Relationship Between Mutations on Precore Region of Integrated HBV DNA and p53 Gene Mutation in Hepatocellular Carcinoma

XiuHai Wang, PeiLin Wang

Department of Biology, Qingdao University Medical College, Qingdao, shandong, 266021, P. R.China

Abstract **Objectives** To investigate the relationship between the gene mutation on precore region of integrated HBV, the mutation of p53 gene and the development of Hepatocellular carcinoma (HCC). **Methods** The integrated HBV DNA, gene mutation on precore region of HBV, and p53 gene mutation were detected in 80 specimens of HCC, paratumor cirrhosis, liver cirrhosis, and normal liver tissue by polymerase chain reaction (PCR) and single strand conformation polymorphism (SSCP) technique. **Results** (1) The positive rates of integrated HBV DNA among tumor liver tissues, paratumor cirrhosis, cirrhotic liver tissues, and normal liver tissues were 96.43%, 96.43%, 66.67%, and 6.66%, respectively. The differences of the positive rates among the four groups were significant ($P<0.005$). (2) The gene mutation rates on precore region of HBV DNA in the four groups were 70.37%, 48.15%, 25%, and 0%, respectively. The differences of the gene mutation rates among the four groups were significant($P<0.05$).(3)The mutation rates(57.14%) of p53 gene in HCC are significantly higher than that in liver cirrhosis (16.67%)($P<0.05$). (4) There was a positive correlation between the p53 gene mutation and the mutation on precore region of integrated HBV DNA in different liver tissues. **Conclusions** (1) The integrated HBV DNA and its mutation on precore region is associated with the genesis and development of HCC. (2) The p53 gene mutation has close relationship with heptocarcinogenesis. (3) The HBV DNA integration and its gene mutation on precore region and p53 gene mutation play synergic roles in the cooperation of development of HCC.

Key Words Liver neoplasm; HBV; p53 gene; polymerase chain reaction–single strand conformation polymorphism (PCR–SSCP)

HCC is one of the most common primary malignant tumors of the liver. Some studies have confirmed the strong association between chronic hepatitis B virus (HBV) infection and the occurrence of HCC [1,2]. However, the precise mechanism of HBV infection resulting in HCC is unknown. It is widely accepted that the process of HBV infection resulting in HCC include many gene changes of HBV genome and cellular genome [3]. Previous studies have described that the gene deletions and mutations of p53 gene in HCC was occurred and the cause of mutation of p53 gene was thought to be infection of HBV and aflatoxin–B1 contamination of food [4]. Moreover, reports about whether HBV DNA integration and its gene mutation on precore region can induced p53 gene mutation are rare. This study was designed to investigated the presence of integrated HBV DNA gene mutation and p53 gene mutation in liver tissues at molecular level, to explore the relationship between integrated HBV DNA gene mutation on precore region and p53 gene mutation and to illustrate molecular mechanism of hepatocarcinogenesis.

MATERIALS AND METHODS

Subjects

All samples were obtained between June 1999 to June 2002 from Department of general surgery of Affiliated Hospital of Qingdao University Medical College. Qingdao, China. 28 patients with HCC served as HCC group (20 males and 8 females, aged 36–70 years old, with average age 51, 26 patients of them were HBsAg positive); the tumor tissues and adjacent tissues (2.5 cm apart from tumor tissues) were obtained during surgical resection. 12 patients with liver cirrhosis (8 males and 4 females, aged 23–73 years old, with average age 51 years; 8 patients of them were HBsAg positive, LC

Dr. XiuHai WANG, male, born in 1959–03 in Qingdao, Shandong province, Han nationality, Graduated from Xian Medical University in 1987, Master Degree, associate professor, majoring in molecular biology research of genetic hepatopathy and genetic disease.
Correspondence to: Dr. Xiu–Hai Wang, Department of Biology, Medical College of Qingdao University, 38 Dengzhou Road, Qingdao 266021, Shandong Province, China Tel: +86.532.3812463; Email. Wangxiuhai@qingdaonews.com

group); 12 cirrhosis tissue were obtained from surgery or hepatic centesis. 15 patients with non-hepatic disease (liver tissues obtained from hepatolithectomy) served as controls (6 males and 9 females, aged 35-71 years old, with average age 45 years, 1 patients of them were HBsAg positive, CON group). HCC and LC were diagnosed by serum AFP, B-ultrasound and confirmed by pathological examination. 25 in 28 adjacent tissues have cirrhosis alter, the cirrhosis degree were graded by Jimenez method, among them, 6 cases were slightly cirrhotic, 10 cases were moderate and 9 cases were severe. All liver tissues of non-hepatic disease were normal by biochemistry and pathological examination.

Reagents

PCR reagents were purchased from Shanghai Sangon Biotech.Co. The primers of PCR were synthesized by the Sangon Biotech. Co. The nucleotide sequences of primers of HBV DNA in precore region were as following: sense, 5´-CAAGCCTCCAAGCTGTGCCTTGGG-3´ (nucleotides 1839-1863), antisense, 5´-GCCTCCCGATACAGAGCAGAGGCG-3´ (nucleotides 1974-1997), the anticipated fragment of amplification was 158 bp. The nucleotide sequences of primers of p53 gene (exon 7) were as following: sense, 5´-TCTCCTAGGTTGGCTCTGACTG-3´; antisense, 5´-GGTGAACGCTCCTGACCTGGA-3´, the anticipated fragment of amplification was 133 bp.

Methods

DNA was extracted from the liver tissues using a modified "salting out" procedure.

PCR for precore region of HBV: The PCR reaction system in each tube consisted of 10×amplification buffer solution 5μl, 4×dNTPs (10 mmol/L for each) 3μl, $MgCl_2$(25mmol/L) 3μl, primer (20 pmol/L for each) 1.5μl, DNA 100ng, Taq DNA polymerase 2U, and added DDW up to 50μl, to mix them together. PCR reactions were performed in the Gene Amp Machine (Perkin Elmer. USA). PCR conditions were: 95℃ for 5 min of initial denaturation, 30 cycles of 94℃ for 45s, 65℃ for 45s, 72℃ for 1.5 min, and then 72℃ for 5 min of final elongation. To exclude the contamination of serum HBV DNA, the DNA of tissues was washed three time with 10 mL of phosphate buffer saline, the washing solution was identified negative of HBV DNA.

PCR for p53 gene The total 25μL PCR reaction system in each tube consisted of 10×amplification buffer solution 5μl, 4×dNTPs (10 mmol/L for each) 1μl, $MgCl_2$ (25mmol/L) 1.5μl, primer (20 pmol/L for each) 0.8μl, DNA 100 ng, Taq DNA polymerase 1U, and added ddH_2O up to 50μl, to mix them together. PCR reactions were performed in the Gene Amp Machine (Perkin Elmer. USA). PCR conditions were: 95℃ for 5 min of initial denaturation, 35 cycles of 95℃ for 60s, 55℃ for 60s, 72℃ for 60s, and then 72℃ for 5 min of final elongation.

Above two amplified products were detected by electrophoresis. The positive products were performed by PCR-based single strand conformation polymorphism (PCR-SSCP) analysis. Each 5μL of amplified products was mixed with an equal volume of stop solution (95% formamide, 20 mmol/L EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol, 10mmol/L NaOH). boiled for 5 min, immediately loaded on the wells of a non-denaturing SSCP gel, 8% polyacrylamide gel (49:1) in 1×Tris borate-EDTA (TBE) buffer, and separated for 3 h in room temperature at a constant 300 v. After electrophoresis, the gel was silver stained. The gene mutation was identified by band mobility shifts of experimental samples as compared with control sample.

Statistical analysis

The comparison between groups was performed using χ^2 test and corelation analysis was performed using degree correlation test.

RESULTS

The positive rates of integrated HBV DNA and gene mutation of precore region in different liver tissues (table 1)

The integrated HBV DNA was detected in all 4 groups liver tissues. The significant difference was found among control groups and other three groups (χ^2=19.52, P<0.005). The positive rate of tumor liver tissues and tumor adjacent liver tissues were highest, the difference were no significance between the two groups (χ^2=0.41, P>0.05). The significant difference was found between HCC and cirrhosis liver tissues (χ^2=4.35, P<0.05), Between HCC and normal liver tissues (χ^2=30.98, P<0.005).

The gene mutation of precore region of integrated HBV DNA was detected in HCC liver tis-

Tab.1 Detection Results of Integrated HBV and Gene Mutation of Precore Region in Different Liver Tissues

Group	n	HBV (−)	DNA (+)	Positive rate (%)	Gene mutation (−)	(+)	Positive rate (%)
Hepatocellar cancer	28	1	27	96.43	8	19	70.37
Paratumor cirrhosis	25	1	24	96.00[a]	13	11	45.83[b]
Liver cirrhosis	12	4	8	66.67[b]	6	2	25[c]
Normal liver tissue	15	14	1	6.66[c]	1	0	0

χ^2–test: [a]$P>0.05$, [b]$P<0.05$, [c]$P<0.01$

Table 2. Detection Results of p53 Gene Mutation in Different Liver Tissues

Group	Number of cases	Normal cases	Mutation Cases	Mutation rate (%)
Hepatocellar cancer	28	12	16	57.14
Paratumor cirrhosis	25	14	11	44.00[a]
Liver cirrhosis	12	10	2	16.67[b]
Normal liver tissue	15	15	0	0.00[c]

χ^2–test: [a]$P>0.05$, [b]$P<0.05$, [c]$P<0.001$

sues, in Paratumor cirrhosis liver tissues and in liver tissues of cirrhosis; but not found in liver tissues of non–liver disease. The positive rates of HBV DNA gene mutation of precore region of integrated HBV DNA in the three groups (HCC, cirrhosis, control) were quite different, significant difference was shown among of them (χ^2=7.81, $P<0.005$). The positive rate was highest in HCC liver tissues, the difference was no significant compared with that of the Paratumor cirrhosis liver tissues (χ^2=2.23, $P>0.05$), but the difference was significant compared with that of the cirrhosis liver tissues (χ^2=3.85, $P<0.05$).

Detection results of PCR–SSCP for p53 gene mutation in different liver tissues

The amplified products of p53 (exon 7) were analyzed by PCR–SSCP. The results were shown in table 2. The difference of the mutation rates of p53 (exon 7) between tumor liver tissues and paratumor cirrhosis was no significant (χ^2=0.29, $P>0.05$), but the difference of that between tumor liver tissues and cirrhosis liver tissues was significant (χ^2=5.5, $P<0.05$).

The relationship between p53 gene mutation and gene mutation of pre–core region of integrated HBV DNA in different liver tissues

There was a positive correlation between p53 gene mutation and gene mutation of precore region of integrated HBV DNA in different liver tissues (rs=1.00, P=0.0) by Statistical analysis using degree correlation test, The results showed the three rates of p53 gene mutation, integrated HBV DNA and gene mutation of pre–core region were much higher and have consistent expression in liver tissues of HCC.

DISCUSSION

Hepatocellular carcinoma is multifactorial in aetiology and its pathogenesis is complex. The chronic infection with HBV is a recognized major risk factor for the development of HCC. Some studies of epidemiological and molecular pathobiology have confirmed the strong association between chronic hepatitis B virus infection and the occurrence of HCC[5].

The HBV DNA not only infect tissue cells with free status but also integrated cellular genome [6]. Our results showed that the infection rates of integrated HBV DNA in HCC liver tissues and tumor adjacent liver tissues were much higher than in cirrhosis and normal liver tissues. The results suggested that the integration of HBV DNA was association with HCC.

The clinical investigations showed that majority of chronic hepatitis B can result in liver cirrhosis, while cirrhosis at last leads to the occurrence of HCC[1]. The possible cause of HBV infection resulting in HCC is considered that the HBV DNA may have developed genetic variation during a long–term infection. The variant virus might escaped immunologic elimination through cytotoxic T–lymphocytes[7、8]. Previous studies have demonstrated that development of hepatic diseases is closely association with the gene mutation of precore region of HBV DNA [9]. Some of the gene mutation types and hot

sites of precore region of HBV DHA have been confirmed in the worldwide[10].

The termination codon mutation in precore region of serum HBV in HCC has been detected, but no difference were found in the mutation rate as compared with control group[11]. Moreover, the reports assessing the relationship between gene mutation of precore region of integrated HBV DNA and HCC development are rare. In our study, the results showed that the gene mutation of precore region of integrated HBV DNA in liver tissues of HCC was obviously higher than other three groups ($P< 0.05$). The results presented here demonstrated that the gene mutation of precore region of integrated HBV DNA have relation not only with development of other hepatic diseases but also and even more closely relation with development and occurrence of HCC.

The molecular mechanism for HBV DNA resulting in HCC has not yet been understood fully, It is widely accepted that some gene abnormal expression of HBV DNA and cellular genome after integration of HBV DNA[12]. In addition the integrated HBV DNA also may damage normal cellular genome and induce activation of proto-oncogene, inactivation or deletion of anti-oncogene, Thus leading to the occurrence of HCC. Recent studies have suggested a important role for the inactivation of tumor suppressor gene and activation of oncogene in the development of a subset of HCCs[13]. P53 gene mutation is often occurred in all tumor tissues. The mutation frequently occurs at exon 7 among the 11 exon of p53 gene. There was a specific site (AGG→AGT transversion), at codon 249 of exon 7 of the p53 gene, its mutation rates was 50% in the HCCs[4, 14].

The p53 gene mutation in liver tissue were detected by PCR-SSCP technique in this study. The results showed that the mutation rates in tumor liver tissues was 57.14%(16/28), the rates was higher than the rates of paratumor cirrhosis (44.00%) and cirrhotic liver tissues (16.67%). These results are in correspondence with other reports and the results indicated that development of HCC is closely associated with the gene mutation of p53.

The relationship between the gene mutation of precore region of HBV DNA and the gene mutation of p53 in liver tissues of HCC were investigated by this study, and indicating that p53 gene mutation, integrated HBV DNA and its gene mutation of precore region in tumor liver tissues have consistent expression. There was a positive correlation between them. This results suggested that gene mutation of p53 may have relating to integrated HBV DNA with mutation of precore region and also suggested that both of which play synergic roles in the development of HCC. However, further investigations of the precise mechanism of cooperation role should be performed.

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