

Detection of HBV, PCNA and GST-p in hepatocellular carcinoma and chronic liver diseases

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Abstract

AIM: To investigate the change of HBV DNA, PCNA and GST- π in chronic liver disease and hepatocellular carcinoma (HCC).

METHODS: Hepatitis B surface antigen (HBsAg), proliferating cell nuclear antigen (PCNA) and glutathione S-transferases (GST- π) were detected by immunohistochemical staining and HBV DNA was detected by *in situ* hybridization (ISH) in formalin-fixed and paraffin-embedded sections with a total of 111 specimens of chronic hepatitis, liver cirrhosis, paratumorous tissue, HCC and normal liver tissue.

RESULTS: The positive rates of HBsAg and HBVDNA were 62.5 % (15/24) and 75.0 % (12/16) in chronic hepatitis, 64.0 % (16/25) and 83.3 % (15/18) in liver cirrhosis, 72.7 % (16/22) and 85.7 % (12/14) in the paratumorous tissue and 45.0 % (14/31) and 64.3 % (9/14) in HCC. The positive HBVDNA granules in chronic hepatitis, liver cirrhosis and the paratumorous tissue were more intense than that in HCC. The positive rates of PCNA and GST- π were 34.8 % (8/23) and 25.0 % (4/16) in chronic hepatitis, 73.7 % (14/19) and 17.6 % (3/17) in liver cirrhosis, 86.7 % (13/15) and 53.3 % (8/15) in the paratumorous tissue, 100 % (15/15) and 60.0 % (9/15) in HCC, respectively, and the positive rate of GST- π in the paratumorous tissue was significantly higher than that in the liver cirrhosis without tumor ($P < 0.05$), but same as that in HCC ($P > 0.05$).

CONCLUSION: The HBV infection may increase expression of PCNA and GST- π . The paratumorous cirrhosis may be a sequential lesion of precancerous cirrhosis around HCC.

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INTRODUCTION

Proliferating cell nuclear antigen (PCNA) is an auxiliary protein of DNA polymerase and is thought to play an important role in the elongation or replication of the DNA chain. It accumulated in the nucleus during the G-1 and S stages of the cell cycle and the percentage of PCNA-positive cell is correlated with the proliferative activity and the prognosis of various malignant tumors^[1-5]. Glutathione S-transferases (GST- π) is closely related with cancer and is increased in blood and tissues of cancer patients, it is now recognized as a tumor marker^[6, 7]. We have detected hepatitis B surface antigen (HBsAg), PCNA and GST- π by immunohistochemical staining and hepatitis B virus DNA (HBV DNA) by *in situ* hybridization (ISH) in patients with chronic hepatitis, liver cirrhosis, the paratumorous tissue, hepatocellular carcinoma (HCC) and normal liver tissue, to see whether there are any changes of HBV, PCNA and GST- π in the above diseases.

MATERIALS AND METHODS

Materials

Specimens obtained from surgical resection, autopsy and needle aspiration biopsy of livers from 1965 to 2001 were fixed in 10 % formalin, embedded in paraffin sections, and stained by routine HE. They were divided into 5 groups: Normal liver tissues used as controls ($n=9$); Chronic hepatitis ($n=24$); liver cirrhosis ($n=25$); The paratumorous tissue ($n=22$); HCC ($n=31$). All specimens were examined by two pathologists. The diagnosis of hepatitis was according to the standard of Xi'an Conference in 2000^[8].

Immunohistochemical staining

Immunohistochemistry S-P method was used to detect HBsAg, PCNA and GST- π . Mouse monoclonal antibody to human HBsAg (ZMHB5), PCNA (PC10), GST- π (353-10) and Immunostaining S-P Kit were purchased from Fuzhou Maxim Biotechnical Company. The main steps were as follows: (1) The tissues were treated with endogenous peroxidase blocking solution at room temperature for 10 minutes and then incubated in normal nonimmune serum at room temperature for 10 minutes. (2) The mouse anti HBsAg, PCNA or GST- π antibody were added to adjacent tissue sections respectively and incubated overnight at 4 °C. (3) Biotin-conjugated second antibody was added to the sections and incubated at room temperature for 10 minutes. (4) S-P complex was added at room temperature for 10 minutes and then DAB was used for the color reaction. The tissue sections were washed with PBS (0.01M, pH 7.4) between each step. Positive and negative controls were simultaneously used to ensure specificity and reliability of the staining process. A positive section was taken as positive control. In negative control, PBS was used to replace the first antibody. The positive result showed brown coloration in the cytoplasm or /and the nucleus and was graded as follows: $<10\%$ -, 10-30 % +, 31-50 % ++, $>50\%$ +++^[9].

In situ hybridization (ISH)

In situ hybridization was used for detection of HBV DNA.

The HBV DNA probed with biotin-labeled and ISH-kit were purchased from Fuzhou Maxim Biotechnical Company. The main steps were as follows: (1) Baked the slides at 60-80 °C for 1 hour till overnight. (2) Deparaffinized by xylene and graded alcohols. (3) Dried at 37 °C for 5 min. (4) Added proteinase K at 37 °C for 10-15 min. (5) Enhancer wash buffer for 5 min. (6) Dehydrated the slides by graded alcohols. (7) Dried at 37 °C for 5 min. (8) Added biotin-labeled probe with coverslip. (9) Denatured at 95 °C for 8-10 min. (10) Hybridized at 37 °C for 1-2 hours in humidity chamber. (11) Soaked off coverslips in PBS. (12) Hybridization wash at 37 °C for 10 min. (13) Protein block at 37 °C for 20 min. (14) Conjugated at 37 °C for 20 min. (15) PBS rinsed enhancer wash buffer for 5 min. (16) The substrate (NBT/BCIP) showed coloration at room temperature for 10-40 min, or till the coloration developed became complete. (17) Distilled water washed for 2-3 times. (18) The slides were counterstained using nuclear fast red. The positive and negative controls were concomitantly used to ensure the specificity and reliability of the staining with a known HBVDNA positive tissue section, the normal liver tissues and hybridization liquids without probe were served as controls. The positive result showed blue coloration in the cytoplasm or/and in the nucleus.

RESULTS

Detection of HBsAg and HBV DNA

HBsAg and HBVDNA were widely expressed in chronic hepatitis, liver cirrhosis, paratumorous tissue cirrhosis and HCC (Table 1). The positive rates of HBsAg and HBVDNA were highest in the paratumorous tissue.

Table 1 Detection of hepatitis B virus, PCNA and GST- π in hepatocellular carcinoma and chronic liver diseases

Group	HBsAg ^a	HBVDNA ^b	PCNA ^c	GST- π ^d
1. Normal liver tissue	0/9 (0)	0/9 (0)	0/5 (0)	0/5 (0)
2. Chronic hepatitis	15/24 (62.5)	12/16 (75.0)	8/23 (34.8)	4/16 (25.0)
3. Liver cirrhosis	16/25 (64.0)	15/18 (83.3)	14/19 (73.7)	3/17 (17.6)
4. Paratumorous tissue	16/22 (72.7)	12/14 (85.7)	13/15 (86.7)	8/15 (53.3)
5. HCC	14/31 (45.2)	9/14 (64.3)	15/15 (100)	9/15 (60.0)

HCC: hepatocellular carcinoma; HBsAg: Hepatitis B surface antigen; HBVDNA: hepatitis B virus DNA; PCNA: proliferating cell nuclear antigen; GST- π : glutathione S-transferases.

^a $P < 0.05$, 5 vs 4; ^b $P < 0.01$, 1 vs 4; ^c $P < 0.01$, 1 vs 4; ^d $P < 0.05$, 3 vs 4, $P < 0.01$, 1 vs 4.

The expression of HBsAg was seen in the cytoplasm (Figure 1). HBVDNA was detected in the cytoplasm and in the nucleus. HBVDNA positive granules in chronic hepatitis, liver cirrhosis and the paratumorous tissue were more intense than that in HCC. HBVDNA positive granules in HCC was mainly expressed in the nucleus but the signals were much weaker (Figure 2).

Detection of PCNA and GST- π

PCNA and GST- π were widely expressed in chronic hepatitis, liver cirrhosis, paratumorous tissue and HCC, the positive rates of PCNA and GST- π increased evidently in the paratumorous tissue and HCC (Figure 3-4). The positive rate of GST- π in paratumorous tissue (53.3 %) was significantly higher than that in cirrhosis without tumor (17.6 %) ($P < 0.05$, $\chi^2 = 6.58$).

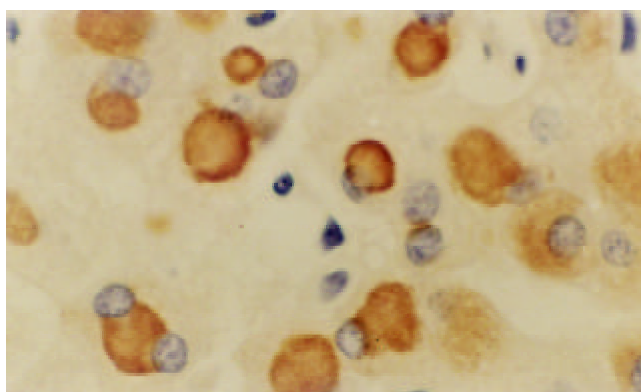


Figure 1 HBsAg was expressed at cytoplasm in chronic hepatitis. $\times 400$.

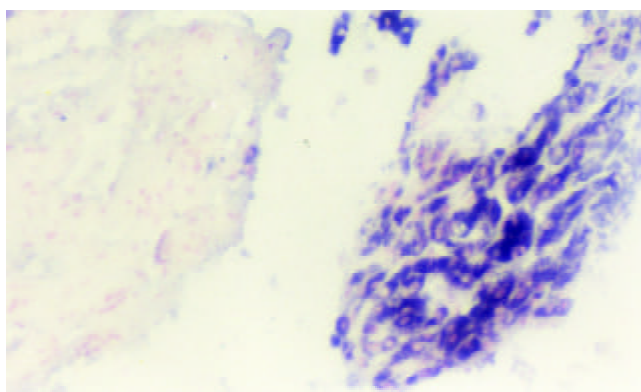


Figure 2 HBV DNA was rich in paratumorous tissue (right) and less in HCC (left). $\times 100$.

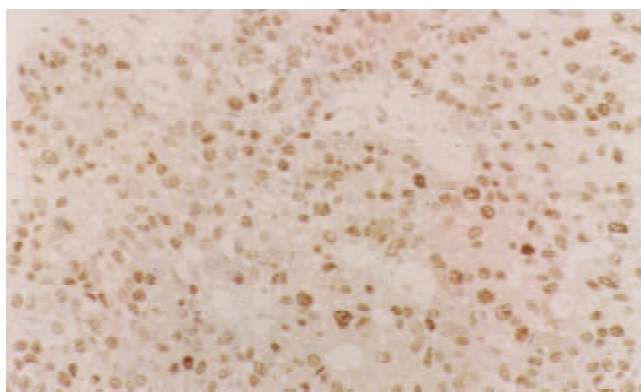


Figure 3 PCNA was expressed in nuclei of HCC. $\times 100$.

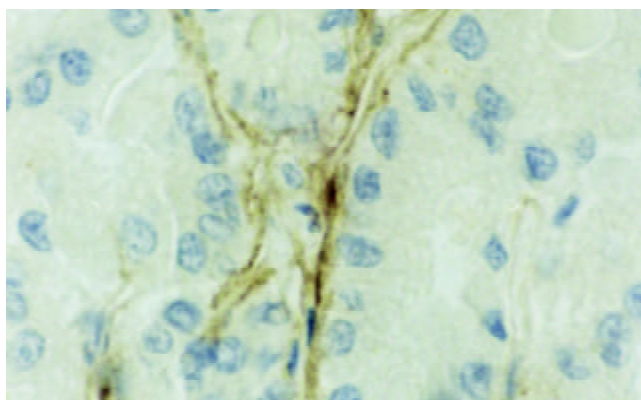


Figure 4 GST- π was expressed on cell membranes of HCC. $\times 400$.

DISCUSSION

HCC is one of the most common malignant tumors in China. In recent decades, the incidence of HCC has been found to be increasing and HCC came ranks the second in cancer mortality since 1990s^[10]. Most of patients with HCC were associated with HBV^[11-15]. The relative risk of HBV carrier developing HCC approaches 200:1, which is the highest relative risks known for HCC^[16]. we have found that there are higher positive rates of HBsAg and HBV DNA in chronic hepatitis, liver cirrhosis, paratumorous tissue and HCC, but the highest positive rate in the paratumorous tissue, supports the view of HBV as the main cause of HCC in China. The positive rate of HBV DNA in HCC was 64.3 %, mainly expressed in nuclei, HBV DNA positive granules were less and weakly stained, perhaps because most of the HBV DNA had been integrated into the genome of HCC tissue. The positive rates of HBV DNA were 85.7 % in the paratumorous tissue, which was mainly expressed in the cytoplasm, HBV DNA positive granules were more intense, because HBV DNA was mainly in the free form and in an active replicative state. Our results were similar to those of most researchers^[17]. The carcinogenesis of HCC was related to integration of HBV DNA into the genome with rearrangement of the chromosome. The integration of viral gene often occurred in the earlier stage of HCC. The integration of HBV might lead to the structural abnormality of the chromosome, which enhanced the transcription of oncogene and lost the its suppressor function, resulting in malignant transformation of hepatic cells by reverse activation of the X gene of HBV^[18]. HBV DNA was integrated into the genome randomly. The integrated HVB DNA was not complete, there were some defect in the virus genome which caused the weaking of HBV DNA hybridization signal in the nuclei of HCC^[19]. The integrated HVB DNA in the hepatic cells could bring about abnormal expression of the gene and abnormal synthesis of protein so that the growth of HCC and the differentiation and regulation of the hepatic cells became out of control. PCNA is a better marker for assessment of cellular proliferative activity, the poorer the differentiation of the cancer, the stronger the proliferative activity and the higher positive rate of PCNA^[20-21]. When the carcinogenesis initiated, the shape and structure of the cells did not have much changes, but the function and the metabolism enzymes had already been abnormal. The expression of GST- π might increase abnormally in the course of the carcinogenesis of many tumors. This change occurred much more earlier than that of the morphology^[22,23]. Our study demonstrated that PCNA and GST- π were all expressed in chronic hepatitis (34.8 % and 25.0 %), liver cirrhosis (73.7 % and 17.6 %), the paratumorous tissue (86.7 % and 53.3 %) and HCC (100 % and 60.0 %). But the positive rates of PCNA and GST- π increased significantly in the paratumorous tissue and in the HCC. These results demonstrated that repeated degeneration, necrosis and hyperplasia occurred in the chronic HBV hepatitis, because of the increase of cell proliferation and the integration of viral genome, caused disturbed proteins synthesis abnormality in metabolism enzymes. It might be a gradual developmental process from quantitation to qualitation change. The paratumorous tissue differs essentially from cirrhosis without tumor, it is rather a precancerous lesion^[24-28], which already has some characteristics of fetal liver or HCC with earlier expression AFP and AFPmRNA^[29,30], also with increased expression of PCNA and GST- π ^[31-39].

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