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Differential Expression of HBx Protein in the Liver from Patients with Chronic Hepatitis, Cirrhosis and Hepatocellular Carcinoma

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Background/aims: Hepatocellular carcinoma is frequently associated with hepatitis B virus infection and HBx protein is suspected to be the main cause of hepatitis B virus related hepatocellular carcinoma. But little is known about the time of the HBx protein expression during hepatocarcinogenesis. These issues, therefore, are addressed in this article.

Methods: We examined HBx protein expression in the chronic active hepatitis, cirrhosis and hepatocellular carcinoma by the immunohistochemical method.

Results: Among the patients with chronic active hepatitis, four (80%) were HBx protein positive in hepatocytes. In contrast, only one (6%) out of 16 liver specimens with hepatocellular carcinoma showed positivity for HBx protein, whereas four (25%) of surrounding cirrhotic tissues showed HBx protein positivity. Thus, differential expression of HBx protein was observed in the livers during hepatocarcinogenesis. HBx protein was expressed mostly in the cytoplasm of hepatocytes. We also examined p53 protein and $p21^{WAF1}$ protein expression in some of the hepatocellular carcinoma. P53 protein was found only in the nucleus of hepatocellular carcinoma cells (38%), and $p21^{WAF1}$ protein was also detected in some hepatocellular carcinoma tissues irrespective of HBx protein and/or p53 protein expression.

Conclusions: These findings suggest that the HBx protein is expressed in the early stage of hepatitis B virus related hepatocarcinogenesis.

Key Words: Hepatitis B virus, HBx protein, Chronic active hepatitis, Cirrhosis, Hepatocellular carcinoma

INTRODUCTION

Hepatocellular carcinoma is known to be one of the leading causes of death in cancer in Korea. More than eighty percents of patients with hepatocellular carcinoma in Korea are associated with hepatitis B virus infection[1]. The product of the hepatitis B virus X gene, HBx protein, has been known as a promiscuous transactivator for various viral and cellular genes[2,3] and introduction of HBx gene into NIH3T3 fibroblast cells has shown to cause cellular transformation[4,5]. The transforming activity of HBx gene is further confirmed by the HBx transgenic mice, which are known to develop hepatocellular carcinoma[6]. Thus, HBx gene is suspected as a main cause for the development of hepatitis B virus related hepatocellular carcinoma. One of the mechanisms by which HBx induces HCC is its ability to bind p53 tumor suppressor protein[7] and, thereby, repress p53-mediated apoptosis[8]. Therefore, it is very likely that expression of HBx protein and its persistency in hepatocytes play an important role in hepatocarcinogenesis.

The expression rate of HBx protein in the chronic active hepatitis, cirrhosis and hepatocellular carcinoma remains controversial. Wang et al. (1991)[9,10] reported that among patients with chronic hepatitis, cirrhosis and hepatocellular carcinoma, most (84~95%) were positive for HBx protein in the hepa-

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tocytes. HBx protein was far more frequently observed than HBsAg (19%) and HBcAg (11%) in patients with hepatocellular carcinoma. They also observed that there was an association between HBx protein and p53 protein in the liver tissues with hepatocellular carcinoma[11]. On the other hand, other researchers have reported the presence of 12～30% HBx protein-positive cells in patients with chronic hepatitis and 40～60% in those with cirrhosis[12,13]. Therefore, it remains unclear when the HBx protein is expressed during hepatocarcinogenesis.

In the present preliminary study, we examined the expression of HBx protein in the liver of patients with chronic hepatitis, cirrhosis and hepatocellular carcinoma in order to investigate the time of HBx protein expression during hepatocarcinogenesis. We also studied the expression of p53 protein in these hepatocytes in order to examine the relationship between the two proteins.

MATERIALS AND METHODS

Tissue collection

Surgically resected liver specimens were obtained from 50 patients with hepatocellular carcinoma between 1994 and 1996 in the Department of Pathology, Ajou University Hospital. In order to compare the expression of HBx protein, sixteen specimens were selected on the basis of two criteria: (1) HBsAg positivity in the serum and (2) hepatocellular carcinoma accompanied with surrounding cirrhosis. Five surgical liver biopsy specimens from patients with chronic active hepatitis were also included in this study.

Tissue preparation

Liver specimens were fixed in 10% formalin for 12 hours, embedded in paraffin and serially sectioned at 5 μm per section. The sections were stained with hematoxylin and eosin, and classified as chronic active hepatitis, cirrhosis and hepatocellular carcinoma.

Immunohistochemistry

The prepared paraffin sections were treated with xylene to remove the paraffin and dehydrated. Primary rabbit anti-HBx antibodies prepared from either GST-HBx fusion protein and HBx synthetic peptide spanning 144～154 amino acid residues were kindly provided by Dr. Yungdae Yun (Mogam Research Institute). Similar immunohistochemical staining patterns were obtained from these two anti-HBx antibodies. Rabbit anti-p53 antibody (DAKO) and mouse anti-$p21^{WAF1}$ antibody (Pharmingen) were also used. Staining was detected by the streptoavidin-biotin complex method using aminoethyl carbazole (LSAB kit, DAKO), and the sections were then counterstained with Meyer's hematoxylin.

Staining specificity: Anti-HBx antibody was tested by Western blot analysis with E. coli lysate from bacteria expressing HBx polypeptide. Anti-p53 antibody was able to detect both wild-type p53 protein[15] in the HepG2 hepatocellular carcinoma cell line and mutant p53 protein[15] in the Huh7 hepatocellular carcinoma cell line.

RESULTS

Clinical background of patients

Among the 16 patients with hepatocellular carcinoma, 13 were male and their mean age was 52.3 years. The average age of three female patients with HCC was 48.0 years, and that of four male and one female patients with chronic active hepatitis was 21.0 years.

Expression of HBx protein in the liver with chronic hepatitis, cirrhosis and hepatocellular carcinoma

Among patients with chronic active hepatitis, four (80%) were HBx protein positive(Table 1 and Fig. 1A). In contrast, only one (6%) out of 16 specimens with hepatocellular carcinoma showed positivity for HBx protein, whereas four (25%) surrounding cirrhotic tissues showed HBx positivity. In spite of the small number of cases examined, there was a clear difference in the expression of HBx protein among the patients with chronic hepatitis, cirrhosis and hepatocellular

Table 1. Expression rate of HBx protein in chronic active hepatitis, cirrhosis, and hepatocellular carcinoma

	HBx protein positive cases (percent)
Chronic active hepatitis(n=5)	4 (80%)
Cirrhosis(n=16)	4 (25%)
Hepatocellular carcinoma(n=16)	1 (6%)

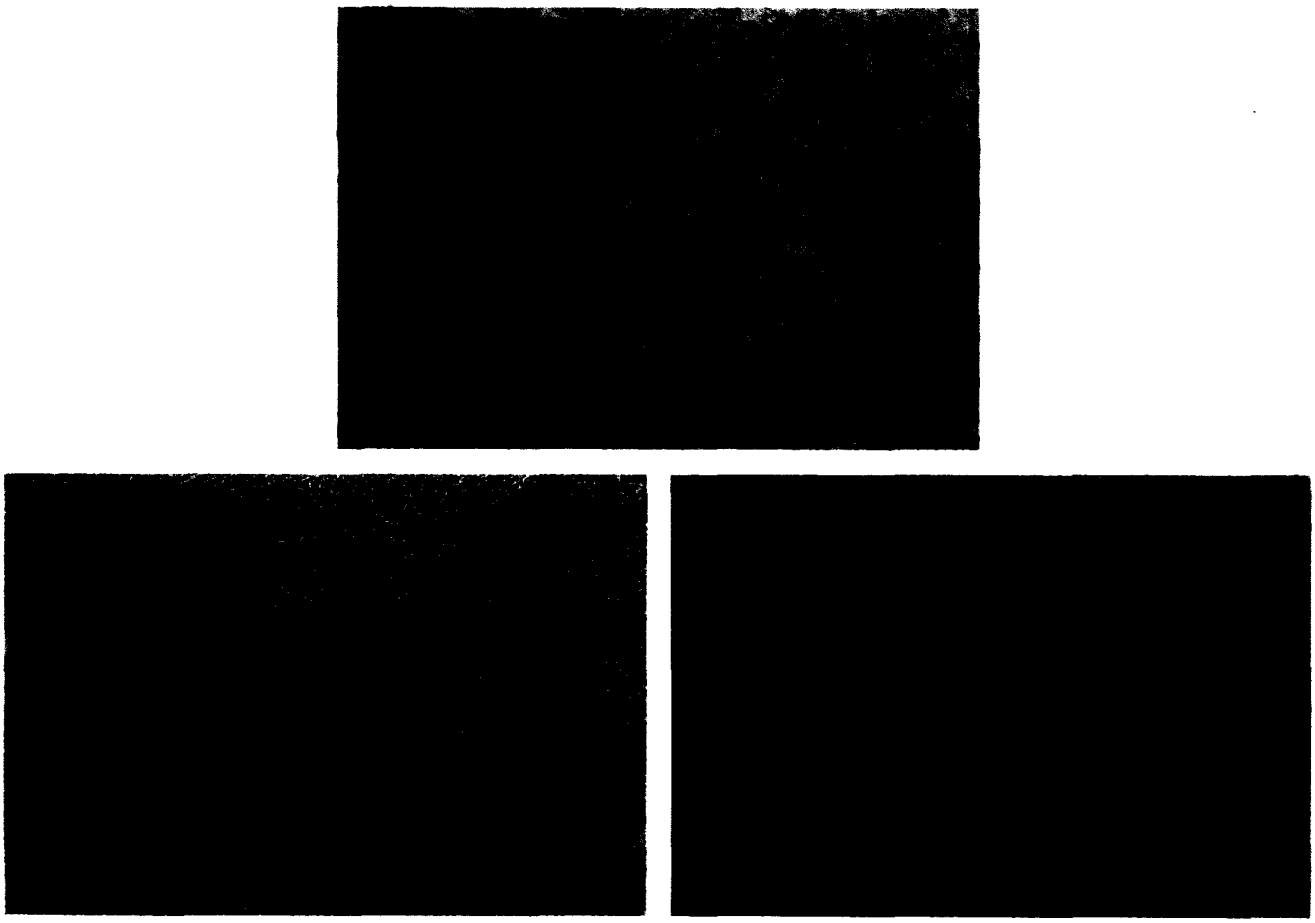

Fig 1. Expression of HBx protein in the liver with chronic active hepatitis, cirrhosis and hepatocellular carcinoma by immunohistochemical staining.
A. Cytoplasmic expression of HBx protein in the liver with chronic active hepatitis (X100)
B. Cytoplasmic expression of HBx protein in the liver with cirrhosis (X40)
C. Nuclear and cytoplasmic expression of HBx protein in the hepatocellular carcinoma (X200)
Cytolasmic and nuclear expressions of HBx protein are indicated by close and open arrows, respectively.

carcinoma. Expression of HBx protein was present throughout the cytoplasm of hepatocytes in chronic hepatitis and cirrhosis (Fig. 1A and 1B). However, both nuclear and cytoplasmic localization of HBx protein was observed in a hepatocellular carcinoma case (Fig. 1C).

Expression of p53 tumor suppressor protein

It was previously reported that HBx protein and p53 protein are associated in the liver tissues from the patients with hepatocellular carcinoma[11]. In addition, the association of p53 protein with HBx protein in HBx transgenic mice caused complete blockade of p53 entry into the nucleus[16]. Thus, we examined whether colocalization of HBx protein and p53 protein might be observed in the HBx-protein positive hepatocytes. Six out of 16 (38%) liver tissues with hepatocellular carcinoma were p53 positive (Table 2), whereas there was no detectable

Table 2. Expression of p53 protein in hepatocellular carcinoma

HBx protein expression	No. of p53 protein expression (percent)
Positive(n=1)	1 (100%)
Negative(n=15)	5 (33%)
Total No.=16	6 (38%)

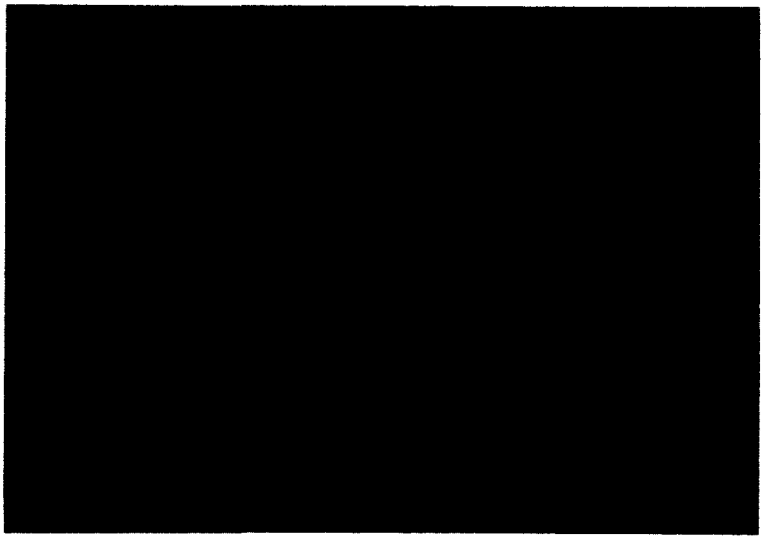

Fig 2. Expression of p53 protein in the nucleus of hepatocellular carcinoma cells (X 200).

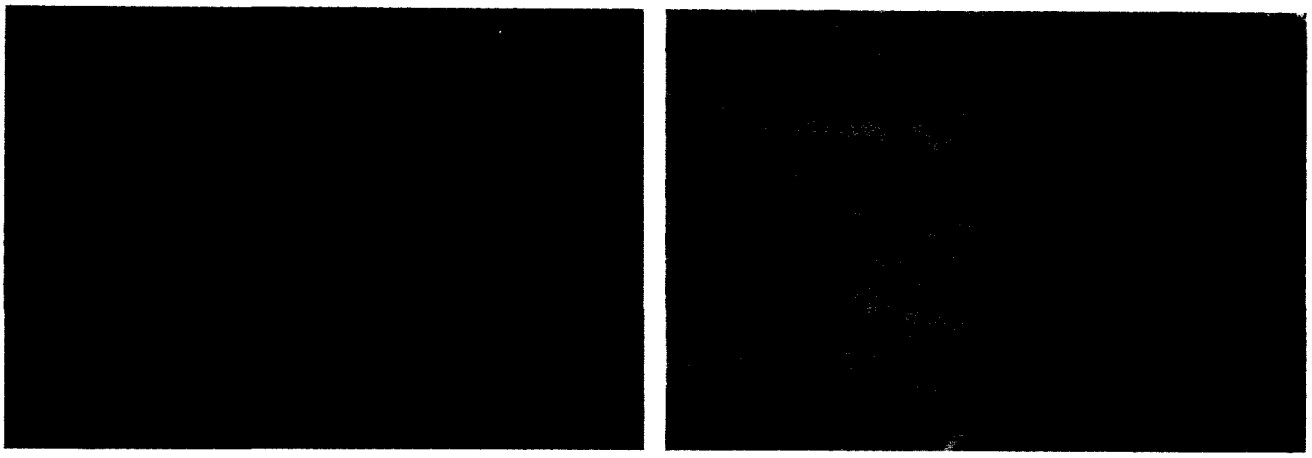

Fig 3. Comparison of p53 (A) and $p21^{WAF1}$ (B) protein expression in the hepatocellular carcinoma (X100).

expression of p53 protein in the surrounding cirrhotic tissues including HBx-protein positive cells. Only one p53-positive hepatocellular carcinoma showed HBx protein expression while the other p53-positive livers with hepatocellular carcinoma did not express HBx protein. The p53 protein expression was observed in the nucleus (Fig. 2) whereas HBx protein was mainly present in the cytoplasm.

Expression of $p21^{WAF1}$ protein

In order to detect molecular changes in HBx protein and/or p53-positive liver cells, we investigted the expression of $p21^{WAF1}$ protein in these cells. Since expression of $p21^{WAF1}$ gene is regulated by p53 protein[17], we suspected that association of HBx protein with p53 or alteration in p53 function might affect the expression of $p21^{WAF1}$ protein. Expression of $p21^{WAF1}$ protein was not detected in any cirrhotic liver tissues but was detected in some hepatocellular carcinoma tissues irrespective of HBx protein and/or p53 protein expression. Fig. 3. shows random distribution of p53 and $p21^{WAF1}$ protein in the same hepatocellular carcinoma tissue.

DISCUSSION

Since the majority of hepatocellular carcinomas in Korea are shown to be associated with hepatitis B virus infection, it is very likely that the expression of HBx protein in hepato-

cytes plays an important role during hepatocarcinogenesis.

We have demonstrated in the present study the differential expression of HBx protein in the livers with chronic active hepatitis, cirrhosis and hepatocellular carcinoma even though the number of cases examined are small. Expression of HBx protein was most abundant in the hepatocytes with chronic active hepatitis whereas it was hardly detected in those with hepatocellular carcinoma (Table 1). In agreement with our results, Henkler et al. (1995)[14] reported that HBx protein was not detected in hepatocytes with hepatitis B virus-related hepatocellular carcinoma by either Western blot analysis, immunoprecipitation or immunocytochemistry. These observations are in contrast to others[9,10] who reported that the majority of the hepatocytes with chronic hepatitis, cirrhosis and hepatocellular carcinoma frequently showed expression of HBx protein. The discrepancy is not only due to the difference in the antibody for HBx protein used, but also to the relatively high nonspecific binding of anti-HBx antibody to cellular proteins because of low antigenicity of HBx protein[1,14]. In our study, two anti-HBx antibodies raised against either c-terminal (144 ~ 154 amino acids) of HBx or GST-HBx fusion protein resulted in the similar pattern of staining, making us to believe that our antibodies are highly specific for the detection of HBx protein. Our results show that expression of HBx protein is differentially expressed during hepatocarcinogenesis. Since the most of the hepatitis B virus related hepatocellular carcinoma occurs in the background cirrhotic liver, the HBx protein seems to play an important role in the early stage of chronic hepatitis-cirrhosis-hepatocellular carcinoma sequence.

We next examined the expression of p53 protein and its relation to HBx protein in these hepatocytes. Expression of p53 protein was detected in 38% of the liver cells with hepatocellular carcinomas but not in the adjacent cirrhotic liver tissues (Table 2). Since the half-life of wild-type p53 protein is short whereas that of mutant p53 protein in other cancers is long[18], it is likely that the wild-type p53 protein in the liver was not detected and only the mutant p53 protein was detectable with the anti-p53 antibody used here. Our results show at least that there is no correlation between HBx protein expression and mutation of p53. In this respect, it is still not clear whether colocalization of HBx protein and wild-type p53 protein may exist in HBx protein positive cells. It is possible that the roles of HBx protein and mutation of p53 during hepatocarcinogenesis are independent. Further studies are necessary to find the precise relation between these two proteins. It should also be added that the expression of p53 protein was found only in the nucleus whereas the most of the HBx protein in the hepatocytes were found in the cytoplasm.

In order to find any molecular changes in HBx protein positive hepatocytes, we also investigated the expression of $p21^{WAF1}$ protein in HBx protein and/or p53 protein positive liver cells. However, expression of $p21^{WAF1}$ protein was detected only in some hepatocellular carcinoma tissues independent of HBx protein and/or p53 protein expression.

In these study, we demonstrate that HBx protein is expressed in the early stage of hepatitis B virus related hepatocarcinogenesis using immunohistochemical procedure.

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