



VIRAL HEPATITIS

COOH-terminal deletion of HBx gene is a frequent event in HBV-associated hepatocellular carcinoma

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associated HCC tissues in China. HBV integration had also taken place in partial HCC tissues. This supporting the hypothesis that deletion and probably integrated forms of the HBx gene may be implicated in liver carcinogenesis.

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Abstract

AIM: To investigate the hepatitis B virus (HBV) x gene (HBx) state in the tissues of HBV-related hepatocellular carcinoma (HCC) in Chinese patients and whether there were particular HBx mutations.

METHODS: HBx gene was amplified and direct sequencing was used in genomic DNA samples from 20 HCC and corresponding non-cancerous liver tissues from HBsAg-positive patients. HBV DNA integration and HBx deleted mutation were validated in 45 HCC patients at different stages by Southern blot analysis and polymerase chain reaction methods.

RESULTS: The frequencies of HBx point mutations were significantly lower in HCC than their corresponding non-cancerous liver tissues (11/19 vs 18/19, $P = 0.019$). In contrast, deletions in HBx gene were significantly higher in HCC than their non-cancerous liver tissues (16/19 vs 4/19, $P < 0.001$). The deletion of HBx COOH-terminal was detected in 14 HCC tissues. A specific integration of HBx at 17p13 locus was also found in 8 of 16 HCC, and all of them also exhibited full-length HBx deletions. Integrated or integrated coexistence with replicated pattern was obtained in 45.5% (20/45) - 56.8% (25/45) tumors and 40.9% (18/45) - 52.3% (23/45) non-tumor tissues.

CONCLUSION: HBx deletion, especially the COOH-terminal deletion of HBx is a frequent event in HBV-

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and a leading cause of death in many countries, mainly in Asia and Africa. The most prominent factors associated with HCC include chronic hepatitis B and C viral infection, chronic alcohol consumption, aflatoxin-B1-contaminated food and virtually all cirrhosis-inducing conditions^[1,2]. The hepatitis B virus (HBV) genomic DNA contains four partially overlapping open reading frames: preC/C, P, preS/S and X gene^[3-5]. The X gene product, HBV X protein (HBxAg) is a trans-activating protein, and a multifunctional regulator that modulates transcription, signal transduction, cell cycle progress, protein degradation pathways, apoptosis, and genetic stability by directly or indirectly interacting with host factors^[6,7]. Transgenic mice created with the X gene have been reported to develop HCC^[8-10]. Although the transactivation properties of the X gene and HBxAg overexpressed selectively in human HCC^[11,12], its precise role in human hepatocarcinogenesis is still unclear^[1,2]. In some studies, X gene containing substantial but different deletions in the COOH-terminal region was found in many HBV-infected HCC patients^[13-16]. To further explore the relationship between nucleotide changes in HBx and the development of HCC, we examined the mutants and

nucleotide sequence of the HBx gene obtained from the tissues of HBV-related HCC in Chinese patients.

MATERIALS AND METHODS

Tissue samples

Twenty HCCs and corresponding noncancerous liver tissues were obtained from surgically resected samples at Changhai Hospital between August 2003 to October 2004. Patients had not received any treatment before. This study included 18 men and 2 women. Their ages ranged from 31 to 69 years (median 50 years). All patients were positive for serum hepatitis B surface antigen (HBsAg). The median tumor size was 5.5 cm (2.0-12.5 cm). Histopathological diagnosis was made according to the World Health Organization histological classification of tumors of the liver and intrahepatic bile ducts^[17]. Three HCC were well differentiated, 14 were moderately differentiated, and 3 were poorly differentiated. Of the 20 patients, 6 had evidence of intrahepatic metastasis (portal vein invasion and/or intrahepatic dissemination). Sixteen HCC cases were accompanied with liver cirrhosis and the remaining 4 cases were with chronic hepatitis.

Polymerase chain reaction and sequence analysis of HBX

Genomic DNA was extracted from 20 frozen HCC tissues and corresponding non-cancerous liver tissues using the standard phenol/chloroform extraction and ethanol precipitation method. To amplify the integrated HBx sequences from tissues, we used an HBx-Alu PCR-based approach^[18]. The sequences of the primers were as follows: 5'-TGCCAAGTGTGTTGCTGACGC-3' (HBV 1176-1195, AY220699), 5'-AAGGAAAGAAGTCAG AAGG-3' (HBV 1960-1978)^[7]. The fragment size is 803 bp. After denaturation at 94°C for 2 min, 36 cycles of DNA amplification were performed at 94°C for 30 s, at 53°C for 60 s, and at 72 °C for 60 s; with a final extension at 72°C for 10 min and stored at 4°C. PCR results were identified by electrophoresis on a 1% agarose gel stained with ethidium bromide.

HBx gene sequence was directly determined by automated DNA sequencing (ABI PRISMTM 3730 DNA Sequencer) after purified on silica columns (QIAquick PCR purification kit, Qiagen, Courtaboeuf, France). At least two independent DNA extractions and PCR reactions were performed for each sample, and four sequencing reactions (two for each strand) were carried out to confirm that the reported sequence reflected the most prevalent HBx in a specific sample. The plasmid pHBXB1 containing the full length of the HBV X gene cloned in the pcDNA3 vector, was used as a positive control.

Detection of HBV DNA integration and HBx deleted mutation

To evaluate HBV DNA integration and HBx deleted mutation in HCC tissues, genomic Southern blot analysis and PCR were performed on carcinoma tissues and the corresponding non-cancerous liver tissues from a set of independent samples in 45 HCC patients at different stages from Northwest of China, a relative low-aflatoxins

exposure area. No patient had received treatment before. Serum hepatitis B surface antigen (HBsAg) was positive in all patients. The non-tumor tissues exhibited cirrhosis in 43 patients.

HBV DNA integration by genomic Southern blot analysis

Genomic DNA was extracted from frozen HCC tissues and corresponding non-cancerous liver tissues using the standard phenol/chloroform extraction and ethanol precipitation method. Each sample of extracted genomic DNA (8 µg) was subjected to restriction by EcoRI and Hind III, separated on agarose gel and blotted onto nylon membrane (Zetabind, Life Sci., Ltd.). Southern blot analysis was carried out using 32P dCTP DNA Labeling and Detection Kit (Amersham Life Sci., Ltd., England) with the 32P dCTP-labeled HBV, HBc, HBx coding region and Pre-S as probes (Dupont).

Polymerase chain reaction

HBc, HBc and HBx genes were amplified from HCC genomic DNA by polymerase chain reaction (PCR). The sequences of those primers were HBc: P1 (1903-1929), 5'-ATGGACATCGACCCTTATAAAGAATTG-3', P2 (2434-2411): 5'-CTAAGATTGAGATCTTCTGAGAC GCGG-3'; HBc and HBx: P2 (2434-2411): 5'-CTAAGA TTGAGATCTTCTGAGACGCGG-3'; P3 (1227-1243): 5'-AGCGCATGCGTGGAACC-3'. The fragment sizes are 531 bp and 1207 bp, respectively. All PCRs were performed using a Thermal Cycler 9600 (Perkin Elmer, CA) under the following conditions: after denaturation at 94°C for 2 min, 38 cycles of DNA amplification was performed at 94°C for 60 s, at 55°C for 60 s, and at 72°C for 120 s; with a final extension at 72°C for 10 min and stored at 4°C. PCR results were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide. To assess the specificity of our results, Southern blot analysis was carried out as described above.

Statistical analysis

All statistical analyses were carried out using the SPSS.11 software. Comparisons between two characteristics were made using Chi-square test or Fisher's exact test. $P < 0.05$ was considered statistically significant in a two-tail analysis.

RESULTS

HBx gene sequencing in HBsAg-positive HCC tissues

To confirm the HBx deletion mutation, HBx gene was amplified and direct sequencing was made in 20 HBsAg-positive HCC genomic DNA. The fragment sizes of PCR products ranged from 150 bp to 800 bp (Figure 1). The results of sequencing were compared with the known sequences in the GenBank database using the BLAST programs (Figure 2). The frequencies of HBx point mutations were significantly lower in HCC than in the corresponding non-cancerous liver tissues (11/19 *vs* 18/19, $P = 0.019$). In 19 available non-tumorous livers, sequencing results revealed 47 different point mutation patterns, including 30 mis-sense mutations, 15 sense mutations and 2 non-sense mutations. The loci with the highest frequency

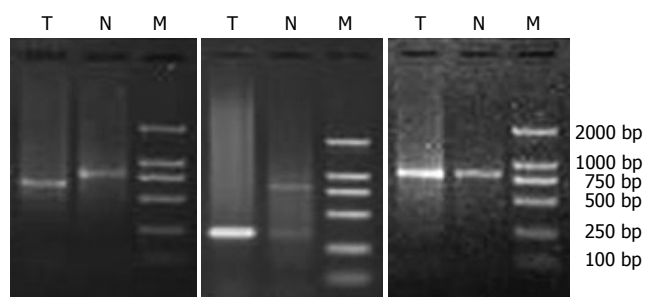


Figure 1 PCR amplified products of HBx-DNA. The fragment sizes of PCR products range from 150 bp to 800 bp. M: Marker; T: Tumor; N: Non-tumor.

of mutation were 67aa (8/19, all were gga→ggt sense mutations, Figure 3A) and 127aa (6/19, four were att→act missense mutations, Figure 3B, 1 was atc→att sense mutations, and 1 was direct linked-point HBV -nt1899 leading to deletions of amino acid at the HBx COOH-terminal end).

In contrast, the frequencies of HBx deletion mutation in HCC were significantly higher than the corresponding non-cancerous liver tissues (16/19 *vs* 4/19, $P < 0.001$). Among them, eight exhibited full-length HBx deletions, the other 8 were deletions with size ranging from 4aa to 150aa. The deletion of HBx COOH-terminal end was also detected in 6 of the latter (Figure 4). The COOH-terminal deletion of HBx was exhibited in 14 HCC samples, which is a major feature of HBx identified in tumor tissues.

In addition, a specific integration of HBx at GA-rich region of 17p13 locus (repeat region 56047..56210/rpt_family = "GA-rich") was also found in 8 of 16 HCC (Figure 5, Table 1). All of these also exhibited full-length HBx deletions. And the specific integration of HBx was more frequent in HCC than in the corresponding non-cancerous liver tissues (8/16 *vs* 1/16, $P = 0.015$). Finally, the translocation occurring between chromosomes 17 and 14 was obtained from HCC tissues from the no. 2 patient. We considered that a mispriming of the HBV primer may have occurred, so we rechecked the primer sequences and found no significant similarities in chromosome 17 using BLAST. The HBx gene was amplified and direct sequencing was duplicated in all samples, and identical results were obtained. A mispriming of HBV primer in the chromosome 17 was eliminated.

Genomic integrated HBV DNA in HCC tissues

To investigate whether the detected HBV DNA was integrated into the genome of the HCC tissues, genomic DNA was extracted and restricted by EcoRI or Hind III prior to Southern blot analysis. Results revealed that the bands of variable sizes were hybridized with the 32P-labeled HBV, HBc, HBx and Pre-S probe. These include the three band patterns: approximately more than 3.2 kb of integrated pattern, replicated pattern of less than 3.2 kb and mixed pattern (integrated coexistence with replicated) (Figure 6A). In 44 of 45 pair samples, the hybridized bands of HBV full gene and fragments were obtained in 45.5%-56.8% tumors and 40.9%-52.3% non-

cancerous tissues, respectively (Table 2). Hybridized bands of HBx and HBc revealed deletion at least in some cases compared to HBV full-length gene probe. None of HBV full gene and fragments showed single replicated pattern in HCC, indicating that HBx integration had taken place in partial HCC tissues.

Deleted mutation of HBc and HBx in HCC

To further confirm that the hybridized bands contained deleted mutation HBx, PCR amplification was carried out using specific primers on DNA extracted from genomic DNA in 45 HCC and their corresponding non-cancerous liver tissues. The fragment sizes include two patterns: approximately 531bp of wild type and less than 531 bp of mutant type in HBc, and approximately 1207 bp of wild type and less than 1207 bp of mutant type in HBc + HBx, respectively (Figure 6B). As shown in Table 3, deleted mutation of HBx was more frequent in HCC than that of HBc. The amplified products were consistent with the genomic Southern blot analysis.

DISCUSSION

HBV infection is a major factor contributing to the development of HCC in China and the mutation in HBx plays an important role in this process^[2,19-22]. However, analysis of HBx sequence in tumor tissues of HCC patients from mainland China has not been done^[14,16,22,23]. A previous study of sera from 67 HCC patients from Taiwan indicates that 52% of samples contain HBx mutations^[23]. Our present study showed that the frequency of the HBx gene mutation in either tumor tissues or the corresponding non-cancerous liver tissue samples of HCC is very high, 57.9% and 94.7%, respectively. The most frequent spots of mutation identified are 67aa (8/20) and 127aa (6/20). The hot spots reported by other groups are nt. 382-389 (codons 128-130) in HCC samples collected from Qidong, China^[14], nt. 204 and 260-264 (codons 68 and 87-88) in HCC samples from Hong Kong^[16] and nt. 93 (codon 31) in HCC samples from Taiwan^[23]. These suggested that the HBx may have its own distinguished patterns of mutation in different geographic regions. As was reported by Chen GG *et al*, the biological consequence may be the same in all these regions^[16].

The most important finding of this study was the identification of HBx deletion mutation in our samples. HBx deletion was detected in 16 HCC samples, and 14 exhibited the COOH-terminal deletion of HBx in HCC tissues. A COOH-terminal deletion in the HBx gene was found in 5 of 9^[24] and 5/6 HCCs^[15]. There may be three regions of the X gene essential for the transactivation function of the X protein (at codons 46-52, 61-69 and 132-139)^[14,25]. All of these results suggest that HBx mutants with a COOH-terminal deletion were significantly correlated with the development and progression of HCC.

Although the mechanism involved in pathogenesis of HBx mutants with a COOH-terminal deletion remains largely unknown, some studies showed that HBx deleted mutants isolated from tumor tissues abrogated both

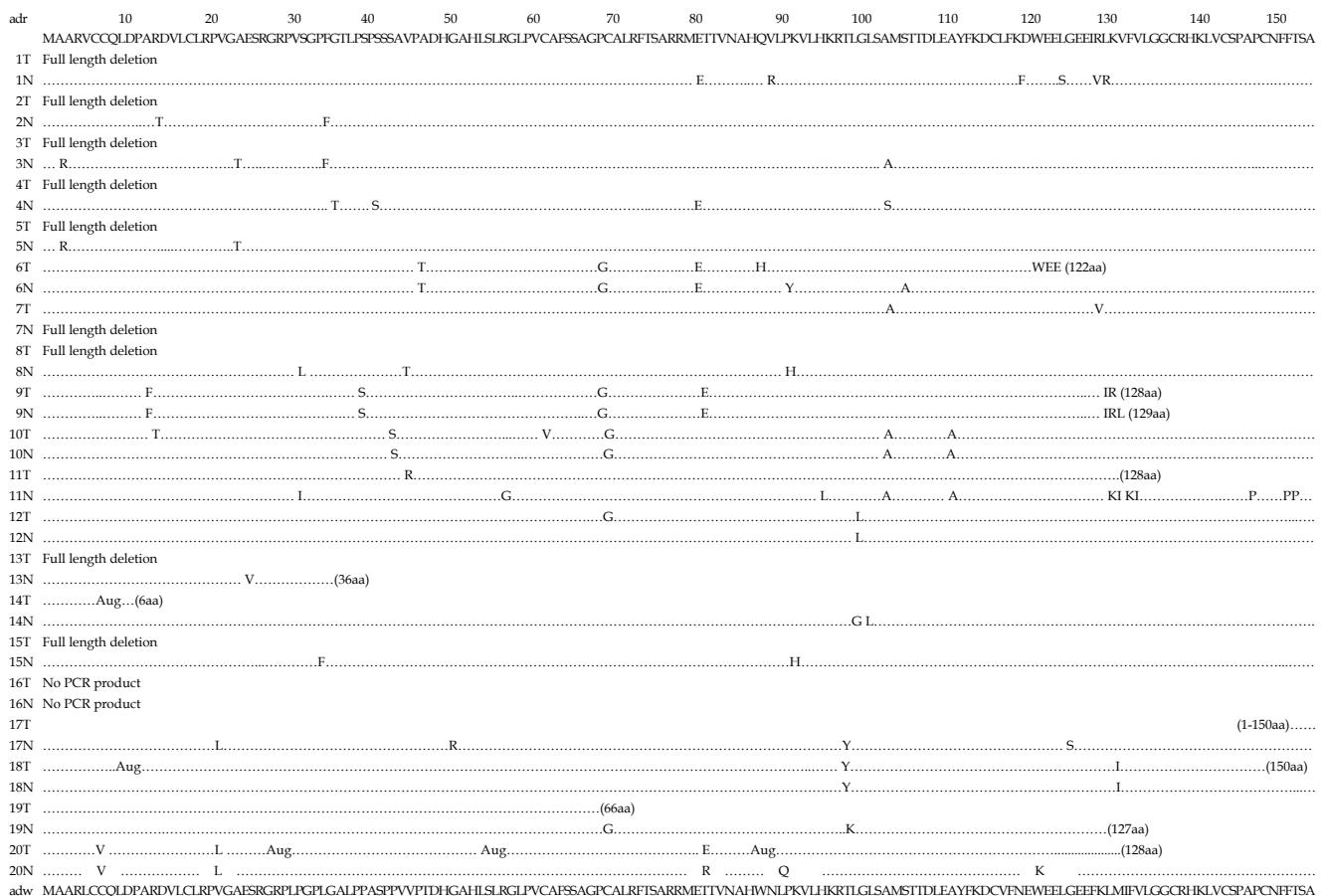


Figure 2 HBx sequencing in cancer and non-cancerous tissues from 20 HBV-associated HCC patients. The amino acid sequences of HBV adr and adw subtypes are shown at the top or the bottom. Identical amino acid residues are represented by dots. The underlined amino acids were deduced from cellular flanking sequences. The frequencies of HBx point mutations were significantly lower in HCC than in the corresponding non-cancerous liver tissues (11/19 vs 18/19, $P = 0.019$). T: Tumor; N: Non-tumor. "...": Represent the corresponding PCR amplified base sequences.

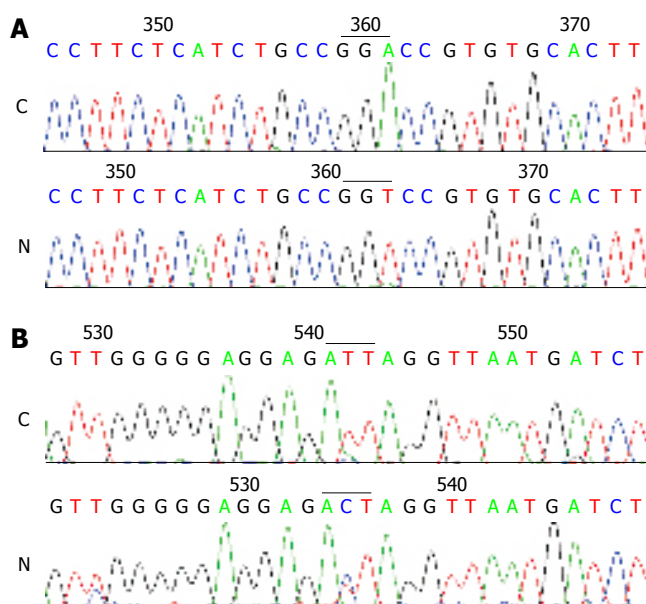


Figure 3 (A) GGA→GGT sense mutations at 67aa and (B) ATT→ACT mis-sense mutations at 127aa in non-cancerous liver tissues. C: Control; N: Non-tumor.

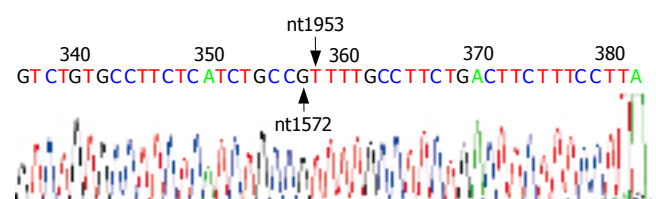


Figure 4 Sequencing of HBx deletion mutation in HCC.

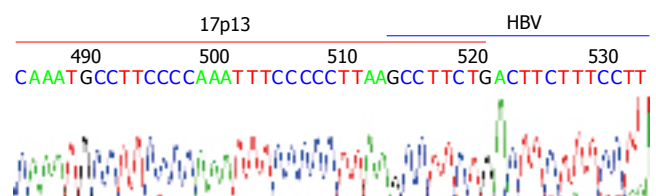


Figure 5 Sequencing of a specific integrated HBV at GA-rich region of 17p13 locus from HCC tissues of no. 2 patient. A: Tumor; B: Non-tumor.

Table 1 Sequences of HBx integrated with Homo sapiens chromosomal 17 nucleotide sequences in 8 HCC cases

Case No.	Homo sapiens chromosome 17, clone RP11-104H15 (PCR sequencing)			HBV gene
	A	B	C	
2	chromosome 14 (39-87)	56455-56249 (224-430)	56194-56158 (485-521)	1957-1976 (514-533)
3	56677-56472 (1-206)	56455-56249 (223-429)	56194-56158 (484-520)	1957-1976 (513-532)
4	56663-56472 (1-192)	56455-56249 (209-415)	56194-56158 (470-506)	1957-1976 (499-518)
5	56676-56472 (1-205)	56455-56249 (222-428)	56194-56158 (483-519)	1957-1976 (512-531)
8	56675-56472 (6-208)	56455-56249 (225-431)	56194-56158 (486-522)	1957-1976 (515-534)
13	56679-56472 (1-208)	56455-56249 (225-431)	56194-56158 (486-522)	1957-1976 (515-534)
15	56669-56472 (1-198)	56455-56249 (215-421)	56194-56158 (476-512)	1957-1976 (505-524)
16	56675-56472 (1-204)	56455-56249 (221-427)	56194-56158 (482-518)	1957-1976 (511-530)

A, B and C represent 3 discontinued sequences at 17p13 and integrated HBV gene sequences.

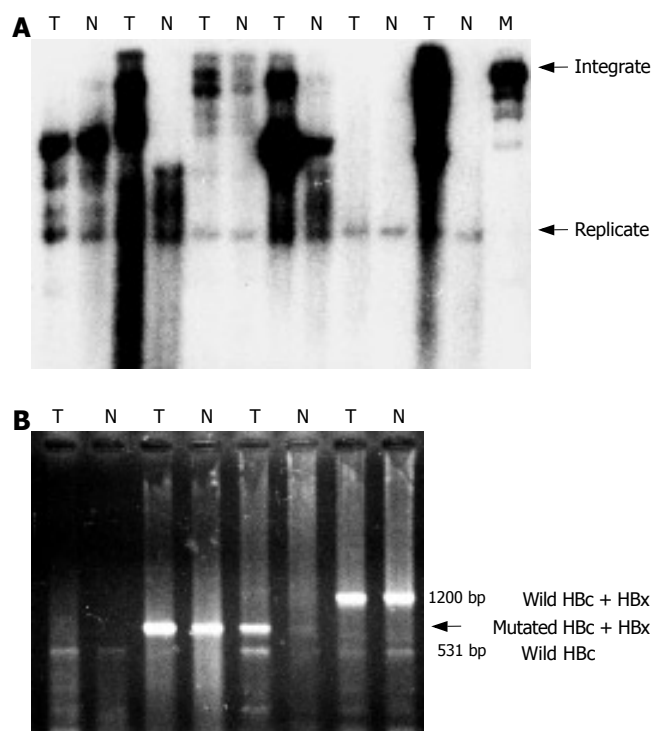
Table 2 Patterns of genomic integrated HBV DNA in HCC tissues by Southern blot

Gene probes	n	Tumor					Non-tumor liver tissues				
		I	R	I + R	Total	%	I	R	I + R	Total	%
HBV-DNA (EcoR I restriction)	44	16	0	9	25	56.8	10	5	8	23	52.3
HBV-DNA (Hind III restriction)	44	18	0	7	25	56.8	13	6	4	23	52.3
HBx-DNA (EcoR I restriction)	44	13	0	7	20	45.0	8	4	6	18	40.9
HBc-DNA (EcoR I restriction)	44	16	0	6	22	50.0	9	5	5	19	43.2
Pre-S (EcoR I restriction)	44	16	0	9	25	56.8	10	5	7	22	50.0

I: Integrated pattern; R: Replicated pattern, I + R: Integrated coexistence with replicated (mixed pattern).

the transactivation and antiproliferative effects of wild type HBx^[24]. When HBx deleted mutant plasmids were transfected to murine and human cell lines, a strongly increased colony formation, accelerated cell cycle progression, and synergetically promoted ras and myc transforming capacity were confirmed^[7,24,26]. Therefore, a COOH-terminal deletion may alter the balance of HBx functional domains in regulating cell proliferation and apoptosis, viability, and transformation. In addition, as previously reported^[15,27], we also noted the coexistence within the same tumor cells of full-length and COOH-terminally deleted HBx sequences, encoded by free or integrated HBV genome sequences. Although we do not exclude the coexistence within the same tumor cells of full-length and COOH-terminally deleted HBx sequences encoded by free or integrated HBV genome, we are investigating the biological implication of the COOH-terminally truncated HBx sequences.

Integration of HBV DNA was found in HCC at

**Figure 6** A: Detection of integrated HBx fragments in HCC tissues by Southern blot analysis; B: HBc and HBx fragment amplified by PCR. M: Marker; T: Tumor; N: Non-tumor.

GA-rich region of 17p13 locus in our study. We have eliminated the mispriming of HBV primer, and identified a specific integration of HBV DNA. This region includes the p53 gene, which is bound to and inactivated by HBxAg prior to tumor formation and then lost during tumor progression^[19]. This region also encodes microRNAs-22, -132, -195, -212^[19]; ubiquitin-conjugating enzyme E2G 1 and ubiquitin specific protease^[28]. We have also noted that most of the samples contained COOH-terminally deleted mutants of the HBx, and the specific integration of HBx was more frequent in HCC than in the corresponding non-cancerous liver tissues, and HBV integration including HBx, occurred in partial HCC tissues by Southern blot. These changes, to the best of our knowledge, have not been described elsewhere. Almost all of the HBV-associated HCCs harbor chromosomally integrated HBV DNA sequences, including chromosome 17p12-13^[4,19,29,30]. HBV integration can induce deletions in the host chromosome at the integration site^[19]. It was also recently confirmed that HBV insertion into cellular genes is a frequent event and that integration can occur in genes regulating cellular signal transduction cascades, proliferation control and cell viability^[7,19]. Thus, the putative HBV-specific integration sites, the biological impacts and the process with functional genomics of HBV associated HCCs need further studies.

In conclusion, HBx deletion, especially the COOH-terminal deletion of HBx, is a frequent event in HBV-associated HCC tissues. HBV integration including HBx, occurred in partial HCC tissues. This supports the hypothesis that the deletion and probably integrated forms of the HBx gene may be implicated in liver carcinogenesis.

Table 3 Deleted mutation of HBc and HBx in HCC by PCR, *n* (%)

Item	<i>n</i>	Tumor			Non-tumor		
		Positive	Wild type	Mutant type	Positive	Wild type	Mutant type
HBc	45	38 (84.4)	28 (73.7)	10 (26.3)	38 (84.4)	31 (81.6)	7 (18.4)
HBc + HBx	45	32 (71.1)	8 (25.0) ^b	24 (75.00) ^b	31 (68.9)	9 (29.0) ^b	22 (71.0) ^b

^b*P* < 0.01.

COMMENTS

Background

The hepatitis B virus (HBV) x protein (HBx) plays a critical role in the molecular pathogenesis of hepatocellular carcinoma (HCC). However, the mechanism remains largely unknown.

Research frontiers

HBx gene containing substantial but different deletions in the COOH-terminal region was found in many HBV-infected HCC patients from Qidong, Hong Kong and Taiwan, China.

Innovations and breakthroughs

The frequencies of HBx point mutations were significantly lower in HCC than their corresponding non-cancerous liver tissues. HBx deletion, especially the COOH-terminal deletion of HBx is a frequent event in HBV-associated HCC tissues in China. Integrated or integrated coexistence with replicated pattern were obtained in HCC and non-cancerous tissues.

Applications

Deletion and probably integrated forms of the HBx gene may be implicated in liver carcinogenesis.

Peer review

This study showed that HBx deletion, especially the COOH-terminal deletion of HBx is a frequent event in HBV-associated HCC tissues in China. This study confirmed their existence of HBx COOH-terminal deletion in human HCC, and provided some new information to clarify the mechanism in hepatocarcinogenesis.

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