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Activating mechanism of transcription factor NF- κ B regulated by hepatitis B virus X protein in hepatocellular carcinoma

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Abstract

AIM: To investigate the mechanism and significance of NF- κ B activation regulated by hepatitis B virus X protein (HBx) in hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC).

METHODS: The expression levels of HBx, p65, I κ B- α and ubiquitin were detected by immunohistochemistry in HCC tissue microarrays (TMA) respectively, and I κ B- α was detected by Western blot in HCC and corresponding liver tissues.

RESULTS: The percentage of informative TMA samples was 98.8% in 186 cases with a total of 367 samples. Compared with corresponding liver tissues (60.0%), the HBx expression was obviously decreased in HBV-associated HCC (47.9%, $u=2.24$, $P<0.05$). On the contrary, the expressions of p65 (20.6% vs 45.3%, $u=4.85$, $P<0.01$) and ubiquitin (8.9% vs 59.0%, $u=9.68$, $P<0.01$) were notably elevated in HCC. In addition, I κ B- α had a tendency to go up. Importantly, positive relativity was observed between HBx and p65 ($\chi^2=10.26$, $P<0.01$), p65 and I κ B- α ($\chi^2=16.86$, $P<0.01$), I κ B- α and ubiquitin ($\chi^2=8.90$, $P<0.01$) in HCC, respectively.

CONCLUSION: Both active and non-active forms of NF- κ B are increased in HBV-associated HCC. Variant HBx is the major cause of the enhancement of NF- κ B activity. The activation always proceeds in nucleus and the proteasome complexes play an important role in the activation.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a malignant tumor with a poor prognosis. Hepatitis B virus (HBV) has been shown to be linked epidemiologically to the HCC development and about eighty percent of the tumors in China are induced by HBV. As a unique non-structure protein, hepatitis B virus X protein (HBx) performs a variety of biological functions, such as gene

transactivation^[1], interaction with p53^[2], interference with host DNA repair^[3], repression of physiological proteolysis^[4], modulation of cell proliferation and apoptosis^[5,6], induction of malignant cell migration^[7,8]. These functions may play an important role in the initiation and development of HCC associated with HBV infection. NF- κ B, a crucial transcription factor, takes part in almost all aspects of cell regulation, including immune cell activation, stress response, proliferation, apoptosis, differentiation and oncogenic transformation. Currently, more attentions have been paid to the carcinogenesis of HBx transactivating NF- κ B^[9]. However, the active state of NF- κ B in HCC has been seldom studied. As a new high-throughput technology introduced in 1999, tissue microarray (TMA) is worthy of popularization. In our study, the expression levels of HBx, p65, I κ B- α and ubiquitin were detected by immunohistochemistry on TMA respectively, as well as I κ B- α was detected by Western blot, in order to investigate the mechanism and significance of HBx activating NF- κ B.

MATERIALS AND METHODS

Tissue samples

Paraffin specimens were prepared from operatively-resected HCC and non-HCC counterparts between 1997 and 2000, including 171 cases of serum HBV-positive HCC, 10 cases of serum HBV-negative HCC and their corresponding liver tissues, 5 cases of normal control liver tissues (Table 1). In addition, 24 couples of fresh HCC and its corresponding liver tissues were collected between March and October in 2001, stored at -80 °C until experiment.

Tissue microarray construction

All formalin-fixed and paraffin-embedded HCC tissues used in this study were sectioned and stained with hematoxylin-eosin (H&E). The H&E-stained sections were carefully diagnosed, and the representative regions of the tumor and its corresponding liver tissue for microarray were defined as well. HCC TMA was constructed according to the procedure described by Kononen *et al*^[10]. Briefly, core tissue specimens, 0.6 mm in diameter, were taken from selected regions of individual donor blocks and precisely arrayed into recipient paraffin blocks (45 mm×22 mm) using a tissue-arraying instrument (Beecher Instruments, Silver Spring, MD, USA). After construction, the recipient paraffin block was incubated at 37 °C for one hour and the surface of the block was smoothed. Five-micrometer consecutive sections of this TMA block were cut with a microtome. The presence and morphology of tumor and liver tissues on arrayed samples were identified by H&E stained sections.

Immunohistochemistry

TMA section was deparaffinized through xylene and dehydrated with graded alcohol. Endogenous peroxidase was then blocked with 0.3% H₂O₂ diluted in methanol for 30 min at room temperature. Antigen retrieval was performed by treating the slide in citrate buffer in a microwave for 10 min. The slide was incubated in a moist chamber with HBx mouse monoclonal

antibody (1:100; Chemicon, USA), p65 mouse monoclonal antibody (Santa Cruz, USA), I κ B- α rabbit polyclonal antibody (Santa Cruz, USA) and ubiquitin rabbit polyclonal antibody (Neomarkers, USA) at 4 °C overnight respectively. After a brief wash in PBS, the slide was treated with goat anti-mouse antibody and goat anti-rabbit antibody (EnVision™ +Kits, DAKO, Denmark), respectively, for 45 min at 37 °C. After a brief wash in PBS, the slide was developed in 0.05% freshly prepared diaminobenzidine solution (DAB, Sigma, St. Louis, MO) for 8 min, and then counterstained with hematoxylin. More than 5% cells stained were identified as a positive result.

Western blot

Western blot was carried out based on the protocol of *molecular clone*^[11]. Briefly, frozen tissues were lysed in a single eradicator buffer (150 mmol/L NaCl, 50 mmol/L pH 8.0 Tris-HCl, 0.02% natriumazid, 1 μ g/ml aprotinin, 100 μ g/ml PMSF, 1% Triton X-100) and quantified by BCA method. The samples were boiled, loaded, separated on 12% SDS gel electrophoresis, transferred to nitrocellulose membrane, and reacted with I κ B- α rabbit polyclonal antibody (1:1 000, Santa Cruz, USA). At last, the membrane was exposed several minutes after ECL substrate incubation.

Statistical analysis

HBx, p65, I κ B- α , ubiquitin expression differences between HBV-associated HCC and corresponding liver tissues were analyzed statistically using *u* test. The relativity between HBx, I κ B- α , p65, ubiquitin was analyzed statistically using χ^2 test or adjusted χ^2 test.

RESULTS

Tissue microarray

In this study, two HCC TMA blocks were constructed which

contained a total of 181 cases with 367 samples. One thousand four hundred fifty-one informative samples were totally detected by immunohistochemistry on arrays and the observed ratio was up to 98.8% (1 451/1 468 samples). HE-stained sections showed that the morphology of tissues and cells could be seen clearly. The HCC array HE-stained sections and several types of the tumor are shown in Figure 1.

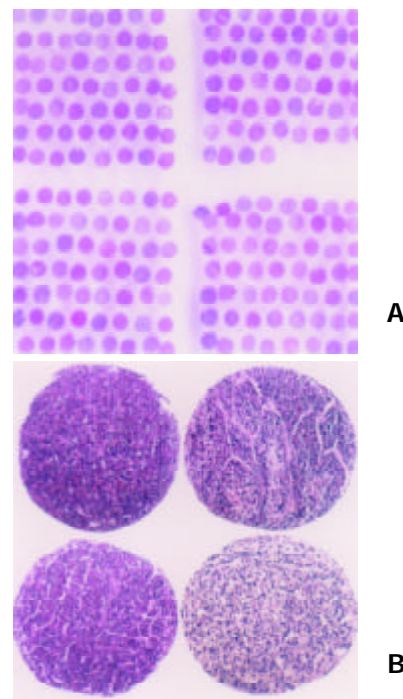


Figure 1 Overview of HCC TMA. A: TMA overview of H&E-staining section, B: HCC morphology on TMA stained by H&E.

Table 1 Clinicopathologic information of patients used in TMA

Group	HBV			Sex		Age	Diameter	Grade			Cirrhosis	
	Total	Positive	Negative	Man	Female	mean \pm SD (year)	mean \pm SD (cm)	II	III	IV	Yes	No
HCC	181	171	10	158	23	49.3 \pm 10.6	7.2 \pm 3.5	28	141	12	166	15
Normal	5	0	5	2	3	42.4 \pm 9.6	9.3 \pm 5.8				0	5

Table 2 Expressions of HBx, p65, I κ B- α and ubiquitin in HCC and corresponding liver tissues

Group	HBx			p65			I κ B- α			Ubiquitin		
	Total	Positive	%	Total	Positive	%	Total	Positive	%	Total	Positive	%
HCC	169	81	47.9	170	77	45.3	170	124	72.9	166	98	59.0
Control	170	102	60.0	170	35	20.6	171	116	67.0	168	15	8.9
Statistics	<i>u</i> =2.24 ^a			<i>u</i> =4.85 ^b			<i>u</i> =1.19			<i>u</i> =9.68 ^b		

^a*P*<0.05, ^b*P*<0.01.

Table 3 Relativity analysis between HBx, I κ B- α , p65 and ubiquitin in HCC and corresponding liver tissues

	HBx		P65		Ubiquitin	
	Negative	Positive	Negative	Positive	Negative	Positive
HBx	Negative		58(52)	30(16)	39(57)	46(8)
	Positive		33(83)	47(19)	28(95)	52(7)
Statistics			$\chi^2=10.26^b(0.60)$		$\chi^2=2.02(1.44)$	
I κ B- α	Negative	29(21)	17(34)	36(46)	9(9)	26(50)
	Positive	59(47)	63(68)	56(89)	68(26)	42(103)
Statistics	$\chi^2=2.28(0.11)$		$\chi^2=16.86^b(1.02)$		$\chi^2=8.90^b(0.04)$	

^b*P*<0.01, vs Corresponding liver tissue.

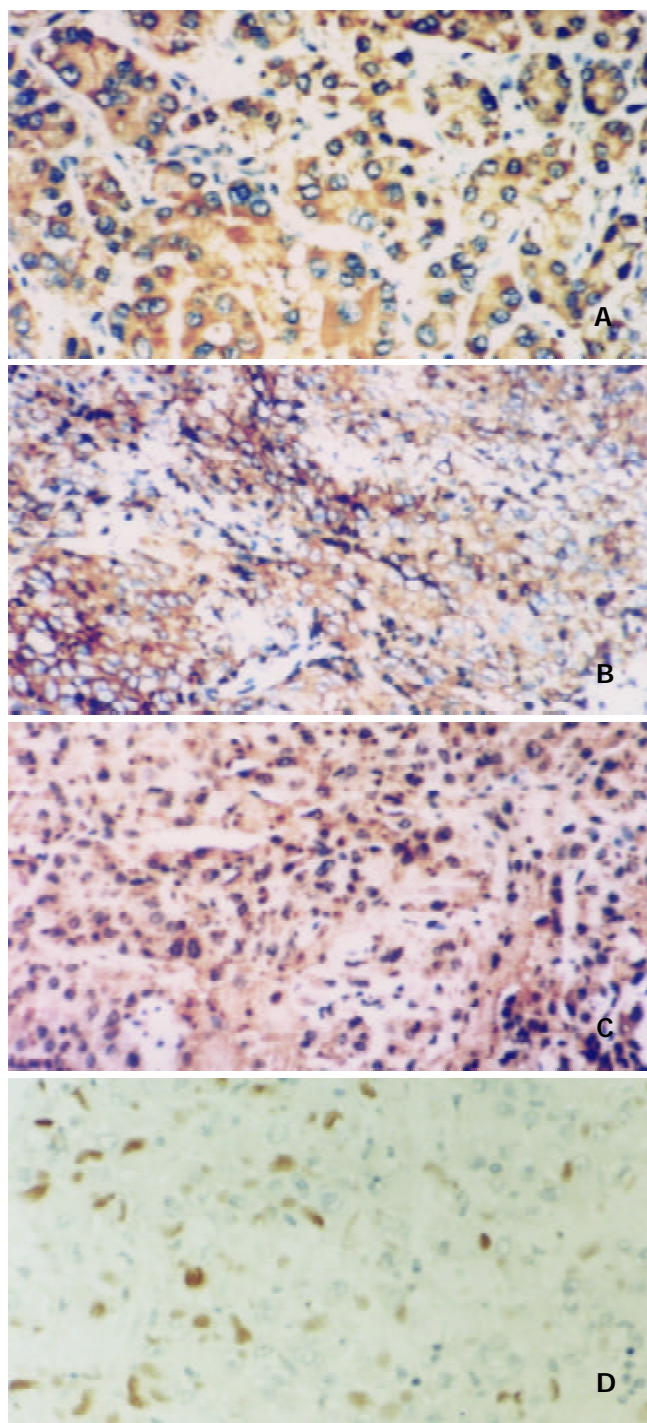


Figure 2 Expressions and locations of HBx, p65, IκB-α and ubiquitin in HCC detected by immunohistochemistry, EnVision $\times 200$. A: HBx expression in cytoplasm, B: p65 immunostaining in cytoplasm and nuclei, C: IκB-α distribution in cytoplasm and nuclei, D: ubiquitin location in nuclei.

Expression differences of four proteins in HCC and corresponding liver tissues

The expression of HBx was restricted exclusively to cytoplasmic location in both HCC and liver tissues. The positive immunostainings of p65 and IκB-α were seen only in cytoplasm of liver tissues, but in both cytoplasm and nuclei of HCC. The positive signal of ubiquitin was distributed predominantly in cytoplasm of liver tissues and in nuclei of HCC (Figure 2).

In serum HBV-positive cases, the HBx expressions in HCC (81/169, 47.9%) were significantly decreased as compared with the corresponding liver tissues (102/170, 60.0%, $P < 0.05$). On the contrary, the expressions of p65 and ubiquitin were notably

elevated in HCC (45.3%, 59.0% respectively) as compared with corresponding liver tissues (20.6%, 8.9% respectively, $P < 0.01$). The positive rate of immunostaining reaction of IκB-α in HCC and corresponding liver tissues was 72.9% and 67% respectively. The difference was not significant, though the staining in HCC was more intense (Table 2).

In serum HBV-negative cases, the expressions of HBx, p65, IκB-α, ubiquitin in HCC were detected in 2/10 cases, 5/9 cases, 7/9 cases and 5/10 cases respectively. In corresponding liver tissues, their expressions were detected in 2/10 cases, 2/9 cases, 7/9 cases and 2/10 cases respectively.

In five normal liver tissues, all expressions of HBx, p65 and ubiquitin were negative, whereas, IκB-α was demonstrated to be weakly positive.

Western blot of IκB-α

Compared with corresponding liver tissues, elevated levels of IκB-α were detected in 10 HCC cases, decreased in 1 case. In the other 13 couples of HCC and corresponding liver tissues, no obvious difference of expression was detected (Figure 3).

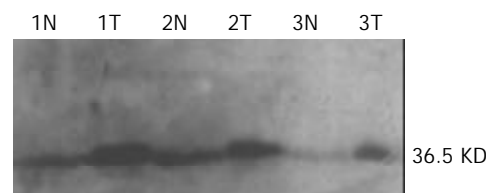


Figure 3 IκB-α expression levels detected by Western blot. IκB-α levels were elevated in 3 cases of HCC compared with their corresponding liver tissues.

Relativity analysis between HBx, IκB-α, p65 and ubiquitin

χ^2 test showed that no relativity existed between HBx, IκB-α, p65 and ubiquitin in corresponding liver tissues, but a positive relativity was observed between HBx and p65, p65 and IκB-α, IκB-α and ubiquitin in HCC (Table 3).

DISCUSSION

TMA is a new technology first introduced in 1999. It contains hundreds or even thousands of small tissue samples arranged into a grid for rapid, cost efficient and high-throughput analysis. TMA has been used widely in gene or protein expression analysis, antibody screening, tissue specificity detection of proteins, phenotype versus genotype analysis, RNA or DNA *in situ*-hybridization^[10,12,13]. Based on TMA, we studied the expressions of HBx, NF-κB, IκB-α, ubiquitin and their interrelationship in HCC.

The result of HBx expression detection in HBV-associated HCC showed that HBx expression was reduced in cancer tissue as compared with the corresponding liver tissue. The result might be ascribed to the existing form of HBV in tumor tissue. Many researches indicated that integration was the major form of HBV in HCC, so the viral copies were far less than that in non-tumor tissues where free virus prevails^[14]. The expression of HBx in samples with serum HBV-negative implicated quite a few patients were once infected with HBV. Moreover, the cytoplasmic location of HBx was consistent with the previous reports^[15].

NF-κB plays a vital role in almost all aspects of cell regulation such as immune cell activation, proliferation, apoptosis, stress response, differentiation and oncogenic transformation. Activated NF-κB can mediate the expression of a large (more than 150) and diverse set of inflammatory and immune response mediators. It has been considered as a central regulator of cellular responses and played a pivotal

role both at the stage of initiation and perpetuation of chronic inflammation^[16-19]. NF- κ B is sequestered in the cytosol of unstimulated cells via non-covalent interactions with a class of inhibitor proteins, called I κ Bs. Signals that induce NF- κ B activity cause the phosphorylation of I κ Bs, their dissociation and subsequent ubiquitination and degradation in 26S proteasome complex, allowing NF- κ B proteins to enter the nucleus and induce gene expression by binding κ B site in DNA. As one of the NF- κ B activation products, I κ B- α could induce NF- κ B export from the nucleus by reuniting NF- κ B and terminating associated gene activation. The negative feedback loop could keep NF- κ B sensitivity to signals by returning rapidly to baseline activity^[20-22].

Because p65 is one of the most common members in NF- κ B family, and I κ B- α is the most important inhibitor, detection of p65 and I κ B- α expression can reflect the state of NF- κ B metabolism. In our test, low expression rate of p65 and weakly positive expression of I κ B- α represented the nonactive state of NF- κ B in non-cancerous tissues, but the activation enhancement of NF- κ B in HCC was in accord with the previous reports^[23,24]. The expression of I κ B- α in HCC and its corresponding liver tissue has not been reported yet. In our study, no statistical difference was found in positive rates between HCC and its corresponding liver tissue, the discrimination of immunostaining intensity was scented. Furthermore, Western blot validated the concentration augmentation of I κ B- α in HCC, while in the meantime, molecular weight alteration was not observed. The results illustrated that I κ B- α regulation gave priority to quantity change in HCC. The location of p65 and I κ B- α represented the activation or non-activation form of NF- κ B. Quite a few cytoplasm locations of p65 and I κ B- α observed in our test accounted for the non-activated NF- κ B increase in HCC, which contradicted with cytoplasm decrease of p65 and I κ B- α observed *in vivo* system^[25]. We ascribed the contradiction of NF- κ B metabolism to the persistence in HCC and the transience *in vivo* system. The lasting activation of NF- κ B can automatically regulate the production of I κ B- α and possibly even p65 or p50. A great deal of non-activated NF- κ B repertory is in favor of lasting activation of NF- κ B.

I κ B- α proteolysis is by the ubiquitin-proteasome pathway, but the exact locality is still not clear. Birbach *et al* expatiated I κ B- α shuttle mechanism between cytoplasm and nucleus^[26]. It is recognized now that besides I κ B- α , up-stream kinases such as NIK and MAPKK can shuttle between cytoplasm and nucleus. The present study is the first to show the distribution and expression of ubiquitin in HCC. The results of nuclear location of ubiquitin and its relativity with I κ B- α indicated the proteolysis of I κ B- α was processed in tumor cell nuclei. Of course, another likelihood was that transcription of ubiquitin would be promoted after NF- κ B activation^[27]. There is no relativity between ubiquitin and HBx, so whether the proteolysis of HBx passes through non-ubiquitin pathway or not remains to be determined.

Positive relativity between HBx and p65 in HCC indicated that HBx existed in tumor tissues was one cause of inducing NF- κ B activation. It has been found in some studies that HBx mutation was common in HCC compared with corresponding liver tissues^[28]. Frequent types of HBx mutation were COOH-terminal truncation and hotspot mutations in certain amino acids^[29-31]. COOH-terminally truncated HBx is encoded by truncated X gene, which usually derives from HBV integrated into host genome. Base mutation and frame-shift are the other causes. Usually, it was considered that HBx activated NF- κ B relied on its transactivation domains, and COOH-terminal transactivation domain was important to its transactivation function^[32,33]. In our study, NF- κ B activation was elevated in HCC with a low HBx expression compared with corresponding liver tissues. Therefore, the results implied that HBx might

activate NF- κ B by other unknown mechanisms but not its transactivation ability. One likeness was that C-terminally truncated HBx lost its ability to suppress proteasome complex and facilitated I κ B- α degradation and NF- κ B activation^[4]. The high percentage of ubiquitin expressions in HCC (59.0%) from our results in part supports the opinion. The detailed mechanism of NF- κ B activation in HBV-associated hepatocellular carcinoma remains to be further studied.

In conclusion, NF- κ B activation induced by HBx could not only facilitate infected cell survival and HBV escape from immune clearance, but also promote liver cell malignant transformation and tumor cell advantageous growth^[34]. Variant HBx plays an important role in potentiating NF- κ B activation.

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