of HBV DNA and point mutations in codon 12 of *K-ras* and codon 249 of *p53*. We used 'nested' PCR for the amplification of HBV because of the expected low incidence of the virus DNA in the samples. PCR was also used for the amplification of *K-ras* and *p53* regions that contain the codons of interest, followed by RFLP analysis for the detection of point mutations. HBV DNA was amplified in 22 cases (53.7%), while 5 cases (12.2%) appeared to carry mutations in codon 12 of *K-ras* and 7 cases (17.1%) had mutations in codon 249 of the *p53* gene. These results further support the correlation between HBV infection and HCC and also indicate an implication of *K-ras* and *p53* genes in hepatocarcinogenesis.

Introduction

Hepatocellular carcinoma is the most frequent primary liver tumor with 5×10^5 to 10^6 new cases a year and hepatitis B virus infection is thought to be one of the major risk factors (1). HBV oncogenic activity is possibly based on the integration of the viral DNA into the cellular genome, disrupting the normal regulation of certain genes, but the precise molecular mechanism remains to be elucidated (2).

K-ras gene is a member of the *ras* family encoding for a 21 Kd protein which appears to play a key role in the signal transduction pathway and is also found activated in human cancers. *K-ras* gene mutations have been found to be infrequent in HCC (3-5).

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p53 gene is thought to act as a tumor-suppressor gene, regulating the entry of the cell into the S phase of the cell cycle. Mutations in *p53* are frequent in many types of human neoplasms. One of the identified 'hot spots' in *p53* is codon 249 presenting G->T transversions in HCC cases, thus giving a possible correlation between *p53* mutations and aflatoxin B1 which is known to cause such transversions in G-C rich DNA regions and is considered as another risk factor for development of HCC in Asian and African countries (6).

The polymerase chain reaction (PCR) is a powerful method for the detection of specific DNA sequences with many clinical applications due to its great sensitivity and specificity and it has been employed successfully before for the detection of HBV (7,8). For the detection of mutations in *K-ras* and *p53* we employed the restriction fragment length polymorphism (RFLP) analysis since it is a rapid, non-radioactive method and it has been used successfully in the past (9,10). Briefly, the detection of a point mutation by RFLP is based on the digestion of the PCR product with a chosen restriction enzyme that recognizes a sequence containing the examined codon. A point mutation in this codon will lead to loss of the restriction site and after the digestion the PCR product will be intact.

Our study on the frequency of HBV DNA and mutations in *K-ras* and *p53* genes in HCC cases in Greece shows that 53.7% carried HBV, 12.2% *K-ras* mutations at codon 12 and 17.1% *p53* mutations at codon 249.

Materials and methods

DNA extraction. Three 10 μ thick sections from paraffin embedded tissues were put in 1.5 ml eppendorf tubes and 300 μ l lysis solution containing 0.1 N NaOH/ 2M NaCl was added. Samples were then boiled for 5 min and microcentrifuged for another 5 min at 4°C. Supernatants were transferred into fresh tubes and DNA was precipitated with the addition of 1 ml of ethanol. DNA was recovered by centrifugation for 15 min at 4°C, washed twice with cold 70% ethanol and resuspended in 20 μ l distilled water.

Oligonucleotide primers and PCR amplification. Two sets of oligonucleotides were used for the amplification of HBV: Primer 1763 (5' GCT TTG GGG CAT GGA CAT TGA CCC GAA TAA 3') and primer 2032 (5' CTG ACT ACT AAT

Table I. Hepatitis B virus DNA and mutations in *K-ras* and p53 genes in hepatocellular carcinomas.

No	Age	Grade	Cirrhosis	HBV	<i>K-ras</i> 2 codon 12 mutation	p53 codon 249 mutation
1	77	II	YES	++	-	+
2	67	ND	ND	++	-	-
3	46	II	YES	+++	-	-
4	50	III	NO	+	-	+
5	ND	ND	ND	++	-	-
6	57	ND	ND	++	+	+
7	ND	II-III	NO	+	-	-
8	78	I-II	NO	+	-	-
9	ND	I	NO	++	+	-
10	ND	II	NO	+	-	-
11	53	III	NO	++	+	-
12	64	II	YES	+	-	-
13	67	II	YES	+	-	-
14	91	I	NO	++	-	-
15	59	III	NO	+	-	-
16	71	II	NO	++	-	-
17	50	III	NO	+	-	-
18	ND	I	NO	+++	-	-
19	60	II-III	NO	+++	-	-
20	72	II	YES	+++	-	-
21	67	II	NO	++	-	-
22	72	I-II	YES	+++	-	-
23	ND	ND	ND	-	-	-
24	70	II	NO	-	-	+
25	56	II	ND	-	-	-
26	50	I-II	NO	-	-	-
27	60	ND	ND	-	-	-
28	75	ND	NO	-	-	-
29	68	III	NO	-	-	-
30	62	II	YES	-	-	-
31	ND	II	NO	-	-	-
32	76	ND	ND	-	-	-
33	ND	ND	ND	-	-	-
34	66	II-III	NO	-	-	-
35	65	I	NO	-	-	+
36	ND	II	NO	-	-	-
37	82	I	NO	-	-	+
38	ND	I	YES	-	-	+
39	ND	ND	ND	-	-	-
40	67	I	NO	-	+	-
41	ND	ND	ND	-	+	-

ND: no data available.

TCC CTG GAT GCT GGG TCT 3') were used for the first amplification and primer 1778 (5' GAC GAA TTC CAT TGA CCC GTA TAA AGA ATT 3') and primer 2017 (5' ATG GGA TCC CTG GAT GCT GGG TCT TCC AAA 3') were used for the nested PCR. The oligonucleotides for *K-ras* codon 12 amplification were K5 (5' ACT GAA TAT AAA CTT GTG GTA GTT GGA CCT 3') and K3 (5' TCA AAG AAT GGT CCT GGA CC 3'). The oligonucleotides for p53 codon 249 were 18004 (5' CTG GAG TCT TCC AGT GTG AT 3') and 18005 (5' GTT GGC TCT GAC TGT ACC AC 3'). 1 µl of the extracted DNA of each sample was amplified in a volume of 50 µl containing 16.6 mM

(NH₄)₂SO₄, 10 mM, β-mercaptoethanol, 6.7 mM MgCl₂, 67 mM Tris-HCl pH 8.8, 1.7 mg BSA, 200 µM of each dNTP, 250 ng of each primer and 2.5 U Taq polymerase (Perkin Elmer Cetus). Amplification was performed in a thermal cycler (Perkin Elmer Cetus) using the following parameters: (i) HBV amplification: 94°C for 35 sec, 42°C for 40 sec, 72°C for 50 sec, 35 cycles (for both first and nested PCR). Reactions for HBV contained 10% DMSO. (ii) *K-ras* amplification: 94°C for 40 sec, 60°C for 45 sec, 72°C for 50 sec, 40 cycles. (iii) p53 amplification: 95°C for 30 sec, 58°C for 30 sec, 72°C for 50 sec, 35 cycles.

RFLP analysis. (i) *K-ras*: 10-20 μ l aliquots of the amplification products were digested for 3 h with 20 U BstNI. (ii) *p53*: 10-20 μ l aliquots of the amplification products were digested overnight with 20 U Hae III. Enzymes were supplied by New England Biolabs and the conditions followed for digestions were those recommended by the supplier. Incubation temperatures were 60°C for BstNI and 37°C for HaeIII. Digestion products were electrophoresed through an 8% native polyacrylamide or a 2% agarose gel. Gels were stained with ethidium bromide and photographed on a UV light transilluminator.

Statistical analysis. Statistical analysis was performed on a Macintosh computer with the help of STATWORK (version 1.2) software.

Results

Forty one cases of HCC, with known serological markers for HBV infection and cirrhosis in some cases, were studied as shown in Table I.

In order to increase the sensitivity of the method for the detection of HBV which characteristically has a low copy number per cell we used 'nested' PCR, performing reamplification of the PCR products with the use of a pair of primers internal to those used in the first PCR. No positive samples were found after the first PCR while 22 positive samples (53.7%) for HBV DNA were found after the nested PCR. These are represented with a 258 bp DNA band on the gel and they were classified as intensely (+++), moderately (++) or weakly (+) positive according to the intensity of the band (Fig. 1).

Five cases carrying mutations in codon 12 of *K-ras* were also detected as judged from the presence of a 143 bp DNA band on the gel (Fig. 2) and 7 cases carrying mutations in codon 249 of *p53* gene as determined from the presence of a 110 bp DNA band on the gel (Fig. 3). Among the examined samples there was an interesting case found positive for HBV and carrying mutations in the both *K-ras* and the *p53* genes. The results are summarized in Table I.

Discussion

The results described in this study indicate that HBV infection is one of the major risk factors for the development of HCC. The statistical analysis showed that the presence of HBV DNA is not correlated ($P < 0.05$) with the tumor grade suggesting that HBV infection can occur as an early event in hepatocarcinogenesis. The presence of point mutations in *K-ras* and *p53* genes further support a possible role of these genes in the development of the disease and provide evidence that liver carcinogenesis is a multistage process.

Some interesting conclusions can be drawn from a comparison of our results to those of previous studies from our group (11,12) in which we studied HBV/tumor markers and the expression of *ras*, *myc* and *erbB-2* oncogenes in 21 out of the 41 tumors presented in this study, using immunohistochemical techniques. The first obvious conclusion is that PCR is much more sensitive in the detection of the virus than immunohistochemical methods. Also, the statistical analysis showed no correlation ($P < 0.05$) between

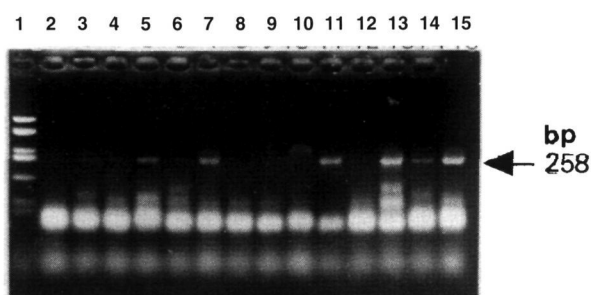


Figure 1. Detection of HBV DNA in hepatocellular carcinomas by nested PCR. Amplification products were electrophoresed through a 2% agarose gel. Lane 1: pUC18/HaeIII molecular marker, lane 2: negative control, lanes 8, 9, 12: negative samples, lanes 3, 4, 6, 10: weakly positive samples, lanes 5, 7, 11, 14: moderately positive samples, lanes 13, 15: intensely positive samples.

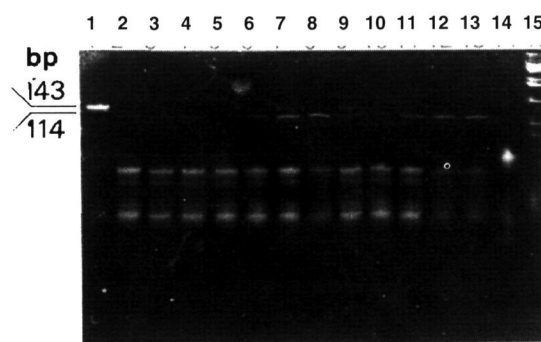


Figure 2. Detection of *K-ras* codon 12 mutations by PCR-RFLP. PCR products after digestion with BstNI were electrophoresed through an 8% acrylamide gel. Lane 1: positive control (SW480 cell line), lanes 5, 9: positive samples, lane 15: pUC18/HaeIII molecular marker.



Figure 3. Detection of *p53* codon 249 mutations by PCR-RFLP. PCR products after digestion with HaeIII were electrophoresed through a 2% agarose gel. Lanes 1, 9: positive controls, lanes 2, 3, 4, 5: positive samples, lanes 6, 7, 8: negative samples, lane 10: pUC18/HaeIII molecular marker.

the presence of HBV DNA and the expression of *ras* and *myc* genes indicating no disturbance of the regulation of these genes by the viral genome in the examined tumors.

Concerning the point mutations in codon 12 *K-ras* gene, we found higher frequency compared to other studies where only 2 out of 43 (5) or no mutations in this codon have been found (4,6).

Furthermore, it is of interest that we have found a 17.1% frequency of p53 codon 249 mutations in HCC in Greece as compared to high mutation frequencies (3,13,14) and low mutation frequencies (15,16) in some African and Asian countries. It has been suggested that aflatoxins could account for this increased p53 mutation frequency (13,14).

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